RNase III-CLASH of multi-drug resistant Staphylococcus aureus reveals a regulatory mRNA 3′ UTR required for intermediate vancomycin resistance

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Treatment of methicillin-resistant Staphylococcus aureus infections is dependent on the efficacy of last-line antibiotics including vancomycin. Treatment failure is commonly linked to isolates with intermediate vancomycin resistance (termed VISA). These isolates have accumulated point mutations that collectively reduce vancomycin sensitivity, often by thickening the cell wall. Changes in regulatory small RNA expression have been correlated with antibiotic stress in VISA isolates however the functions of most RNA regulators is unknown. Here we capture RNA–RNA interactions associated with RNase III using CLASH. RNase III-CLASH uncovers hundreds of novel RNA–RNA interactions in vivo allowing functional characterisation of many sRNAs for the first time. Surprisingly, many mRNA–mRNA interactions are recovered and we find that an mRNA encoding a long 3′ untranslated region (UTR) (termed vigR 3′ UTR) functions as a regulatory ‘hub’ within the RNA–RNA interaction network. We demonstrate that the vigR 3′ UTR promotes expression of folD and the cell wall lytic transglycosylase isaA through direct mRNA–mRNA base-pairing. Deletion of the vigR 3′ UTR re-sensitised VISA to glycopeptide treatment and both isaA and vigR 3′ UTR deletions impact cell wall thickness. Our results demonstrate the utility of RNase III-CLASH and indicate that S. aureus uses mRNA–mRNA interactions to co-ordinate gene expression more widely than previously appreciated.
Staphylococcus aureus is a highly adaptable opportunistic pathogen capable of causing a wide range of infections. S. aureus is a leading cause of osteomyelitis, infective endocarditis, and bacteremia. Methicillin-resistant S. aureus (MRSA) has become common in both community and healthcare settings with global prevalence ranging from 13 to 74% of S. aureus infections.[4]-[2]. Mortality rates are high among patients that develop MRSA bacteremia[2] and treatment options are often limited to last-line antibiotics[1]. The treatment of choice for patients with MRSA bacteremia is the glycopeptide vancomycin[1,3]. Vancomycin resistant S. aureus (VRSA, MIC > 8 μg/mL) is relatively rare and vancomycin treatment failure is most commonly associated with S. aureus strains that have vancomycin-intermediate resistance, termed VISA (MIC 4–8 μg/mL).[3] The genetic determinants that lead to VISA are heterogeneous and incompletely defined, although it is clear that these strains do not acquire additional genetic elements. Genome sequencing has identified single nucleotide polymorphisms (SNPs) that arise during repeated vancomycin exposure and commonly lead to cell wall thickening, reduced autolysis, and reduced acetate catabolism.[1,2]. Cell wall thickening is thought to reduce diffusion of vancomycin through the division septum however the underlying mechanism of cell wall thickening remains unclear.

In this study, we have used the MRSA isolate JKD6009 which was isolated from a patient in New Zealand with bacteremia. Over 42 days of treatment with vancomycin, a second isolate from the patient presented a VISA phenotype (designated strain JKD6008)[4]. These two isolates belong to ST239, a sequence-type representing a global multi-drug resistant, persister cell development[8].

Regulatory small RNA (sRNA) are approximately 50-500 nucleotide gene regulators that form base-paired interactions with mRNAs and inhibit or activate mRNA translation, transcription, and stability through a range of mechanisms. Using a collection of VSSA and VISA isolates, Howden et al. demonstrated that sRNA expression is correlated with exposure to last-line antibiotics, suggesting that sRNAs may constitute an acute antibiotic response[5]. More recently, Dejoies et al. identified a coordinated expression profile of specific sRNAs under acute biocide stress[6]. The transcriptome of S. aureus contains hundreds of sRNAs, most of which have unknown biological functions. The sRNA SprX represses the transcriptional regulator SpoVG and alters intermediate glycopeptide tolerance[7]. More recently, the non-coding antisense RNA, SprF1, was shown to bind ribosomes and reduce protein synthesis to favour ciprofloxacin and vancomycin persistor cell development[8].

In S. aureus, the double-stranded RNA-specific endonuclease RNase III plays a major role in RNA maturation and mRNA processing. RNase III typically binds 22-nt RNA duplexes[9] with a preference for at least one GC or CG base pair at cleavage sites[10]. RNA duplexes can be formed in cis or trans and many sRNA-mRNA pairs in S. aureus are processed by RNase III. Interestingly, RNase III also recognises a co-axially stacked interaction between RNAIII and coa mRNA that pairs through a short kissing loop interaction[11,12]. This interaction mimics an extended RNA duplex and is processed by RNase III indicating that shorter regions of complementarity may also be RNA III substrates.

In this work, we have adapted our proximity-dependant RNA ligation technique (RNase-CLASH) for profiling sRNA-mRNA interactions[13] to MRSA and captured RNA–RNA interactions associated with RNase III. RNase III-CLASH identifies RNase III binding sites enriched within the untranscribed regions (UTRs) of mRNAs and recovers many mRNAs that act as regulatory ‘hubs’. We reveal the in vivo targets for many uncharacterised sRNAs providing insight into their function. We also utilise differential RNA-seq (dRNA-seq) and Term-seq to accurately map the 5' and 3' RNA boundary ends and identified many sRNAs not previously identified in S. aureus. We use this RNA interaction network to identify regulatory RNAs that may contribute to intermediate vancomycin resistance and find that a previously unknown regulatory mRNA, here termed vigR, is required for vancomycin-intermediate tolerance. The vigR mRNA encodes an unusually long 3'UTR that acts in trans to positively regulate isaA and fold mRNA through direct base-pairing interactions. IsaA is a lytic transglycosylase involved in cell wall peptidoglycan turnover and expansion, and we demonstrate that it also plays a role in intermediate glycopeptide tolerance. Deletion and CRISPRi knockdown of isaA significantly reduces cell wall thickness of the VISA isolate JKD6008, while the vigR 3'UTR deletion has a modest reduction in cell wall thickness. Our study has uncovered a previously unknown but important mechanism of intermediate vancomycin resistance through a regulatory mRNA 3'UTR that promotes cell wall thickening in a clinical VISA isolate.

Results
Transcriptome architecture of methicillin-resistant Staphylococcus aureus JKD6009. The 5' and 3'UTRs of mRNAs are often sites of regulatory RNA interactions, but they are poorly predicted in silico. To facilitate accurate mapping of RNA–RNA interactions to genomic features and identify novel regulatory RNAs in S. aureus JKD6009 we utilised dRNA-seq[14] and Term-seq[15] to map RNA 5' and 3' boundaries, respectively. Total RNA was extracted from cells grown in liquid Mueller–Hinton (MH) to an OD₆₀₀ ~0.6 and sequenced using the dRNA-seq and Term-seq protocols (Supplementary Methods). Analysis of the dRNA-seq data using the ANNOgesic workflow[16] identified 1399 transcription start sites (TSS) and 17 RNA processing sites (Fig. 1a). Primary, secondary, antisense, and internal promoters were also identified (Fig. 1a) and are detailed in Supplementary Data 1. Motif analysis of our TSS identified a canonical -10 element (Prbnow box) (Supplementary Fig. 1A) and our primary 5' ends are in agreement with previously published dRNA-seq data from the MRSA isolate USA300[17], and TSS-EMOTE analysis of isolate MW2[18] supporting the veracity of our data (Supplementary Fig. 1B). The 5'UTRs of mRNAs had a median length of 41-nts, with 281 5'UTRs (24.3%) longer than 100 nts (Supplementary Fig. 1C).

