LETTERS

Self versus non-self discrimination during CRISPR RNA-directed immunity

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All immune systems must distinguish self from non-self to repel invaders without inducing autoimmunity. Clustered, regularly interspaced, short palindromic repeat (CRISPR) loci protect bacteria and archaea from invasion by phage and plasmid DNA through a genetic interference pathway[1–9]. CRISPR loci are present in ~40% and ~90% of sequenced bacterial and archaeal genomes, respectively[10], and evolve rapidly, acquiring new spacer sequences to adapt to highly dynamic viral populations[1,11–13]. Immunity requires a sequence match between the invasive DNA and the spacers that lie between CRISPR repeats[1–9]. Each cluster is genetically linked to a subset of the *cas* (CRISPR-associated) genes[14–16] that collectively encode >40 families of proteins involved in adaptation and interference. CRISPR loci encode small CRISPR RNAs (crRNAs) that contain a full spacer flanked by partial repeat sequences[2,17–19]. CrRNA spacers are thought to identify targets by direct Watson–Crick pairing with invasive 'protospacer' DNA[2,3], but how they avoid targeting the spacer DNA within the encoding CRISPR locus itself is unknown. Here we have defined the mechanism of CRISPR self/non-self discrimination. In *Staphylococcus epidermidis*, target/crRNA mismatches at specific positions outside of the spacer sequence license foreign DNA for interference, whereas extended pairing between crRNA and CRISPR DNA repeats prevents autoimmunity. Hence, this CRISPR system uses the base-pairing potential of crRNAs not only to specify a target, but also to spare the bacterial chromosome from interference. Differential complementarity outside of the spacer sequence is a built-in feature of all CRISPR systems, indicating that this mechanism is a broadly applicable solution to the self/non-self dilemma that confronts all immune pathways.

S. epidermidis strain RP62a (ref. 20) contains a CRISPR locus that includes a spacer (*spc1*) that is identical to a region of the *nickase* (*nes*) gene found in nearly all sequenced staphylococcal conjugative plasmids (Fig. 1a and Supplementary Fig. 1a), including those that confer antibiotic resistance in methicillin- and vancomycin-resistant *Staphylococcus aureus* strains[21–23]. The *S. epidermidis* CRISPR system limits conjugation between staphylococci by an *spc1*-directed interference pathway[3]. As in other species[2,7,17–19,24,25] the *S. epidermidis* CRISPR locus is transcribed and processed into crRNAs[3], and northern analysis indicates that most *spc1*-containing crRNAs are ~49 nucleotides long (Supplementary Fig. 1b). During CRISPR interference in *S. epidermidis*, target specificity seems to be achieved by direct pairing of the *spc1* crRNA with the plasmid DNA[3]. DNA targeting most likely underlies CRISPR interference in many other bacterial and archaeal species as well[2,26]. Because a sequence match also exists between the crRNA and the CRISPR locus DNA that encodes it, a central issue in CRISPR interference is therefore how the crRNA identifies bona fide targets without attacking the CRISPR locus in the host chromosome.

We demonstrated previously that CRISPR interference can prevent transformation of pC194-based plasmids that contain a target sequence[3]. To test whether CRISPR spacers have an intrinsic ability to evade interference, we cloned the repeat/spacer sequences of the *S. epidermidis* CRISPR locus, along with ~200 base pairs (bp) from either side of the repeats and spacers, into pC194 (Fig. 1a). The resulting plasmid, pCRISPR(wt), which contains three possible interference targets (*spc1*, *spc2* and *spc3*), was transformed into wild-type and Δ*crispr* cells. Unlike the *nes* protospacer-containing plasmid, pCRISPR(wt) transformation efficiency was similar in both strains (Fig. 1a; Supplementary Table 1 shows the transformation efficiency values for all plasmids described here). These results indicate that the potential targets present in the CRISPR locus are specifically exempted from CRISPR interference.

