A single natural nucleotide mutation alters bacterial pathogen host tropism

David Viana1,9, Maria Comos2,9, Paul R McAdam3,9, Melissa J Ward4, Laura Selva1, Caitriona M Guinane3, Beatriz M González-Muñoz5, Anne Tristan6, Simon J Foster5, J Ross Fitzgerald3,10 & José R Penadés7,8,10

The capacity of microbial pathogens to alter their host tropism leading to epidemics in distinct host species populations is a global public and veterinary health concern. To investigate the molecular basis of a bacterial host-switching event in a tractable host species, we traced the evolutionary trajectory of the common rabbit clone of Staphylococcus aureus. We report that it evolved through a likely human-to-rabbit host jump over 40 years ago and that only a single naturally occurring nucleotide mutation was required and sufficient to convert a human-specific S. aureus strain into one that could infect rabbits. Related mutations were identified at the same locus in other rabbit strains of distinct clonal origin, consistent with convergent evolution. This first report of a single mutation that was sufficient to alter the host tropism of a microorganism during its evolution highlights the capacity of some pathogens to readily expand into new host species populations.

The capacity for pathogens to switch host species leading to epidemic spread in new host populations is a major veterinary and public health concern, but understanding of the genetic basis of host adaptation is very limited. Many animal- and plant-associated microorganisms coevolve with a single host species leading to complex mutualistic, commensal or pathogenic relationships, and switches between host species must overcome innate host-specific barriers to colonization and subsequent transmission. However, successful host jumps provide an opportunity for the microorganism to expand into new ecological niches, and anthropogenic events such as domestication, industrialization of agriculture and globalization have provided increased opportunities for the transmission of bacteria between humans and animals and their subsequent dissemination. Recent studies have established that the host shift and onward transmission of influenza virus requires several mutations in multiple genes2–4, but the precise primary mechanisms have not been completely deciphered. The mechanisms underlying successful bacterial host-switching events are even less well understood, but it is generally assumed that more complex genetic adaptations affecting multiple pathways would underlie adaptation to distantly related members of the animal kingdom.

S. aureus is a major human pathogen that is also associated with economically important infections of livestock, including dairy cows, sheep, poultry and rabbits5. Previously, it has been demonstrated that the major clones of S. aureus associated with ruminant and poultry infections evolved as the result of human-to-animal host jumps, leading to host-specialized endemic clones responsible for disease6–9. Several studies have demonstrated an association of specific mobile genetic elements (MGEs) with strains infecting particular host species, suggestive of a role for MGEs in host specificity7,8,10–12. However, the molecular basis for S. aureus host adaptation and the underlying genetic events involved are unclear.

RESULTS S. aureus clonal complex CC121 has a multi-host tropism

A common cause of human skin and soft-tissue infections such as staphylococcal scalded skin syndrome and life-threatening infections such as necrotizing pneumonia is the multi-locus sequence typing (MLST)-defined clone ST121 (ref. 13). Recently, the industrialization of rabbit farming in the developed world has coincided with the emergence of a highly virulent epidemic clone of S. aureus, also ST121, which is associated with skin abscesses and mastitis infections of rabbits in commercial rabbitries14. However, the basis for this association with multiple host species is unknown. We hypothesized that the association of ST121 strains with multiple hosts could be explained either by the existence of host-specific subtypes of ST121 or an innate capacity of ST121 strains to infect both human and rabbit hosts. To address this question, we carried out experimental intradermal infections of rabbits with representative ST121 isolates of rabbit (n = 3) and human (n = 3) clinical origin. The rabbit ST121 strains caused infections resulting in skin abscesses, which are characteristic of clinical symptoms seen in naturally occurring rabbit skin infections, at doses as low as 300 colony-forming units (CFU; Fig. 1a–c). In remarkable
contrast, human ST121 strains failed to cause clinical infections with overt symptoms, even at an inoculum of $10^6$ CFU (Fig. 1a,b). These data indicate that, in contrast to human ST121 isolates, rabbit ST121 strains have the capacity to cause disease in experimental infection of rabbits at very low inoculum levels.