We next used Term-seq[15] to identify 3' RNA ends. Our Term-seq analysis identified 2385 statistically significant RNA 3' ends, including 798 that overlapped a predicted Rho-independent transcription terminator. A stem-loop RNA structure was identified within our RNA 3' ends consistent with the presence of intrinsic terminators and stabilising stems at many of these sites[19] (Fig. 1b). Our RNA 3' ends defined 1031 mRNA 3'UTRs that had a median length of 75-nts, and 346 3'UTRs (33.5%) that were longer than 100-nts (Supplementary Fig. 1D).

Our dRNA-seq and Term-seq data also allowed identification of novel non-coding regulatory RNA elements. Using combined RNA-seq, dRNA-seq, and Term-seq data as input for ANNOgesic[16], we identified 141 potential sRNAs which included 50 previously reported in S. aureus[20] (Supplementary Data 2). Of these predicted sRNAs, 57 had RNA 5'UTRs and 3' ends defined by both dRNA-seq and Term-seq (Supplementary Data 2). An additional 357 trans-encoded S. aureus sRNAs have been previously reported[20] and we included these elements in our subsequent analyses.

Collectively, the RNA 5' and 3' end data provide a high resolution, condition-specific map of transcript boundaries,
**Fig. 1 RNase III interactions with the ANNOgesic annotated S. aureus JK06009 transcriptome.**

**a** Transcription start sites and putative promoter regions identified through the ANNOgesic workflow. Nucleotides are represented as coloured dots specifying the nucleotide percentage identity.

**b** Consensus 10-nt stem-loop RNA structure identified within our RNA 3' boundary ends. UTRs were classified as RNase III-bound if they contained >10 reads in two independent experiments. **c** Heatmap showing the two-sided Pearson's correlation between replicate RNase III CRAC datasets. **d** Proportion of RNase III-bound reads mapping to genomic features for replicate datasets. **e** Cumulative count of RNase III-bound reads mapping across CDSs. Each CDS was divided into 100 bins and the read depth within each bin is indicated. **f** Cumulative count of RNase III-bound reads at 5' UTRs and 3' UTR ends. Read counts for each individual UTR were normalised to 1 to prevent biases from abundant sites. **g** Distribution of RNase III-bound UTR lengths (nts) (left) and structure (expressed as the free energy of the folded UTR, kcal/mol) (right). UTRs were classified as RNase III-bound if they contained >10 reads in two independent experiments. p-values were calculated using a two-sided t-test. For each boxplot, centre represents the median, box minima and maxima represent the 25th and 75th percentile respectively, and whiskers represent 1.5× the interquartile range.
defining mRNA 5’ and 3’UTRs, and 92 novel regulatory sRNAs in the MRSA isolate JKD6009.

**UV-crosslinking reveals RNase III bound transcripts.** In our previous analysis of the enterohaemorrhagic *E. coli* RNA interaction network, we used the endoribonuclease RNase E (rne) as bait to capture sRNA–RNA interactions using an UV-crosslinking and RNA proximity-dependant ligation technique termed CLASH13. While *S. aureus* does not encode an orthologue of *rne*, many sRNA–mRNA pairs are substrates for the double-stranded RNA-specific endoribonuclease RNase III11,21-25 that interacts with the sRNA–mRNA duplex. To facilitate high-stringency purification of UV-crosslinked RNA-RNase III complexes we created a translational fusion of the chromosomal copy of *S. aureus* HTF (called JKD6009) and RNase III is not essential in *S. aureus*26 and JKD6009 rnc-HTF produced wild-type levels of mature 16S and 23S ribosomal RNA (rRNA) (Supplementary Fig. 2A), demonstrating that the fusion protein is functional. IMAC purification of RNase III-HTF and α-His Western blotting revealed a single protein at the expected mass of 34 kDa (Supplementary Fig. 2B).

RNA-RNase III complexes were covalently crosslinked using UV-C in wild-type (untagged) and rnc-HTF tagged cultures grown in brain heart infusion (BHI) media to OD578nm 3.0. BHI was stranded RNA-specific RNase III11,21-25 that interacts with the sRNA–mRNA duplex. To facilitate high-stringency purification of UV-crosslinked RNA-RNase III complexes we created a translational fusion of the chromosomal copy of RNase III (rne) with the dual affinity tag, His6-TEV-FLAG (HTF) called JKD6009 rnc-HTF. RNase III is not essential in *S. aureus*26 and JKD6009 rnc-HTF produced wild-type levels of mature 16S and 23S ribosomal RNA (rRNA) (Supplementary Fig. 2A), demonstrating that the fusion protein is functional. IMAC purification of RNase III-HTF and α-His Western blotting revealed a single protein at the expected mass of 34 kDa (Supplementary Fig. 2B).

RNase III crosslinked RNA fragments were mapped to the JKD6009 transcriptome and had a good correlation between replicate experiments (Pearsons = 0.57–0.95) (Fig. 1c). The majority of reads (61.5–90%) mapped to rRNAs consistent with RNase III processing of this abundant species (Fig. 1d)28,29. Coding sequences (3–29%), 3’UTRs (1.5–3.8%), sRNAs (0.9–2.6%), and 5’UTRs (0.4–2.3%) were the next most abundantly recovered RNA classes (Fig. 1d). Plotting the cumulative read count across all CDS indicated that RNase III bound strongly to the 5’ end of mRNAs (Fig. 1e) and plotting RNase III binding relative to the start of 5’UTRs and the end of 3’UTRs indicated enrichment of RNase III within the UTRs of mRNAs (Fig. 1f). RNase III-bound 5’ and 3’UTRs were generally longer and more structured compared with UTRs that did not bind RNase III, consistent with recognition of double-stranded RNA structures formed in cis within the UTR (Fig. 1g). These results are also consistent with analyses of RNase III in *S. pyogenes* where RNase III preferentially cleaves UTRs30. We additionally recovered RNase III interactions with the known substrates cspA and RNAIII, confirming that our analysis recovers bona fide targets (Supplementary Fig. 2C)31,24. We were not able to recover a statistically significant RNA sequence representing a binding motif within the RNase III read peaks, potentially reflecting broad read peaks recovered by RNase III UV-crosslinking and/or a significant role of in trans RNA–RNA interactions in forming the double-stranded RNA substrate for RNase III binding.