We proposed that differences between flanking regions of spacers and targets — that is, the presence or absence of repeats — could provide the basis for self/non-self discrimination. To test this, we replaced 15 bp from either side of the *nes* target with the corresponding *spc1*-flanking repeat sequences. The resulting plasmids (pNes(5′DR,15) and pNes(3′DR,15)) were tested for CRISPR interference by transformation into wild-type and Δ*crispr* cells (Fig. 1b and Supplementary Fig. 2a, b). Only pNes(5′DR,15) escaped interference, indicating that repeat sequences upstream of a target (that is, adjacent to the 5′ end of the crRNA spacer sequence) can protect that target. Similar experiments narrowed the protective region further to the eight base pairs closest to the target (Fig. 1b and Supplementary Fig. 2b). This region contains five mutations in the *nes* upstream sequence, because the 5′-AGA-3′ sequence from position −5 (that is, 5 bp upstream of the start of the *nes* protospacer) to −3 are shared with *spc1* 5′ flank (Supplementary Fig. 2a). Each of these five mutations was individually tested for its effect on CRISPR interference (Fig. 1c). Only the guanosine-to-adenosine change at position −2 (G-2A) conferred protection. These results demonstrate that repeat sequences upstream of spacers prevent CRISPR interference, and point to position −2 as an important determinant of this effect.

Short (2–4 bp), conserved sequences called 'CRISPR motifs'[12,24,25] or 'protospacer adjacent motifs' (PAMs)[27] have been found near protospacers in other CRISPR systems, and mutations in these motifs can compromise interference[12,25]. A CRISPR motif has not been defined in *S. epidermidis* protospacers, of which only two have been specified[3]. To test whether G-2 is part of a conserved motif important for interference, we tested plasmids carrying the mutations G-2C and G-2T. Surprisingly, unlike the G-2A mutation, C and T transversions had no effect on transformation efficiency (Fig. 1c). This result was corroborated in a conjugation assay[3] using pG0400 G-2A or G-2C mutants (Supplementary Fig. 3) and excludes the possibility that a G at position −2 is simply a crucial CRISPR motif residue. Instead, this observation indicates that only an A at position −2, that is, the nucleotide present in the repeats, allows protection, and that any deviation from this nucleotide enables interference.

In light of this observation, we considered the complementarity of crRNAs with target protospacers and with CRISPR sequences:

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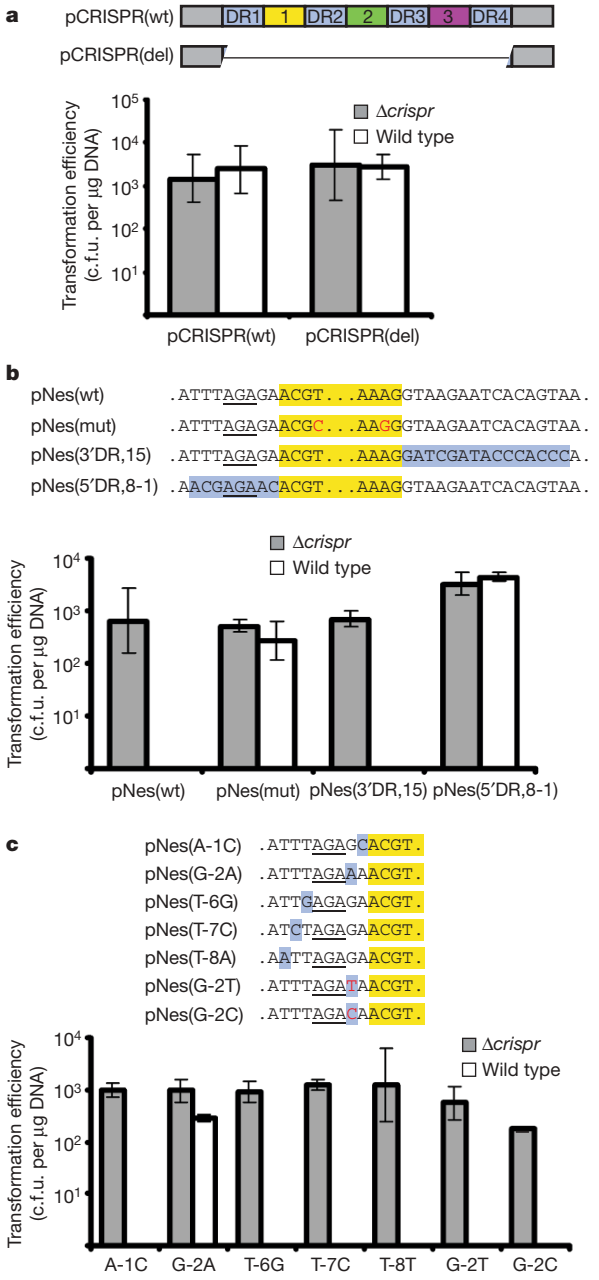