**A human-to-rabbit *S. aureus* host jump occurred over 40 years ago**

Given the existence of a subset of ST121 strains with the capacity to cause clinical disease in rabbits, we investigated the evolutionary history of these strains. Accordingly, we compared the whole-genome sequences from a total of 23 different ST121 strains that broadly represent the breadth of spatial and temporal diversity in the ST121 lineage, including 14 newly sequenced *S. aureus* ST121 isolates obtained from humans ($n = 8$) and rabbits ($n = 6$) in 8 countries (from 3 continents) over a 50-year period (Supplementary Table 1), in addition to 11 diverse publicly available genome sequences for human ST121 strains. Of note, one of the rabbit strains (DL190) was sequenced to completion, resulting in a whole-genome sequence that could be used as a reference for comparison to all other ST121 sequences. We then applied a Bayesian coalescent method in the program Bayesian Evolutionary Analysis Sampling Trees (BEAST) employing a relaxed molecular clock model to reconstruct the phylogeny of the ST121 human and rabbit strains and estimate the rate of evolution of the ST121 clone.16 The resulting phylogenetic tree indicates a high level of diversity for human strains, represented by long branches in the ST121 phylogenetic tree, in contrast to a single clade comprising all rabbit strains that were isolated 20 years apart in four different countries (Fig. 2 and Supplementary Table 1). Using the calculated rate of molecular evolution for the ST121 sequences examined ($1.83 \times 10^{-6}$ substitutions per site per year; 95% highest posterior density (HPD) = $9.84 \times 10^{-7}$ to $2.26 \times 10^{-6}$), we estimated the date of the most recent common ancestor (MRCA) of the rabbit clade to be 1976 (1942–1990) and the MRCA of the rabbit clade with the most closely related human strain F to be 1909 (1804–1958). Taking into account the topology of the ST121 tree and the previously inferred human ancestry of the *S. aureus* species, the most likely explanation for the existence of the ST121 rabbit clade is a single human-to-rabbit host jump that occurred more than 38 years ago leading to the emergence of a new clone associated with epidemics at rabbit farms (Fig. 2).

**Diversification of the rabbit *S. aureus* ST121 core genome**

Considering previous studies that have suggested an important role for MGEs in adaptation to human, ruminant and avian hosts,7,8,10–12, we hypothesized that the ST121 clone had adapted to infect rabbits using a similar strategy. Comparative analysis of the accessory genomes of ST121 strains showed that the majority of human strains contained MGEs, which encode potent toxins involved in human disease pathogenesis such as Panton-Valentine leukocidin (PVL) and exfoliative toxins (ETs), and all except one contained a $\beta$-converting phage encoding the human-specific immune evasion cluster (IEC).17 None of the rabbit strains contained PVL- or ET-encoding MGEs, indicating their dispensability for *S. aureus* infection of rabbits (Fig. 2). Unexpectedly, the rabbit strains did not contain any MGEs that were unique to rabbit *S. aureus* (Fig. 2), implying that the host tropism of the rabbit ST121 clade is not mediated by a niche-specific accessory gene pool as previously reported for *S. aureus* clones associated with poultry and ruminants.5 Accordingly, we concluded that the capacity of ST121 to infect rabbits must have evolved through mutational events in the core genome of an ST121 progenitor of the rabbit clade. Core genome analysis identified mutations common to all rabbit strains and absent from human strains, including the creation of a total of 9 predicted pseudogenes resulting from nonsense mutations leading to premature stop codons or indels leading to frameshifts (Supplementary Table 2) and 254 nonsynonymous mutations in a variety of gene loci (Supplementary Table 3). Of the genes associated with loss-of-function mutations, four had previously been implicated in *S. aureus* virulence, including genes that encode urease, lipase and EsaB and the global gene regulator *rot* (encoding repressor of toxins).18–20 In addition, a single pseudogene caused by the insertion of a prophage into the hisB gene (encoding $\beta$-toxin) was identified in all human ST121 strains, but the gene was intact in the rabbit strains examined. Further, a SNP in the dltB gene encoding the $\delta$-alanine lipoteichoic acid and wall teichoic acid esterification protein resulted in the conversion of the stop codon into a tyrosine
residue and extended the length of the predicted protein by a single amino acid. In addition, there were two further nonsynonymous SNPs in the dltB coding sequence (Supplementary Fig. 1). Finally, an additional single 717-bp gene (splE) encoding a predicted serine protease was identified in each of the rabbit strains in the vSaβ genomic island (Supplementary Table 2).