**RNase III-CLASH captures RNA–RNA interactions.** We have previously shown that our UV-crosslinking approach facilitates proximity-dependant ligation of RNA–RNA interactions13. We used the software package hyb31 to extract hybrid reads that represent RNA–RNA interactions and filtered for interactions with an FDR < 0.05 (detailed in Supplementary Methods). We collated our hybrid reads with additional RNase III-CLASH data generated in a parallel study by McKellar et al.32 utilising TSB and RPMI-1640 media (Supplementary Data 3). We recovered 13,530 unique hybrid reads (21,680 in the collated dataset), representing 822 statistically significant unique RNA–RNA interactions (1,420 in the collated datasets), including 133 sRNA–mRNA interactions (Supplementary Data 3). Consistent with our earlier dataset13 many interactions are recovered in a single experiment with 117 interactions recovered in multiple independent CLASH experiments. We recovered 7 individual hybrid reads mapping to the previously identified interaction between spoVG mRNA and the sRNA SprX7. RNA interactions were recovered for a broad range of RNA classes (Fig. 2a). The RNase III-CLASH dataset contains a high proportion of RNA interactions that map to multiple sites in the transcriptome and were removed by our mapping pipeline (Supplementary Methods). These interactions potentially represent RNA structure or background signal from these abundant RNAs. We recovered 543 statistically significant mRNA–mRNA interactions with 15 interactions recovered in multiple independent experiments (Fig. 2b) suggesting that many mRNAs may be able to exert regulatory functions in trans. RNA interactions were enriched at start codons, in line with canonical regulatory interactions that occlude the ribosomal binding site (RBS) (Fig. 2c). RNA–RNA interactions recovered by RNase III-CLASH had a significantly lower free energy than randomly shuffled RNA pairs, as did sRNA-target RNA interactions, consistent with hybrid reads representing in vivo RNA–RNA interactions (Fig. 2d).

The collated (BHI, TSB, and RPMI-1640) *S. aureus* sRNA interactome contains 287 nodes and 256 sRNA interactions (Fig. 3 and Supplementary Data 3). We independently verified a subset of sRNA–mRNA interactions within the RNase III-CLASH network using a two-plasmid system for high-level, constitutive expression of both sRNA and mRNA7. sRNA–mRNA interactions were chosen from the collated interaction network that had varying degree of hybrid counts. Using a SpoVG-GFP translational fusion we demonstrated that SprX2 (the second copy of SprX that contains 6 SNPs and a single nucleotide deletion) is able to repress spoVG expression consistent with earlier work7. Interestingly, the sRNA SprD is required for intermediate vancomycin tolerance in VISA. We have independently confirmed this interaction21,34. Point mutations within the predicted seed regions of either RsA or MgrA significantly reduced repression, and repression could be partially restored when the complementary point mutations were expressed together, indicating a direct interaction between RsA and MgrA in vivo (Fig. 2f). Many novel sRNA–mRNA interactions were positioned within mRNA coding sequences such as sRNA11-agrA and RNAIII-murQ. Using qRT-PCR to measure transcript abundance we confirmed that sRNA11 promotes agrA mRNA accumulation (Fig. 2g) and RNAIII destabilises murQ mRNA (Fig. 2h) indicating that these RNase III-CLASH interactions identified are functional.

These data indicate that RNase III-CLASH detects in vivo RNA–RNA interactions including functional sRNA–mRNA interactions providing a condition-specific snapshot of the *S. aureus* sRNA interactome.

A novel regulatory RNA is required for intermediate vancomycin tolerance in VISA. We next asked if sRNA interactions identified within our collated RNase III-CLASH network
contributed to increased susceptibility to vancomycin in the VISA strain JKD6008. Regulatory RNA interactions with mRNAs that are linked to the development of vancomycin tolerance, or upregulated during vancomycin treatment were selected for further analysis. CRISPR interference (CRISPRi) was used to knockdown expression of six putative regulatory RNAs and the reduced expression was confirmed by Northern blot (Supplementary Fig. 3A). An agar spot dilution assay was used to assess vancomycin-sensitivity of each regulatory RNA knock-down in JKD6008. In the absence of vancomycin, the CRISPRi knock-downs had comparable growth to the vector-only control (Fig. 4ai). However, in the presence of a sub-inhibitory concentration of vancomycin (3 μg/mL), growth of the regulatory RNA knockdown annotated as sRNA275 (here termed vigR 3’UTR) was reduced 1000-fold (Fig. 4aii). Broth microdilution indicated that the vigR 3’UTR CRISPRi knockdown was vancomycin sensitive with an MIC of 2–4 μg/mL (Supplementary Fig. 3B). In liquid MH, growth of the vigR 3’UTR CRISPRi knockdown was severely attenuated (1.91-fold increase in lag phase, p = 0.0012; 1.25-fold decrease in maximum OD600nm, p = 0.00054) in the presence of vancomycin, similar to VSSA strain JKD6009 (Fig. 4b and Supplementary Fig. 3C). These data show that vigR 3’UTR...
Fig. 2 RNase III-CLASH recovers RNA–RNA interactions in *S. aureus* JKD6009. a Histogram of the RNA classes recovered by RNase III-CLASH expressed as the number of hybrid reads (left) and number of unique RNA–RNA interactions (multiple hybrid reads can represent one RNA–RNA interaction) (right). b Distribution of the number of hybrid reads representing each RNA–RNA interaction recovered by RNase III-CLASH. Each RNA–RNA interaction type is indicated below and the number of independent experiments containing the interaction is indicated by the size of the data point. c Cumulative count of RNA–RNA interactions at start codons (indicated by the red dashed line). d Cumulative distribution function of RNA–RNA interaction strength (ΔG, kcal/mol) for all RNA–RNA interactions recovered (left), or ncRNA–RNA interactions (right). Pairs of interacting RNAs were randomly shuffled and the distribution of interaction strength of randomly paired RNAs is shown in red. A two-sided Kolmogorov–Smirnov test was used to calculate p-values. e–h RNA–RNA interactions recovered by RNase III-CLASH are functional. Constitutively transcribed GFP translational fusions to SpoVG (e) and MgrA (f) were expressed in *S. aureus* RN4220 with or without transcription of cognate sRNAs (indicated below) and median fluorescence intensity (MFI) measured using flow cytometry. Histogram heights represent mean MFI and error bars represent the standard deviation (SD) from *n* = 3 biological replicates. Significance was calculated using a two-sided t-test. *p* < 0.05. Predicted interactions between RNAs recovered in hybrid reads are indicated (right). Blue boxes indicate the ribosomal binding site and start codon for each mRNA. f Compensatory point mutations (M1) were introduced into mgrA and RsaA (indicated by arrows). MFI was measured for combinations of mgrA and RsaA M1 mutants (indicated below histogram). For interactions within the CDS, qRT-PCR was used to quantify target mRNA abundance (relative to *gapA*) in *S. aureus* RN4220 constitutively transcribing the sRNAs sRNA11 (g) or RNAIII (h) from the vector pICS3 (indicated below plot). The mRNAs agrA (g) and murQ (h) are expressed from the chromosomal loci. Histogram heights represent mean relative abundance and error bars indicate standard error from *n* = 3 biological replicates. p-values were calculated using a two-sided t-test. Predicted interactions between RNAs recovered in hybrid reads are indicated (right).