Figure 1 | Protection of *nes* target by spacer flanking sequences. **a**, Direct repeats (DR1–4, purple boxes) and spacers (1–3, coloured boxes) of the *S. epidermidis* RP62a CRISPR locus were cloned into pC194 generating pCRISPR(wt) and its deletion variant, pCRISPR(del). **b**, pNes(wt) and pNes(mut) contain wild-type and mutated *nes* target sequence of pG0400 (highlighted in yellow, with mutations in red). In pNes(5′DR,8-1) and pNes(3′DR,15) *nes* target flanks were replaced by repeat sequences present upstream and downstream of *spc1* (highlighted in purple), respectively. **c**, Individual nucleotides upstream of *nes* target were replaced by those present upstream of *spc1* (highlighted in purple). The G at position −2 was also changed to C and T (in red). The AGA sequence (underlined) is shared by both the *nes* target and spacer 5′ flanking sequences. All plasmids were transformed into *S. epidermidis* RP62a and its isogenic Δ*crispr* mutant. The average of at least three independent measures of the transformation efficiency (determined as colony forming units (c.f.u.) per µg DNA) is reported and error bars indicate 1 s.d.

although the spacer region of a crRNA can pair with target DNA (Fig. 2a) and CRISPR DNA alike, only the CRISPR DNA will be fully complementary with the CRISPR repeat sequences at the crRNA termini. We therefore proposed that specific base pairs in the crRNA/DNA heteroduplex outside of the spacer enable protection, thereby providing a mechanism to avoid autoimmunity. To explore