A single nonsynonymous mutation is required for *S. aureus* rabbit infectivity

To distinguish host-adaptive mutational events among neutral or mildly deleterious mutations associated with genetic drift, we reversed mutations that were associated with encoded proteins of different size in all rabbit strains (resulting from frameshift, nonsense or length-extension mutations) in representative rabbit strains (strains I and J) and introduced them into human ST121 strain F by allelic replacement. In addition, we deleted the single gene *splE* found in the rabbit strains from strains I and J. We then compared the infectivity and severity of experimental infection for wild-type and derived mutant strains in a rabbit skin abscess infection model. For mutations in 10 of the 12 genes examined, reversion to the human ancestral gene state in the rabbit strains and the introduction of the mutations to human ST121 strain F had no effect on infectivity or severity (Supplementary Table 2). However, reversion of the three identified *dltB* SNPs (*dltB^P^*) in rabbit strains I and J resulted in complete loss of infectivity, even at an infectious dose of 10^6^ CFU (equivalent to a wild-type human strain; Fig. 3a). In addition, reversion of the nonsense mutation in the global virulence regulator *rot* (*rot*) significantly (*P < 0.05*) reduced the severity and infectivity of the rabbit strains (Fig. 3a).

To examine the role of specific *dltB* mutations in rabbit infectivity, each of the three *dltB* mutations, in addition to the *rot* nonsense mutation, was individually introduced into human ST121 strain F. Remarkably, introduction of the most 5′ nonsynonymous SNP at the *dltB* locus from the rabbit strains (encoding p.Thr113Lys substitution) into human ST121 strain F conferred the capacity to infect the rabbit host at an inoculum of 300 CFU (Fig. 3b), causing lesions that were highly similar to those caused by wild-type rabbit strain J (Fig. 3c,d). To confirm that the infective phenotype of ST121 strain F *dltB* Thr113Lys was not due to unrelated mutations that had occurred elsewhere in the genome during the synthesis of the mutant, we restored the *dltB* gene to its original, wild-type state in strain F and observed

the expected loss of infectivity (data not shown). In contrast, neither of the other two SNPs in the *dltB* gene (encoding p.Tyr250His and p.^*405*Tyr substitutions) nor the *rot* mutation (*rot^*) increased the infectivity of human clone F when introduced individually. Of note, introduction of the *dltB* mutation encoding p.Thr113Lys resulted in lesions in 40% of inoculated rabbits, although we recovered bacteria from only about 20%. However, introduction of all three *dltB* mutations resulted in infected lesions with 30% recovery, with an infectivity that was equivalent to that of the rabbit strain with a functional *rot* gene (human ancestral state) (Fig. 3b). These data suggest that additional *dltB* mutations promote the persistence of *S. aureus* ST121 in infected lesions. Notably, a combination of the *rot* nonsense mutation with the three *dltB* SNPs in the human F strain resulted in a strain whose infectivity was nearly indistinguishable from that of the wild-type rabbit strains (Fig. 3c), resulting in lesions with highly similar gross and microscopic histopathologies (data not shown). Finally, we also verified that rabbit strain J with a deleted *rot* gene demonstrated no further attenuation in infectivity (data not shown), suggesting that loss of *rot* gene function rather than allelic diversification of the *rot* gene is responsible for the attenuation. Taken together, the data indicate that a single naturally occurring nucleotide mutation of *dltB* was sufficient to confer the capacity of *S. aureus* ST121 to cause intra-dermal infections of rabbits and that two additional nonsynonymous mutations of *dltB* and loss of function of *rot* elevated the infectivity and bacterial recovery relative to wild-type *S. aureus* rabbit strains.