Fig. 3 Small RNA interactome of *S. aureus* JKD6009. Individual RNAs are indicated as nodes (circles) and coloured according to RNA class (top right). RNA–RNA interactions are indicated as edges (lines) and coloured according to FDR adjusted probability (middle right). The discrete probability P(X = n) of recovering each hybrid was modelled using a binomial distribution and combined between replicate experiments using the Fishers method. A detailed description of the statistical analysis performed on the RNase III-CLASH dataset is presented in Supplementary Methods. The line thickness of RNA–RNA interaction edges are weighted to indicate the number of hybrid reads captured for each interaction (bottom right). RNA–RNA interactions that are experimentally validated in the text are labelled at the node and edges are coloured blue.

(Previously annotated as sRNA275) is required for vancomycin intermediate resistance in JKD6008, and that knocking-down expression of vigR 3' UTR reverts the strain to a vancomycin-sensitive phenotype.

**vigR** is a regulatory mRNA that controls a glycopeptide-specific intermediate tolerance. Term-seq identified a transcription termination site at the 3' end of the long vigR 3' UTR and our dRNA-seq data indicated a primary TSS upstream of the uncharacterised YtxH-domain protein, *E0E12_RS09390* (vigR) (Fig. 4c). We did not identify additional TSSs, processing sites, or termination sites within *E0E12_RS09390* or the UTR boundaries. Northern blot analysis probing for vigR 3' UTR identified a long transcript (~1.1 kb) indicating that sRNA275 is the 3' UTR of the *E0E12_RS09390* mRNA (Fig. 4d). Based on these results and later observations we have named this mRNA the vancomycin-intermediate and glycopeptide resistance mRNA (vigR). Northern blot analysis indicated that vigR expression is up-regulated in the VISA strain JKD6008 compared to JKD6009, consistent with a role in intermediate vancomycin tolerance (Fig. 4d and Supplementary Fig. 3D). Northern blot analysis also indicated that vigR...
transcript levels are increased between mid-log and stationary phase during growth in BHI (Supplementary Fig. 3D), but appear consistent throughout the growth phases in RPMI-1640 media (Supplementary Fig. 3E). We also observed a single transcript band (~1.1 kb) in RPMI-1640 media, confirming no processing events or independent transcription of the vigR 3′UTR in this infection-relevant condition (Supplementary Fig. 3E).

Our results suggested that the vigR 3′UTR may confer vancomycin tolerance through cis regulation of the VigR protein. To determine the relative contribution of each region to intermediate-vancomycin tolerance, clean deletions of both the 3′UTR (vigRΔ3′UTR) and CDS (vigRΔCDS), and a chromosomally repaired vigRΔ3′UTR (vigRΔ3′UTR-repair, restoring the wild type genotype) were constructed in JKD6008 (schematic representation of constructs in Supplementary Fig. 3F). These strains were confirmed using Northern blot analysis (Fig. 4d), qRT-PCR (Supplementary Fig. 3G) and whole genome sequencing. We find that the vigR 3′UTR is required for vigR CDS stability (CDS transcript levels are 37.5% c.f. WT, Supplementary Fig. 3Gi). The 3′UTR of vigR is more stable in the absence of the CDS (68.2% c.f. WT; Supplementary Fig. 3Gi).

The vigRΔCDS strain had a slight growth defect in liquid MH and grew to similar levels in the presence of 3 μg/mL vancomycin (Fig. 4e and Supplementary Fig. 4A). In contrast, the vigRΔ3′UTR
deletion grew similar to the parent VISA strain in liquid MH but was sensitive to vancomycin and this could be partially restored by repairing the vigRΔ3′UTR deletion (vigRΔ3′UTR-repair, Fig. 4e and Supplementary Fig. 4A). Deletion of the vigR 3′UTR also sensitised the VSSA strain JKD6009 to vancomycin (Supplementary Fig. 4B), indicating that vigR also contributes to vancomycin tolerance in the MRSA background, albeit at a lower MIC.

We next assessed if the vigR 3′UTR deletions had altered susceptibility to other antibiotics used to treat S. aureus infections. The vigR 3′UTR deletion, but not the CDS deletion, was sensitive to an intermediate level of the last-line antibiotic teicoplanin (2 μg/mL) in both the VISA (Fig. 4e and Supplementary Fig. 4A) and VSSA backgrounds (Supplementary Fig. 4B), and was fully restored in the repaired strains. Neither vigR 3′UTR deletions were significantly more sensitive to fosfomycin, tigecycline, or ampicillin than their respective parents strains (Supplementary Fig. 4B, C). Collectively, these results indicate that the vigR 3′UTR controls a glycopeptide-specific response, independent of the VigR protein.

**vigR 3′UTR regulates the expression of cell wall metabolism genes.** Our RNase III-CLASH analysis indicated that vigR 3′UTR acts as a regulatory hub (Fig. 5a). To understand how the expression of vigR target RNAs were controlled and to gain further insight into the mechanism of intermediate glycopeptide tolerance, we used RNA-seq to measure RNA abundance in both the vigRΔ3′UTR and vigR 3′UTR CRISPRi knockdown. These analyses identified 117 transcripts including 16 sRNAs that were differentially expressed in both the vigR 3′UTR deletion and knockdown (FDR ≤ 0.05). The addition of sub-inhibitory vancomycin (2 μg/mL) for 10 min did not reveal additional transcriptional changes (Supplementary Fig. 5). Ontological clustering of differentially expressed transcripts indicated that terms associated with ‘carbohydrate transport and metabolism’, ‘amino acid transport and metabolism’, and ‘cell wall, envelope, and membrane biogenesis’ were enriched (Fig. 5b). Differentially expressed transcripts involved in cell wall and envelope biosynthesis included the downregulation of *daa*<sup>36</sup>, *lytM*<sup>37</sup>, the *dltXABC*<sup>78</sup> operons and the lytic transglycosylases *isaA* and *sceD*, along with the upregulation of *mur*<sup>36</sup> (Fig. 5c). Notably, *isaA, sceD,* and *lytM* also belong to the WalKR regulon<sup>40</sup> suggesting that the vigR mRNA and WalKR regulons at least partly overlap. There was no change in transcript abundance of *walKR* or *walHI* in the vigR<sup>3′UTR</sup> or knockdown strain, indicating that cross-regulation does not occur at the transcriptional level.

The vigR CLASH targets *folD* and *isaA* were reduced in the vigRΔ3′UTR and/or vigR knockdown strain indicating that vigR mRNA–mRNA interactions stabilise these transcripts (Fig. 5a, c). The *folD* mRNA (reduced in the knockdown strain, FDR = 0.0047, log<sub>FC</sub> = −1.21), involved in folate metabolism, produces tetrahydrofolate which forms as a key metabolite for amino acid (histidine) and nucleotide (purine) biosynthesis. The vigR and *folD* mRNAs are predicted to form an extensive 145-nt RNA–RNA duplex including 63-nts of perfect Watson–Crick base-pairing (Fig. 5d). We used EMSA to confirm that the vigR 3′UTR stabilises *folD* mRNA through a direct interaction. Titrating the vigR 3′UTR with either a 32P-labelled full-length *folD* mRNA or a 32P-labelled *folD* sub-fragment incorporating the 145-nt duplex site shifted the 32P-labelled *folD* to a slower migrating species consistent with the formation of a vigR–*folD* RNA duplex (Fig. 5e).

Collectively, these results indicate that vigR mRNA has profound effects on the abundance of transcripts required for cell wall metabolism, and confirm the novel interaction with *folD* mRNA identified by RNase III-CLASH that increases the abundance of this transcript.