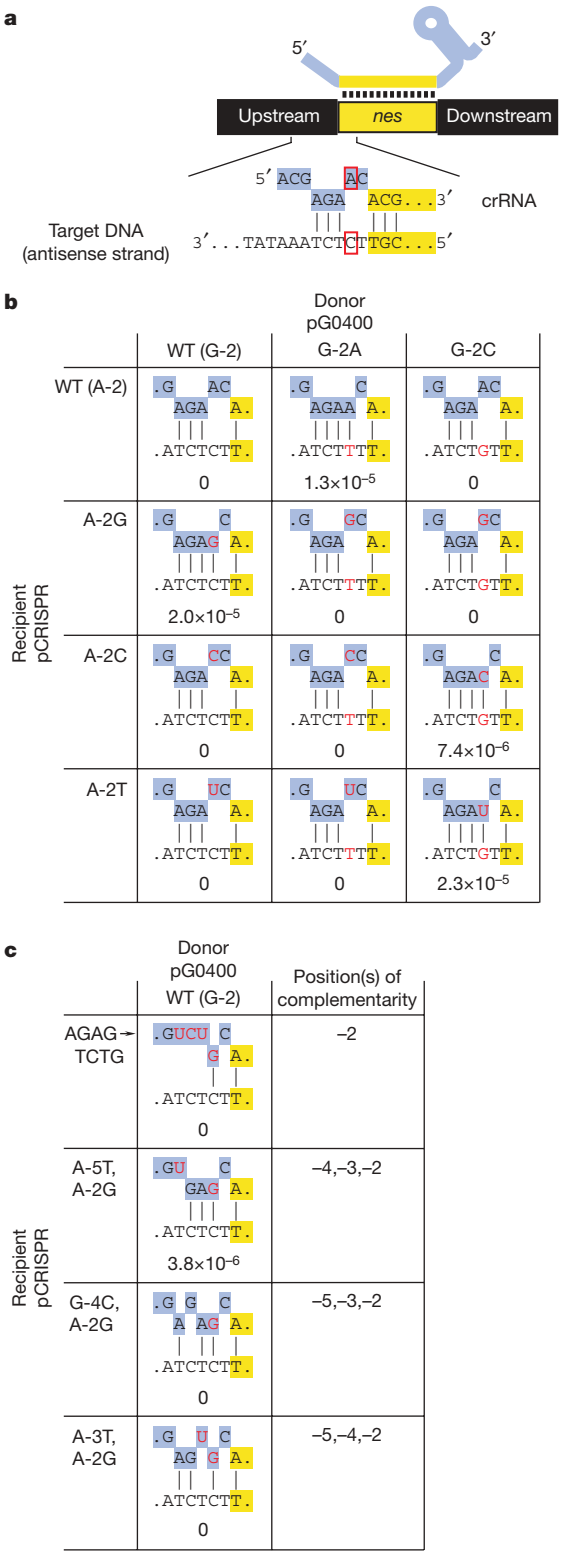

Figure 2 | Complementarity between crRNA and target DNA flanking sequences is required for protection. **a**, Schematic of the complementarity between the flanking sequences of crRNA (top, highlighted in purple) and target DNA (bottom). The red boxes indicate the nucleotides mutated in the experiments shown in **b**. **b**, **c**, Conjugation assays of pG0400 and its mutant variants, using as a recipient the Δ*crispr* strain harbouring different pCRISPR plasmids. Mutations are shown in red. Conjugation efficiency was determined as transconjugant c.f.u. per recipient c.f.u.; the average of at least three independent experiments is reported. WT, Wild type.

this possibility we introduced compensatory mutations in the crRNA (Fig. 2a, b) by changing sequences upstream of *spc1* in pCRISPR(wt), which can complement the interference deficiency of the Δ*crispr* strain. The wild-type *spc1* crRNA contains an adenosine at position −2, so we generated the A-2G, A-2C and A-2T mutants. We then tested each for interference with conjugation of wild-type pG0400 as well as its G-2A and G-2C mutant derivatives. In agreement with our proposal, the *nes* target was protected from interference only when pairing was possible at position −2: rA-dT, rG-dC and rC-dG Watson–Crick appositions each resulted in evasion of the CRISPR system by the conjugative plasmid (Fig. 2b). All crRNA mutants were functional and therefore correctly processed[2], because all pCRISPR plasmids were able to restore interference in Δ*crispr* cells with pG0400 derivatives that were mismatched at position −2 (Fig. 2b). Interestingly, a rU-dG wobble apposition also protected the conjugative plasmid from interference whereas a rG-dT wobble apposition did not, despite the greater stability of rG-dT pairs in an otherwise Watson–Crick-paired heteroduplex[28]. These results provide strong evidence that crRNA/target non-complementarity at position −2 is important for interference. Further mutagenesis of the −5 to −2 sequence of *spc1* crRNA indicated that the minimal complementarity required for protection involves positions −4, −3 and −2 (Fig. 2c and Supplementary Fig. 4). Altogether these results indicate that protection of the *nes* target during conjugation in *S. epidermidis* requires complementarity between crRNA and target upstream flanking sequences at these positions, and strongly indicate that the mechanism of protection requires base-pair formation in this region (Fig. 4a). Furthermore, the base pairing implied by our compensatory analyses reinforces our earlier conclusion[3] that the target of the *S. epidermidis spc1* crRNA is the antisense strand of the *nes* DNA locus, not the sense-oriented mRNA.

If base pairs at positions −4, −3 and −2 confer protection on an otherwise susceptible target, then abolition of base pairing in the same region should confer susceptibility on an otherwise protected CRISPR locus. Deletion analyses in pCRISPR (Fig. 3a and Supplementary Fig. 5a) demonstrated that sequences immediately upstream of spacers prevent autoimmunity. We then tested the effect of substitutions on the direct repeat upstream of *spc1* (*DR1*) on CRISPR protection (Fig. 3b, c). To avoid the expression of mutant crRNAs that would complicate our analyses, we generated pDR1(wt), a deletion variant of pCRISPR(wt) that lacks the 200 bp preceding the CRISPR locus. This plasmid is unable to produce functional crRNAs (data not shown), but is protected from interference in wild-type cells (Fig. 3b, c). In contrast, plasmid pDR1(AGAA→TCTG), which contains mutations at positions −5 to −2, was subject to CRISPR interference. This indicates that this region is critical for protection of the CRISPR locus. Mutagenesis of this and other regions of *spc1* upstream flanking sequences (Fig. 3b, c and Supplementary Fig. 5b) indicated that at least two consecutive mismatches from positions −4 to −2 are required to eliminate protection of the CRISPR locus (Fig. 4b).