**dltB** mutation–related d-alanine levels do not correlate with rabbit infectivity

DltB is an integral membrane protein encoded by the *dltABCD* operon that is likely responsible for the translocation and incorporation of d-alanine into teichoic acids and lipoteichoic acids in *S. aureus*.[21] Although understanding of the function of teichoic acids is incomplete, the addition of d-alanine causes a positive charge that is essential for resistance to cationic peptides and for virulence.[21,22] In view of this, we speculated that the activity of the mutated form of DltB might be enhanced, leading to increased d-alanine substitution. Notably, we confirmed that the variant DltB protein from rabbit strain J was fully functional, as a *dltB* deletion mutant was incapable of infecting the rabbit at a dose of 10^6^ CFU. Although d-alanylation of wall teichoic acids, as well as resistance to cationic peptide–mediated
The percentage of rabbits that developed skin lesions from inoculated bacteria is also shown in b. Yates’ $\chi^2$ test was used to compute $P$ values for between-group comparisons: differences that are statistically significant are indicated by asterisks ($^{*}P < 0.05$) and all other comparisons did not show significant differences. WT, wild type. (c) Evolution of the skin lesions produced by rabbit strain J and by the human clone carrying the dltB Thr113Lys allele was indistinguishable. Rabbits were inoculated intradermally with 300 CFU of either the wild-type rabbit or mutant human clone (Online Methods). Images are shown for a representative rabbit. The earliest gross changes were observed 24–48 h after infection, consisting in a slight increase in size and erythema but evolving to form skin abscesses up to 2 cm in diameter (7 d after inoculation). Scale bars, 1.5 cm. (d) Transversal sections of skin lesions. Lesions produced by the rabbit and mutant human clones were characterized by dermal abscesses (DA) up to 2 cm in diameter. Bottom, hematoxylin and eosin staining of sections of skin inoculated with the rabbit or mutant human ST121 clone. Scale bars, 0.5 cm. Microscopically, we observed areas of purulent material surrounded by fibrosis that were infiltrated by inflammatory cells.

Figure 3 A single mutation is sufficient to confer rabbit infectivity to a human S. aureus strain. (a,b) Percentage of rabbits infected with the different rabbit (a) or human (b) ST121 mutants (day 7 after inoculation; $n = 20$ per strain in a, $n = 30$ per strain in b). Of note are data from the killing assays showing increased resistance of the F dltB clone to killing in comparison to the wild-type F clone (Supplementary Fig. 5). However, as the mutated rabbit clone J dltB that is uninfective for rabbits did not demonstrate reduced resistance to killing in comparison to the wild-type J strain and the survival of the J dltB clone in blood was greater than that observed with the F dltB' clone, we concluded that the ability to infect rabbits is not likely to depend on resistance observed in these killing assays. In conclusion, these data demonstrate that, although we detected phenotypic differences between the human and rabbit clones, they were not attributable to the dltB mutations (Supplementary Figs. 5 and 6). Of note are data from the killing assays showing increased resistance of the F dltB clone to killing in comparison to the wild-type F clone (Supplementary Fig. 5). However, as the mutated rabbit clone J dltB that is uninfective for rabbits did not demonstrate reduced resistance to killing in comparison to the wild-type J strain and the survival of the J dltB clone in blood was greater than that observed with the F dltB' clone, we concluded that the ability to infect rabbits is not likely to depend on resistance observed in these killing assays. In conclusion, these data demonstrate that, although we detected phenotypic differences between the human and rabbit clones, they are not dependent on the dltB mutations. This implies that the effect of the mutated DltB protein on rabbit infectivity is due to alternative functions of DltB during infection. In support of this hypothesis, it has recently been proposed that DltB is a member of a new superfamly of proteins, named MBOAT (for membrane-bound O-acyltransferases), that transfer organic acids, typically fatty acids, onto hydroxyl groups, that have a role in signaling.21 However, thus far, O-acyltransferase enzymatic activity has not been demonstrated experimentally for DltB, and the mechanism underlying the role of the dltB mutations in adaptation to rabbits is unclear.