**isaA mRNA is directly regulated by the vigR 3′UTR.** The abundance of *isaA* was reduced in the vigR 3′UTR deletion (FDR = 0.00012, log<sub>FC</sub> = −1.02) and knockdown strain (FDR = 0.033, log<sub>FC</sub> = −0.69) (Fig. 5c), and we confirm this result using Northern blot analysis (Fig. 6a). To confirm vigR 3′–3′UTR interactions with *isaA*, we employed a two-plasmid system to constitutively transcribe vigR 3′UTR and an *isaA*-GFP translational fusion<sup>2</sup>. This construct does not include the *isaA* promoter and high-level, constitutive transcription of *isaA*-GFP from *P<sub>syn</sub>* uncouples expression from native transcriptional regulation. In the presence of the vigR 3′UTR, translation of *isaA*-GFP was increased 58% (p = 0.0001), confirming that the vigR 3′UTR promotes *isaA* mRNA expression independently of transcriptional regulation (Fig. 6b). EMSA was used to verify a direct interaction between vigR 3′UTR and *isaA* mRNA in vitro. A slower migrating vigR–*isaA* duplex was formed when titrating 3P-labelled *isaA* or vigR 3′UTR against each other (Fig. 6c, d). To determine the interaction site, we titrated radiolabelled vigR 3′UTR against sub-fragments of *isaA* (Fig. 6e). These analyses indicated that the vigR 3′UTR interacts with the 3′ region of the *isaA* coding sequence (*isaA* frag-C, Fig. 6f), consistent with the position of the RNA–RNA hybrid captured by RNase III-CLASH. A fainter vigR–*isaA* frag-A complex was also formed (Supplementary Fig. 6a). Labelled vigR 3′UTR was not able to shift *isaA* frag-B (Supplementary Fig. 6b) indicating that the *isaA* fragment C interaction is specific. IntraRNA analysis of vigR 3′UTR–*isaA* frag-C identified an imperfect 86-nt interaction duplex (50-nt of complementarity) that included the GC-rich RNAIII-CLASH hybrid read (Fig. 6g). To further resolve the interaction site, competitor oligonucleotides antisense to either vigR 3′UTR (C1–C4) or *isaA* frag-C (C5–C8) tiled across the duplex site were added into the EMSA reaction (50 nM excess concentration). This analysis revealed that competitor oligonucleotides at the C1 and C5 site antisense to vigR and *isaA*, respectively, were able to effectively compete away radiolabelled vigR 3′UTR–*isaA* frag-C duplex formation (Fig. 6h). Additionally, the competitor oligonucleotides at the C2 site antisense to vigR 3′UTR was also able to effectively compete away radiolabelled vigR 3′UTR, indicating that the 3′ end of the 3′UTR duplex (position +1069-1038) is crucial in vitro for vigR 3′UTR–*isaA* frag-C duplex formation. To complement the EMSA, we titrated *isaA* frag-C with vigR 3′UTR in the presence of RNase T1 and ShortCut RNAIII followed by 32P-labelled-primer extension (Supplementary Fig. 6c). In agreement with the competitor oligonucleotides at the C1 and C5 site, binding of *isaA* frag-C induced ShortCut RNAIII cleavage most notably at position C +1063 (Fig. 6g and Supplementary Fig. 6d).

To understand the role of RNase III in the regulation of *isaA*, a deletion of *ircn* was constructed in JKD6009. Using qRT-PCR, we confirm the increased abundance of the *isaA* transcript in the Δircn strain (Fig. 6i). qRT-PCR showed a statistically significant increase of 35.5% (± 3.9, p = 0.00046; Fig. 6i) relative to WT, consistent with RNase III processing of *isaA*.

These results indicate that the vigR 3′UTR is able to post-transcriptionally promote *isaA* expression in vivo and directly interacts in vitro with the 3′ end of *isaA* identified by RNase III-CLASH.

**vigR 3′UTR regulation of *isaA* impacts cell wall thickness and glycopeptide-intermediate tolerance.** To determine the impact of *isaA* regulation on intermediate vancomycin resistance, an *isaA* deletion and CRISPRi knockdown strain was constructed in VISA strain JKD6008. *isaA* has previously been shown to confer salt resistance and we independently verified this phenotype for our Δ*isaA* strain in both BHI and MH media<sup>41</sup> (Supplementary Fig. 6f). Our RNase III-CLASH analysis indicated that the formation of a vigR–3′UTR stabilises *isaA* mRNA through a direct interaction. Titrating the vigR 3′UTR with either a 32P-labelled full-length *isaA* mRNA or a 32P-labelled *isaA* sub-fragment incorporating the 145-nt duplex site shifted the 32P-labelled *isaA* to a slower migrating species consistent with the formation of a vigR–*isaA* RNA duplex (Fig. 5e).

Collectively, these results indicate that vigR mRNA has profound effects on the abundance of transcripts required for cell wall metabolism, and confirm the novel interaction with *folD* mRNA identified by RNase III-CLASH that increases the abundance of this transcript.
A common feature of clinical VISA isolates is a thicker cell wall. We used TEM to quantify the cell wall thickness of JKD6009 (VSSA), JKD6008 (VISA), vigilR Δ3′ UTR, ΔisaA, and JKD6008 pSD1 and pSD1-isaA knockdown. We confirmed the increased cell wall thickness reported for VISA isolates (25.84 c.f. 22.77 nm; Fig. 6j and Supplementary Fig. 7B). Interestingly, cell wall thickness measurements of the vigilR Δ3′ UTR strain revealed a decrease in cell wall thickness to 24.13 nm (p = 0.058) when compared to the isogenic VISA parent strain, suggesting that vigilR 3′ UTR influences cell wall architecture in S. aureus (Supplementary Fig. 7C, D).

Interestingly, cell wall thickness measurements of the vigilR Δ3′ UTR strain revealed a decrease in cell wall thickness to 24.13 nm (p = 0.058) when compared to the isogenic VISA parent strain, suggesting that vigilR 3′ UTR influences cell wall architecture in S. aureus (Supplementary Fig. 7C, D).

Most notably, both the isaA deletion and knockdown strain had a significantly reduced cell wall thickness when compared to their isogenic parent strains (Fig. 6j and Supplementary Fig. 7B). Deletion of isaA also sensitised JKD6008 to vancomycin but did not completely recapitulate the acute vancomycin sensitivity of the vigilR Δ3′ UTR strain indicating that additional regulatory effects contribute to vancomycin sensitivity (Supplementary Fig. 7E). However, the ΔisaA strain was sensitive to teicoplanin, indicating that activation of isaA is likely responsible for teicoplanin sensitivity in the vigilR Δ3′ UTR strain (Supplementary Fig. 7E). These results demonstrate that isaA contributes to cell wall thickening that is partly responsible for the intermediate vancomycin resistance of VISA isolate JKD6008.