CRISPR systems show a high degree of diversity in *cas* gene content[4,9,15,16], and therefore mechanistic differences between different CRISPR/*cas* subtypes are likely to exist. However, differential crRNA pairing potential with CRISPR loci and invasive targets outside of the spacer region (Fig. 4c) is intrinsic to all CRISPR systems, and therefore the mechanism of self/non-self discrimination that we have defined in *S. epidermidis* could apply broadly. The specific base pairs that are monitored could vary between CRISPR/*cas* systems, and we speculate that protein components of the system may 'proofread' paired versus unpaired structures at these sites in a manner that either aborts or enables later steps in interference, respectively. Our findings also highlight the importance of the previously noted 5′-terminal homogeneity of crRNAs[2,3,17], which consistently contain ~eight nucleotides of upstream repeat sequences. Finally, our results are inconsistent with a critical role for the CRISPR motif[12,25,27] during the interference phase

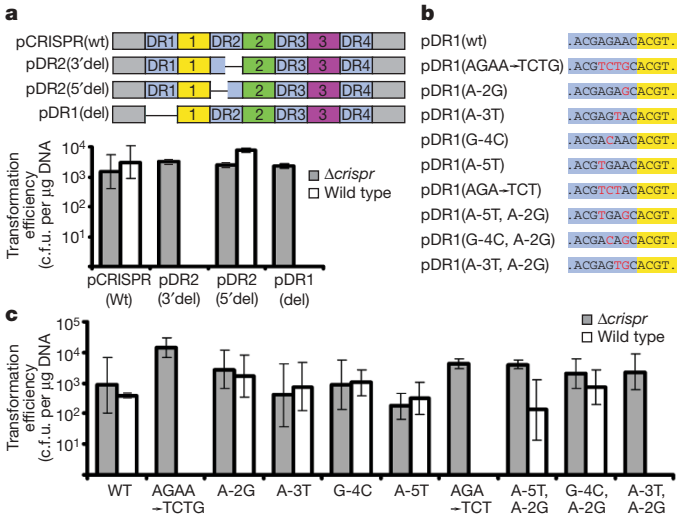

Figure 3 | Mutations in upstream flanking sequences of CRISPR spacers elicit autoimmunity. a, Deletions were performed in the flanking repeats of *spc1*: the 3′ half of DR2, the 5′ half of DR2, and all of DR1 were deleted from pCRISPR(wt) in pDR2(3′del), pDR2(5′del) and pDR1(del), respectively. **b**, **c**, Substitutions (red) were introduced in the 5′ flanking sequence (highlighted in purple) of *spc1* (highlighted in yellow), generating different pDR1 variants that were tested by transformation. All plasmids were transformed into *S. epidermidis* RP62a and Δ*crispr* strains. The average of at least three independent measures of the transformation efficiency (determined as c.f.u. per μg DNA) is reported and error bars indicate 1 s.d.

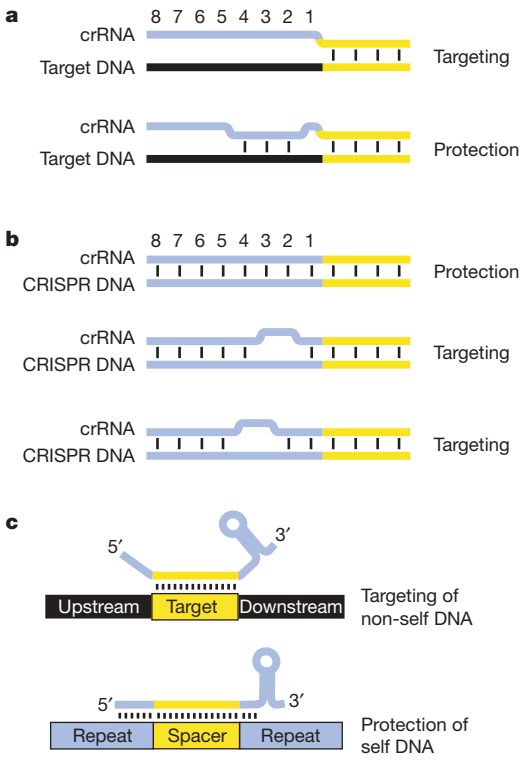

Figure 4 | Requirements for targeting and protection during CRISPR immunity. a, In *S. epidermidis*, CRISPR interference is enabled by mismatches between target DNA and crRNA sequences upstream of the spacer. Formation of at least three base pairs at positions −4, −3 and −2 eliminates targeting. **b**, Complementarity between the *S. epidermidis* CRISPR locus and the crRNA 5′ terminus protects it from interference. Disruption of base pairing at positions −4, −3 or −3, −2 eliminates protection. **c**, General model for the prevention of autoimmunity in CRISPR systems. The ability of crRNA termini (5′, 3′, or both) to base pair with potential targets enables discrimination between self and non-self DNA during CRISPR immunity.