Convergent evolution of S. aureus at the dltB locus

Finally, we hypothesized that, if a single mutation of dltB was essential to change the host tropism of ST121, then similar mutations would have occurred in other S. aureus strains infecting rabbits and perhaps
in other bacterial species. Phylogenetic analysis of rabbit S. aureus strains based on concatenated MLST sequences indicated that gain of a capacity to infect rabbits has occurred on numerous occasions, presumably through human-to-rabbit host-jump events (Fig. 4). To investigate the possibility that the adaptive mutation associated with the dltB locus in ST121 rabbit strains was a widespread S. aureus host-adaptive mechanism for rabbits, we sequenced the dltB gene of representative rabbit isolates from S. aureus ST1, ST8, ST9, ST45, ST96, ST133 and ST398, lineages that each include isolates of both human and rabbit clinical origin (Fig. 4 and Table 1), and compared them to dltB sequences from diverse human and animal isolates. dltB was highly conserved among the great majority of human, ruminant and poultry strains of S. aureus, with only 8 of 445 strains examined (1.8%) containing one or more nonsynonymous mutations (Supplementary Fig. 1). However, 39 of 39 (100%) rabbit strains examined from diverse clonal lineages contained one or more nonsynonymous SNPs in dltB, suggestive of convergent evolution (Fig. 4 and Table 1). Of note, the majority of the alterations were also predicted to be extracellularly exposed or proximal to the outer surface of the membrane (Supplementary Fig. 4). To further examine the convergent host-adaptive evolution of rabbit S. aureus clones, we tested the role in virulence of the distinct dltB allele associated with the S. aureus ST96 rabbit clone (encoding differences at two amino acid residues; Fig. 4). Introduction of the ST96 rabbit dltB allele into a rabbit ST121 strain conferred a level of infectivity similar to that observed with the ST121 rabbit dltB allele (10 of 15 rabbits infected with the ST121 allele versus 7 of 15 rabbits infected with the ST96 allele; $P = 0.46$, Yates’ $\chi^2$ test). Taken together, these data support the conclusion that distinct dltB mutations occurring in different S. aureus strain backgrounds underpinned independent host-jump events from humans to rabbits. In contrast to the allelic diversity of dltB identified among rabbit strains, all rabbit strains except ST121 contained an intact rot gene and the majority contained the $\beta$-converting phase, resulting in non-functional $\beta$-toxin, indicating that mutations affecting these determinants in ST121 are not essential for adaptation to the rabbit host. Taken together, the data are consistent with a critical role for mutations of dltB in S. aureus rabbit host adaptation.

Finally, we identified strains of other bacterial species that had nonsynonymous dltB mutations and predicted DltB proteins of extended length. These included a soil-adapted subtype of the plant bacterium Bacillus ankyloficus, which had several predicted nonsynonymous mutations and an additional histidine residue at the C terminus of DltB that were absent in the plant root-adapted strain (Supplementary Fig. 7a). In addition, analysis of publicly available Streptococcus pneumoniae genome sequences from a study examining resistance to vaccine and therapeutic pressures in humans identified additional predicted residues at the C terminus of the DltB protein in several unrelated strains (Supplementary Fig. 7b). Taken together, these data suggest a potential general role for allelic variants of dltB in adaptation to specific environmental or host-specific niches.