Discussion
Methicillin-resistant Staphylococcus aureus has become increasingly common in both community and healthcare settings. Treatment of MRSA infections is limited to last-line antibiotics and vancomycin is the drug of choice for severe MRSA sepsicaemia. Intermediate vancomycin resistance is the most common cause of vancomycin treatment failure and arises through a heterogeneous collection of point mutations that often lead to reduced autolysis and cell wall thickening which reduces vancomycin permeability. Previous transcriptome profiling experiments suggested that regulatory RNA responses may play critical roles in resistance to last-line antibiotics. Here we have used RNase III-CLASH to identify an mRNA that is required for
intermediate vancomycin resistance and functions as a regulatory RNA ‘hub’ in our network. CRISPRi knockdown of sRNA275 (here termed vigR 3’UTR) reverted the clinical VISA isolate JKD6008 to vancomycin-sensitive. Northern analysis, dRNA-seq, and Term-seq mapping of RNA 5′ and 3′ ends demonstrated that sRNA275 is the 3′UTR of the upstream coding sequence E0E12_RS09390 (SAA6008_01724) encoding a hypothetical YtxH-domain protein (here termed VigR). The vigR 3′UTR is unusually long for a prokaryotic 3′UTR at 657-nt (median 40-nt Bacillus subtilis, 75-nt in this study, and 88-nt in Methanosarcina mazei)42, and is substantially longer than the 378-nt CDS encoded within the vigR mRNA. We demonstrate that the 3′UTR, but not the CDS is required for intermediate vancomycin tolerance indicating that the mRNA, and not the protein mediates these regulatory effects. Bacterial 3′UTRs can have both cis-43–45, and trans-acting regulatory activity. Examples of trans-acting regulatory sRNAs encoded within 3′UTRs have recently been described46,47. These 3′UTR sRNAs can be transcribed as independent transcripts or released from the mRNA by RNase cleavage, and function independently of the parent mRNA48. Here we find that the vigR 3′UTR is neither independently transcribed, nor processed from the vigR mRNA indicating that it is a regulatory mRNA. To our knowledge, vigR is the fourth example of a bacterial mRNA that has trans-acting regulatory functions. In S. aureus, the CDS region of gdpS was shown to directly interact and stabilise the 5′UTR of sarS, however, the mechanism was not determined49. In the gram-positive pathogen, Listeria monocytogenes, interactions between the 3′UTR of hly and the 5′UTR of prsA block exonucleolytic digestion of prsA mRNA by RNase J50. In Streptococcus mutans, the 5′UTR of irvA interacts with the CDS of gbpC where it occludes an RNase J2 endonucleolytic cleavage site51. In both cases, the mRNA-mRNA interaction stabilise the target mRNA by protecting the transcript from ribonuclease processing or degradation. In a slight variation on this Gram-positive theme, we show that the vigR 3′UTR interaction with the CDS of folD and isaA mRNAs...
Fig. 6 The lytic transglycosylase IsaA is regulated by the 3′ UTR of vigR. a Northern analysis of isaA abundance in VISA (JKD6008) and isogenic vigRΔ3UTR, vigRΔ3UTR-repair, along with JKD6008 pSD1 and pSD1-vigR 3′ UTR. Sybr Green II stained 235 and 165 rRNA are shown as loading controls. Quantification of the ratio of 23S rRNA to isaA by densitometry is indicated below. b Quantification of expression of a constitutively transcribed IsaA-GFP fusion (pCN33:isaA-gfp) with or without expression of vigR 3′ UTR (pIC53:vigR) (indicated below). Median fluorescence intensity (MFI) is reported on the y-axis. Histogram heights represent the mean MFI and error bars indicate standard error from n = 5 biological replicates. The p-value is calculated using a two-sided t-test. c EMSA analysis of interactions between vigR 3′ UTR and full-length isaA mRNA. 50 fmol of radiolabelled isaA mRNA was titrated against increasing concentrations of vigR 3′ UTR (top). d 50 fmol of radiolabelled vigR 3′ UTR was titrated against increasing concentrations of full-length isaA mRNA. e Schematic of the RNase III-CLASH hybrid read within isaA mRNA and vigR mRNA. The isaA mRNA was synthesised as sub-fragments (frag A–C, wavy lines) ~300-nt in length to be used for EMSA. f isaA fragment C (frag-C). Concentrations of isaA are indicated (top). Black arrowheads indicate migration of free, radiolabelled RNA and open arrowheads indicate slow migrating vigR-isaA duplexes. g Predicted interaction between RNAs within the isaA-vigR hybrid read. The start and end positions of the RNA-RNA duplex are indicated below the respective mRNA. The antisense oligonucleotide competitor used for EMSAs are detailed above and below their respective mRNAs (C1–C3). The cleavage sites of RNase T1 and ShortCut RNA III detected by primer extension are indicated by the grey and black arrows, respectively (also see Supplementary Fig. 6C, D). h EMSA analysis of interactions between vigR 3′ UTR and isaA fragment C (0.0 or 1.25 μM concentration). Black arrowheads indicate migration of free radiolabelled vigR 3′ UTR RNA. Antisense competitor oligonucleotides C1–C8 (indicated on top) were spiked in at 300× excess concentration. Open arrowhead indicates slow migrating vigR-isaA fragment C duplexes. I Quantitative RT-PCR for isaA mRNA abundance (relative to gapA) in VSSA and Δmc strain grown in BHI to an OD 578 nm of 3.0. Histogram height represents the mean and error bars indicate the standard deviation (SD) from n = 3 biological replicates. The p-value is calculated using a two-sided t-test. J Histogram of cell wall thickness for VSSA, VISA, and isaA deletion and knockdown strains (left). Error bars represent standard error of the mean (SEM) from n = 100 measurements. The p-value is calculated using a two-sided t-test. *p < 0.005. (right) Representative transmission electron microscopy (TEM) images of VSSA (JKD6009), VISA (JKD6008), and JKD6008 derivatives. The average cell wall thickness and standard error are shown below.

also stabilise these transcripts likely by blocking RNase III endonucleolytic processing within these mRNAs.