of CRISPR immunity. No significant similarity exists between the two known targets of the *S. epidermidis* CRISPR locus (Supplementary Fig. 5c) and therefore we cannot determine if the nucleotides that are important for discrimination are also part of a CRISPR motif. However, our mutagenesis experiments indicate that no specific flanking nucleotides are required for interference: the decisive characteristic is non-complementarity with the crRNA rather than nucleotide identity *per se*. By extension, it is conceivable that CRISPR motif mutants previously shown to evade interference[12,25] could reflect a failure of self/non-self discrimination via crRNA/target base pairing, rather than a requirement for specific nucleotide identity within the CRISPR motif. We therefore suspect that CRISPR motifs are more important during the acquisition of new spacers[27]. In summary, our results reveal the mechanism of self/non-self discrimination in this recently discovered immune system and will facilitate efforts to exploit CRISPR interference for biotechnological applications.

METHODS SUMMARY

Bacterial strains and growth conditions. *S. epidermidis* wild-type (RP62a, ref. 20) and Δ*crispr* (LAM104, ref. 3) and *S. aureus* (RN4220, ref. 29) strains were grown in brain-heart infusion (BHI) and tryptic soy broth (TSB) media, respectively. When required, the medium was supplemented with antibiotics as follows: neomycin ($15\,\mu g\,ml^{-1}$) for selection of *S. epidermidis*; chloramphenicol ($10\,\mu g\,ml^{-1}$) for selection of pC194-based plasmids; and mupirocin ($5\,\mu g\,ml^{-1}$) for selection of pG0400-based plasmids. *Escherichia coli* DH5α cells were grown in LB medium, supplemented with ampicillin ($100\,\mu g\,ml^{-1}$) or kanamycin ($50\,\mu g\,ml^{-1}$) when necessary.

Conjugation and transformation. Conjugation and transformation were performed as described previously[3] with the following modification: transformations of *S. epidermidis* were recovered at 30 °C in 150 μl of BHI for 6 h. Corroboration of the presence of the desired plasmid in transconjugants or transformants was achieved by extracting DNA of at least two colonies or agar of empty plates, performing PCR with suitable primers and sequencing the resulting PCR product.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions L.A.M. designed experiments with input from E.J.S.; L.A.M. conducted experiments. L.A.M. and E.J.S. analysed data, interpreted experiments and wrote the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to L.A.M. (l-marraffini@northwestern.edu) and E.J.S. (erik@northwestern.edu).

METHODS

DNA cloning. All plasmids used in this study were constructed by cloning CRISPR or *nes* sequences into the HindIII site of pC194 (ref. 30). Inserts were generated by PCR using primers and templates described in Supplementary Table 2. Supplementary Table 3 contains primer sequences. To introduce mutations, internal primers carrying the desired substitution were used with external primers, thus generating two PCR products that were then joined by nested PCR using the external primers. Amplified DNA was cloned into pCR2.1 vector (Invitrogen), the resulting plasmid purified and the insert sequenced. Inserts were cut from pCR2.1 with HindIII, purified and ligated into pC194. Ligation products were transformed into *S. aureus* OS2 cells[31] and colonies containing the desired insert were identified by PCR using primers P86/P87.

pG0400 mutagenesis. Mutations were introduced by allelic exchange as described previously[3]. P15 and P18 primers were used with reverse and forward primers containing the desired mutation, respectively, to amplify 1 kb of *nes* sequence. PCR products were then joined by nested PCR using P15/P18 primers and recombined into pKOR1 (ref. 32). Recombination products were transformed into *E. coli* DH5α, and the resulting plasmids were purified, sequenced and transformed into *S. aureus* RN4220 (pG0400) (ref. 33) for allelic exchange. pG0400(G-2A), pG0400(G-2C) and pG0400(G-2T) mutants were generated using the knockout constructs pLM397, pLM406 and pLM405, respectively. Inserts contained in these plasmids were generated by joining the PCR products obtained with the following primer pairs: pLM397, P15/P190 and P191/P18; pLM406, P15/P208 and P209/P18; pLM405, P15/P206 and P207/P18. See Supplementary Table 3 for primer sequences.

Northern blot analysis. RNA extraction of *S. epidermidis* and oligonucleotide labelling was performed as indicated in ref. 3. Northern blot analysis was carried out according to ref. 34 using ultraviolet crosslinking and a hybridization temperature of 37 °C.

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