**DISCUSSION**

The molecular basis of the host adaptation of viral pathogens such as influenza and HIV has been intensively investigated. Several key residues such as influenza PB2-627 and HIV Gag-30 have been identified as sites of critical adaptive mutations. However, adaptation to mammalian hosts by both influenza and HIV appears to involve mutations at multiple genes. For bacterial host adaptation, horizontal acquisition of a single gene regulator in the bacterial squid symbiont Vibrio fischeri was demonstrated to be an essential step in adaptation to its host. In addition, previous seminal work demonstrated that, in a mouse model of Listeria monocytogenes infection, a single amino acid change in the mouse E-cadherin receptor increased L. monocytogenes cell internalization in vitro and, conversely, two amino acid changes in the listerial invasion protein InIA increased in vivo infectivity by enhancing bacterial affinity for mouse E-cadherin. However, very high inoculum doses were still required for infectivity, suggesting that for L. monocytogenes infections to occur naturally additional adaptations would be required to facilitate a host shift.

Thus far, the fundamental biological question of how animal-bacteria partnerships are established has been difficult to dissect via established animal models of infection. In the current study, the fact that the bacterial host jump being examined involves rabbits, an experimentally tractable host species, facilitated for the first time, to our knowledge, a dissection of the evolutionary genetic trajectory of a naturally occurring switch in host species by a bacterial pathogen. Using this approach, we traced the evolutionary genetic events that led to the emergence of a major animal clone of S. aureus responsible for disease epidemics in farmed rabbits on a global scale. In contrast to all characterized human and animal clones of S. aureus, adaptation to the rabbit host did not involve acquisition of MGEs from a host-specific accessory gene pool. Remarkably, we report that a single mutation, which occurred naturally, was sufficient to allow a radical change in bacterial host tropism. The mutation in the dltB gene was sufficient and required to convert a human S. aureus strain, which was incapable of causing infections in rabbits, into one that had the capacity to cause epidemics in farmed rabbit populations. Two additional mutations in
the same locus enhanced infectivity and bacterial proliferation within rabbit skin lesions. In spite of apparently numerous host-jump events into rabbits by other \textit{S. aureus} clones, ST121 is by far the dominant clone in the countries sampled. As such, we suggest that, in addition to the initial adaptations required for survival in the new host species, other mutations likely contribute to the transmissibility and successful spread of the infectious clone.

In summary, our results reporting a single naturally occurring mutation associated with a bacterial host-switching event represent a paradigm shift in the understanding of the minimal adaptations required for a bacterium to overcome species barriers and establish itself in new host populations. This discovery has important public and veterinary health implications. It should lead to a closer examination of future threats posed by bacterial pathogen host-switching events.

METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. The sequences reported in this paper have been deposited in the GenBank database (accessions ERS400826–ERS400839). See Supplementary Table I for details.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Bacterial strains and growth conditions. The bacterial strains used are listed in Supplementary Tables 1 and 4. *S. aureus* strains were grown at 37 °C on TSA agar medium, supplemented with antibiotics as appropriate. Broth cultures were grown at 37 °C in TSB broth with shaking (240 rpm). LB agar and LB medium were used for *Escherichia coli* strains, supplemented with antibiotics as appropriate. Procedures for transduction and transformation in *S. aureus* were performed as described previously.12,32

DNA methods. General DNA manipulations were performed by standard procedures. To introduce specific mutations into *S. aureus* strains, we used plasmid pMAD33 for allelic exchange as previously described. Plasmid constructs (listed in Supplementary Table 5) were prepared by cloning PCR products obtained with the oligonucleotide primers listed in Supplementary Table 6, and all clones were sequenced by the Institute Core Sequencing Lab.