Deletion of the vigR 3′UTR led to a modest reduction in cell wall thickness and notable changes in the transcriptome. RNA-seq analysis of the vigRΔ3UTR and knockdown strain indicated differential expression of 117 transcripts with many that clustered within the ontological term ‘cell wall, membrane, and envelope biogenesis’. The addition of sub-inhibitory concentrations of vancomycin did not significantly alter the mutant transcriptome and quantification of the ratio of 23S rRNA to 16S rRNA intermediates and cation of expression of a constitutively transcribed IsaA-GFP fusion. Its abundance in VISA (JKD6008) and isogenic vigRΔ3UTR, vigRΔ3UTR-repair, along with JKD6008 pSD1 and pSD1-vigR 3′ UTR. Sybr Green II stained 23S and 16S rRNA are shown as loading controls. Quantification of the ratio of 23S rRNA to isaA by densitometry is indicated below. b Quantification of expression of a constitutively transcribed IsaA-GFP fusion (pCN33:isaA-gfp) with or without expression of vigR 3′ UTR (pIC53:vigR) (indicated below). Median fluorescence intensity (MFI) is reported on the y-axis. Histogram heights represent the mean MFI and error bars indicate standard error from n = 5 biological replicates. The p-value is calculated using a two-sided t-test. c EMSA analysis of interactions between vigR 3′ UTR and full-length isaA mRNA. 50 fmol of radiolabelled isaA mRNA was titrated against increasing concentrations of vigR 3′ UTR (top). d 50 fmol of radiolabelled vigR 3′ UTR was titrated against increasing concentrations of full-length isaA mRNA. e Schematic of the RNase III-CLASH hybrid read within isaA mRNA and vigR mRNA. The isaA mRNA was synthesised as sub-fragments (frag A–C, wavy lines) ~300-nt in length to be used for EMSA. f isaA fragment C (frag-C). Concentrations of isaA are indicated (top). Black arrowheads indicate migration of free, radiolabelled RNA and open arrowheads indicate slow migrating vigR-isaA duplexes. g Predicted interaction between RNAs within the isaA-vigR hybrid read. The start and end positions of the RNA-RNA duplex are indicated below the respective mRNA. The antisense oligonucleotide competitor used for EMSAs are detailed above and below their respective mRNAs (C1–C3). The cleavage sites of RNase T1 and ShortCut RNA III detected by primer extension are indicated by the grey and black arrows, respectively (also see Supplementary Fig. 6C, D). h EMSA analysis of interactions between vigR 3′ UTR and isaA fragment C (0.0 or 1.25 μM concentration). Black arrowheads indicate migration of free radiolabelled vigR 3′ UTR RNA. Antisense competitor oligonucleotides C1–C8 (indicated on top) were spiked in at 300× excess concentration. Open arrowhead indicates slow migrating vigR-isaA fragment C duplexes. I Quantitative RT-PCR for isaA mRNA abundance (relative to gapA) in VSSA and Δmc strain grown in BHI to an OD 578 nm of 3.0. Histogram height represents the mean and error bars indicate the standard deviation (SD) from n = 3 biological replicates. The p-value is calculated using a two-sided t-test. J Histogram of cell wall thickness for VSSA, VISA, and isaA deletion and knockdown strains (left). Error bars represent standard error of the mean (SEM) from n = 100 measurements. The p-value is calculated using a two-sided t-test. *p < 0.005. (right) Representative transmission electron microscopy (TEM) images of VSSA (JKD6009), VISA (JKD6008), and JKD6008 derivatives. The average cell wall thickness and standard error are shown below.

Methods

Bacterial strains, plasmids, and culture conditions. The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Supplementary Data 4. S. aureus RN4220 and the JKD6009/JKD6008 (VSSA/VISA) pair strains were routinely cultured at 37 °C on solid or in liquid brain heart infusion (BHI, Merck) or Mueller–Hinton (MH, Merck) media. Antibiotics were routinely used in this study to select for plasmids in S. aureus at 10 μg/ml chloramphenicol and/or 10 μg/ml erythromycin, unless otherwise specified. E. coli DH5α and IM08B strains were routinely cultured at 37 °C on solid or in liquid Luria–Broth (LB) media. Antibiotics were routinely used to select for plasmids in E. coli at 100 μg/ml ampicillin and 15 μg/ml chloramphenicol, unless otherwise specified. All bacterial strains were stored at ~80 °C as stationary phase cultures with 16% (v/v) glycerol.

Strain modifications. S. aureus deletions for vigRΔ3′UTR and isaA and chromosomal repair for vigRΔ3′UTR were constructed using the plMY-Z vector and allelic exchange56. At least 500–nt flanking regions using primer pairs detailed in Supplementary Data 4 were amplified from the respective JKD6008 or JKD6009 gDNA using Phusion Hot Start Flex Polymerase (NEB). All amplified flanking regions were annealed together using splicing by overlap extension (SOE) PCR56,57. Mutants were passed and selected on solid BHI and confirmed using allele-specific PCR (Supplementary Data 4). Loss of the plMY-Z vector was confirmed by 15 μg/ml chloramphenicol sensitivity and plasmid-specific PCR (Supplementary Data 4).

CRISPR interference (CRISPRi) transcriptional knockdown constructs in S. aureus were constructed using the pSD1 vector system58. Knockdown primer pairs (Supplementary Data 4) were annealed by heating to 94 °C for 2 min and then cooling 1.5 °C per min for 1 h. The annealed oligonucleotides were cloned into pSD1 at the SapI site using 10 U of T4 DNA ligase (Thermo) and transformed into chemically-competent E. coli DH5α. Constructs were confirmed by Sanger sequencing, transformed into electroporant competent E. coli IM08B, and then transformed into electroporant competent S. aureus JKD6008.

Differential RNA-Seq (dTRNA-Seq). An overnight stationary culture of S. aureus JKD6009 was diluted 1/100 and grown in liquid MH at 37 °C with 200 rpm shaking to an OD600nm of 0.6. Vancomycin (2 μg/ml) was added to antibiotic-treated cultures and allowed to grow for a further 10 min. Media-based cultures were additionally grown for a further 10 min at 37 °C to reflect the treated samples. Growth was halted by the addition of RNAProtect bacterial reagent (Qiagen) and incubated on ice for 10 min. Cells were harvested by centrifugation (3200 × g for 10 min) and underwent GTC-phenolchloioform RNA extraction procedures69. cDNA libraries were prepared for Vertis Biotechnologie (Freising, Germany) as described previously40 and sequenced on an Illumina NextSeq500 platform (75-cycle single-end reads) (Vertis Biotechnologie, Freising, Germany).

Term-Seq. S. aureus JKD6009 was prepared for total RNA extraction as described above, with the exception of using cold transcriptional stop solution (1:9
phenol:ethanol) to halt culture growth. Total RNA was DNase-treated using 10 U of RQI RNase-free DNase (Promega) at 37 °C for 30 min and ethanol precipitated. cDNA libraries were prepared by Vertis Biotechnologie (Freising, Germany) as described previously and sequenced on an Illumina NextSeq500 platform (75-cycle single-end reads) (Vertis Biotechnologie, Freising, Germany). Detailed protocols for the analysis of the Term-seq data are presented in Supplementary Methods.

**Annotation of *S. aureus JKD6009.** The ANNOgesic pipeline was used to integrate transcriptomics data generated from RNA-seq, DNA-seq and, Term-seq to provide a detailed annotation of the *S. aureus* JKD6009 genome. In brief, the transcription start sites (TSS) were identified using the TSSPredator module within the ANNOgesic pipeline. Rho-independent transcription termination sites were identified from the Term-seq data using custom R scripts and the ‘peakPick’ R library. The ANNOgesic pipeline was used to analyse the transcriptomics data and the analyses resulted in a GFF file containing detailed annotations of *S. aureus* JKD6009 transcriptome. This GFF file was used in subsequent CLASH data analyses. The scripts from TermPick pipeline for the analysis of the Term-seq data are available in the following Github repository (https://github.com/IgnatiusPang/TermPick). Detailed protocols for the annotation of the JKD6009 genome using ANNOgesic are presented in Supplementary Methods.

**RNase III-CLASH.** The chromosomal copy of *RNA III (rnc)* in *S. aureus* JKD6009 was tagged with the dual affinity tag Hist-TEV-FLAG (HTF). RNase III-CLASH was performed as described previously using the JKD6009 WT and rnc-HTF strains with a total of six replicates. For a detailed description of RNase III-CLASH performed on initial replicates 3–6 (protocol A) and then replicates 1–2 (protocol B) and modifications to Waters et al. refer to Supplementary Methods. Briefly, JKD6009 and rnc-HTF strains were used to inoculate pre-warmed broth and grown at 37 °C to an OD600nm 2.5 with 200 rpm shaking. Cultures were crosslinked using the MFI of test cultures. A two-sided Student’s t-test assuming unequal variance was used to determine significance.

**Northern blot.** Total RNA was purified using the GTC-phenol:chloroform extraction method as above. At least 3 μg of RNA was treated with a 5:1 ratio of RNasin and RQ1 RNase-free DNase. The plate was incubated and analysed with a Bio-Rad Gel documentation system. Relative gene expression was determined using the GTC-phenol:chloroform extraction method as above.

**Flow cytometry.** Co-transformed RN4220 GFP translation fusion constructs were streaked out onto solid BHI supplemented with 5 μg/mL erythromycin and 5 μg/mL chloramphenicol. RN4220 strain was chosen as it provided better fluorescence intensity within *S. aureus* clones. Individual colonies were used to inoculate 1 mL of 0.45 μm filtered liquid BHI and grown at 37 °C with 200 rpm shaking. The CMPtrack of *S. aureus* RN4220 was transformed with either the gfp gene or *sprX2*, *sprD*, or *sprX2*Δ*rglA* expression plasmids. Bacterial cultures were grown at 80 °C as stationary phase cultures with 16% (v/v) glycerol. The transformed strains were then co-transformed and transfected a second time with the alternative vector, resulting in co-transformed cultures. These were then stored at 80 °C as stationary phase cultures with 16% (v/v) glycerol.

**Quantitative real-time PCR (qRT-PCR).** *S. aureus* JKD6009 and derivative cultures were diluted 1:100 into 10 mL fresh liquid BHI supplemented at 37 °C with 200 rpm shaking to OD600nm 3.0. Cells were harvested by spinning at 3,800 × g for 10 min at 4 °C. A total of 5 μL of RNA was reverse-transcribed using SensiFAST SYBR Hi-ROX SuperScript III (Thermo), according to the manufacturer’s instructions. qRT-PCR was performed on a RotorGene Q (Eppendorf) using SybrGreen. The PMT was adjusted so that background fluorescence was detectable and within range. The median fluorescence intensity (MFI) for the entire population for each test and control culture was determined using the GTC-phenol:chloroform extraction method as above. Relative gene expression was recorded in the MFI. The MFI from *S. aureus* RN4220 (without GFP expressing plasmid) was used to determine background fluorescence and was subtracted from the MFI of test cultures. A one-sided Student’s t-test assuming unequal variance was used to determine significance.

**Growth curves.** JKD6008, JKD6009, and strain derivatives were plated onto solid MH supplemented with the appropriate antibiotics and incubated at 37 °C for 16 h. Single colonies were used to inoculate 5 mL of pre-warmed liquid MH and cultures incubated at 37 °C with 200 rpm shaking for a further 16 h. A volume of 300 μL of fresh liquid MH was added into each well of a sterile 100-well honey-comb microtiter Bioscreen plate (Thermo) in triplicates to give a starting OD600nm ~0.02. Vancomycin (2–3 μg/mL, where specified), teicoplanin (2 μg/mL), fosfomycin (2 μg/mL), ampicillin (5 μg/mL) and tigecycline (2 μg/mL) were added to assess sensitivity. Strains containing the pS1D vector were also supplemented with 100 ng/mL atc and 5 μg/mL chloramphenicol. The plate was incubated and analysed with a Bioscreen C spectrophotometer (Growth Curves USA) at 37 °C for 20 h with continuous low shaking, measuring OD600nm at 20 min intervals. Growth curves were obtained by plotting biological triplicates. The DMFit (DM: Dynamic Modelling, version 3.3) growth curve modelling software was used to obtain values for the lag phase, growth rate (μ) and maximum OD.
probe (oligonucleotide probes are detailed in Supplementary Data 4) for 16 h at 42°C. Membranes were washed three times in 2× sodium chloride sodium phosphate buffer (SSPB) (pH 7.4) with the addition of 0.1% SDS for 15 min at 42°C and imaged using a BAS-MP 2040 phosphorscreen on a FLA9500 Typhoon (GE Healthcare). For CRISPRi RNA northern blots (Supplementary Fig. 3A) at least 2 μg of total purified RNA was resolved on an 8% polyacrylamide TBE-urea gel and transferred onto a nylon membrane for 16 h at 30 V in 0.5x TBE. The membrane was then crosslinked with a Stratagene Auto-Crosslinker with 1200 mJ UV-C and treated as identical to above.

Transmission electron microscopy. S. aureus JKD6009, JKD6008, and JKD6008 derivative constructs were streaked onto Columbia horse blood agaro and grown at 37°C for 16 h. Colonies were scraped from the agar and resuspended in 1 mL of PBS. Cultures were then centrifuged (50 × g for 5 min) and pellets resuspended in paraformaldehyde fixation solution and prepared for TEM as described previously by Howden et al.69. Cells were viewed and imaged on a FEI Tecnai G2 20 microscope at 15,000-22,000× magnification, with specific images taken at >100,000× magnification to focus on the cell wall. To determine cell wall thickness, 100 measurements of individual horizontally-planar cells were recorded using Imaged and the mean and SEM reported. A two-sided Student’s t-test assuming unequal variance was used to determine significance.

RNA-seq and analysis of nucleotide data. S. aureus JKD6009, JKD6008, and JKD6008 derivative constructs were streaked onto Columbia horse blood agaro and grown at 37°C for 16 h. Colonies were scraped from the agar and resuspended in 1 mL of PBS. Cultures were then centrifuged (50 × g for 5 min) and pellets resuspended in paraformaldehyde fixation solution and prepared for TEM as described previously by Howden et al.69. Cells were viewed and imaged on a FEI Tecnai G2 20 microscope at 15,000-22,000× magnification, with specific images taken at >100,000× magnification to focus on the cell wall. To determine cell wall thickness, 100 measurements of individual horizontally-planar cells were recorded using Imaged and the mean and SEM reported. A two-sided Student’s t-test assuming unequal variance was used to determine significance.

RNA-sequencing data generated in this study have been deposited as NCBI GEO accession number GSE158830. Data source is provided with this paper.

Code availability

Scripts used to analyse our Term-seq data are available at https://github.com/IgnatiusPang/TermPick. The snakemake pipeline used to analyse our CRAC and CLASH datasets is available at https://github.com/IgnatiusPang/Hyb-CRAC-R.

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J.J.T. and T.P.S. initiated the project and D.G.M., J.L.W., M.R.W., B.P.H., T.P.S., S.G., and J.J.T. designed the experiments. D.G.M., J.L.W., W.G., S.M., C.N.I.P., S.W., W.W., B.S., I.R.M., and J.M.B. performed the experiments. D.G.M., J.L.W., C.N.I.P., W.W., and J.J.T. analysed the data. C.N.I.P. developed the pipeline and provided statistical analysis of CLASH data. S.G. developed the analysis pipeline for paired end CRAC data. D.G.M. and J.J.T. drafted the manuscript and all authors reviewed the manuscript and approved the final version.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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