Whole-genome sequencing and alignment. Isolates were sequenced using a combination of single- and paired-end technologies on the Roche 454 platform and assembled using the Roche GS De Novo Assembler v2.6 or, alternatively, using the Illumina HiSeq platform with reads assembled using Velvet v1.2.08. Contigs were aligned using the progressive Mauve algorithm and filtered for locally collinear blocks over 1,000 nucleotides in length, and gap positions were removed. The alignment was assessed for recombination using BRATNextGen39, with predicted recombinant regions excluded from further phylogenetic analyses. Genes specific to rabbit ST121 isolates were identified by comparing the genome sequences for the rabbit isolates to the human ST121 sequences using the print Novel_contigs function of cortex_var v1.0.5.20 (ref. 36).

Bayesian evolutionary analysis. On the basis of core genome Mauve alignment (at 6,208 variable sites), the phylogeny of ST121 was reconstructed using BEAST v1.6.1 (ref. 16) under the general time-reversible (GTR) model of nucleotide substitution with a gamma correction for rate heterogeneity and a skyline coalescent tree prior. Simultaneously, we used an uncorrelated log-normal distribution to model the rate of evolution and constrained the tips of the phylogeny to their dates of isolation to calibrate the rate.37 Posterior probabilities were estimated from 3 independent Markov chain Monte Carlo (MCMC) samples from 3 independent analyses each run for 1 × 10^8 iterations, with sampling at every 1,000 generations and 10% discarded as burn-in.

Identification of pseudogenes and nonnonsynonymous mutations. Single-end 454 sequence reads were aligned to the ED133 genome (NCBI, NC_017337.1), and an annotated draft version of the DL190 genome was generated using the BWA long-read aligner.38 Point mutation and insertion or deletion variants were called at sites with average mapping and base quality scores greater than 30. Mutations predicted to result in pseudogenes were further inspected manually using the Integrative Genomics Viewer. Variation in gene content between strains was examined using the GeneFamily method of the Pangene Analysis Pipeline with default parameters. Presence or absence of virulence factors was confirmed through manual inspection of the output of BLAST searches.

Rabbit skin infection model. Two-month-old albino hybrid rabbits of either sex were used for the skin infection model. Rabbits were sedated with a combination of ketamine and xylazine and were shaved on their back and inoculated by intradermal injection (usually with 300 CFU of *S. aureus* in 100 μl of PBS). Each rabbit was inoculated in duplicate with 2 strains (generally wild type and mutant), and each strain was tested in groups of at least 20 rabbits. After 7 d, rabbits were euthanized by intravenous injection of barbiturate (Dolethal; Vélouqinol). In each experiment, an additional group of rabbits was inoculated with vehicle (PBS) and served as a negative control. In addition, to exclude the possibility of contamination, bacteria recovered at the end of the experimental period were analyzed by sequencing of strain-specific gene alleles. The experimental protocol was approved by the ethical committee of the Universidad CEU Cardenal Herrera and by the Conselleria d’Agricultura, Pesca i Alimentació, Generalitat Valenciana (permit number 2011/010). Sample sizes were not predetermined.

Macroscopic and histological examination of lesion tissues. Rabbits were examined daily, and development of infection was followed visually and by palpation of lesion tissue. Lesion length and width were measured to calculate the area of the lesions. In addition to measuring lesion size, we also examined skin to evaluate the presence or absence of epidermal necrosis. After postmortem examination, skin abscesses were routinely processed for histological examination and stained with hematoxylin and eosin and with Gram’s method. Before tissue fixation, swab samples were taken from the lesion and cultivated on blood agar (BioMérieux) to confirm the presence of *S. aureus*.

Cell wall and β-alanine analyses. Cell walls were isolated, wall teichoic acids and lipoteichoic acids were purified, and β-alanine content was measured as previously described.31,41. Peptidoglycan composition and structure in the different strains were analyzed by Cecolabs, as described previously.

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