Supplementary Figure S1. Construction of the sa2056 mutant [1]. sa2056 was excised by a three-step procedure developed by Bae et al. [2]: First, the temperature-sensitive plasmid pCQ30 was integrated at 43 °C either up- or down-stream of sa2056 by homologous recombination. Only the resulting chromosomal organization of the recombination symbolized on the left is given. Next, the plasmid was allowed to excise together with the sa2056 gene at permissive temperature (30 °C). Finally, bacteria were selected for plasmid loss.
Supplementary Figure S2. (a) Amino acid sequence of SA2056. Transmembrane (TM) regions predicted by THMMH and C-termini of fragments (F) fused to PhoA are indicated. For TM2 and TM3, predictions of additional programs are depicted. Extra amino acids added to F3 are indicated (F3a–e). (b) Activity of fusion proteins was measured in biological and technical triplicates; mean values for each clone are given and the standard deviation is indicated. SA2056 fragments directing PhoA to the exoplasm were expected to produce values at least five times higher than the background levels (dashed line) measured in the \textit{phaA}-negative \textit{E. coli} strain CC118 (control).
VYLILVIFKGLAPFTILFLPFTVIGVIIALLITGETISVPSLIGMLM 950

LIGIVVNLIDRVIINNEQQGMEMEAIEAGGTRRPILMTAIATIC 1000

ALVPLLFQDSSILIKGLAATVIGGLISSTLTLVVVPVIYEILFTLKK 1050

RFTKR

| TM2 | 369–391 | 379–392 | 376–398 | 372–391 | 375–391 |
|-----|---------|---------|---------|---------|---------|
| TM3 | 398–415 | 399–416 | 402–424 | 398–415 | 398–415 |

(b)
Supplementary Figure S3. Localisation of SA2056. Exponentially grown *S. aureus* expressing SA2056-GFP under the control of the *sa2056* promoter was visualised by (a) phase contrast or (b) fluorescence microscopy as described below. Arrows indicate examples of dividing bacteria with visible septa and SA2056-patches. Bars indicate the size of 1 μm.

![Image](a.png) ![Image](b.png)

The 3'-region of SA2056 (SA2056$_{666nt}$) was amplified from genomic DNA using primers listed in supplementary table T2. The SA2056$_{666nt}$ fragment was cloned to the 5' end of *gfpmut1* in pSG5082 using the XhoI and HindIII restriction sites, yielding pCQ44 [3]. Following the transformation of pCQ44 into *E. coli* DH5α (CQ44), the suicide vector was integrated into *S. aureus* RN4220 (CQ48). To confirm correct integration, a PCR with subsequent sequencing of the region was performed.

CQ48 was grown in tryptic soy broth (TSB, Difco) until exponential phase, washed once in PBS (8 g NaCl, 0.2 g KCl, 2.68 g Na$_2$HPO$_4$·7H$_2$O, 0.24 g KH$_2$PO$_4$, pH 7.4) and resuspended therein. A drop of bacterial suspension was spotted on a microscope slide overlaid with a thin layer of 1 % agarose in PBS and covered with a cover slip. Cells were visualised using a Zeiss Axio Observer.Z1 microscope and the Metamorph v. 7.5 software (Molecular Devices). Pictures were acquired with the Photometrics CoolSNAP HQ2 camera (Roper Scientific), which was connected to the microscope. Pictures were analysed with the ImageJ software [4].
**Supplementary Table S1.** Resistance profiles of strains Newman and *sa2056*.

| Substance                        | Newman | *sa2056* |
|----------------------------------|--------|----------|
| **Cell wall synthesis inhibitors** |        |          |
| Cefoxitin                        | 6      | 6        |
| Oxacillin                         | 0.38   | 0.38     |
| Teicoplanin                      | 4      | 6        |
| Vancomycin                       | 5      | 6        |
| Lysostaphin                      | 0.125–0.25 | 0.125–0.25 |
| D-cycloserine                    | 8      | 8        |
| Fosfomycin                       | 0.25   | 0.25     |
| Ramoplanin                       | 1      | 1        |
| Nisin                            | 4      | 4        |
| Mersacidin                       | 32     | 32       |
| Bacitracin                       | 8      | 8        |
| **RND substrates**               |        |          |
| Acriflavine                      | 8      | 8        |
| EtBr                             | 1–2    | 1–2      |
| SDS                              | 64     | 64       |
| **Others**                       |        |          |
| Daptomycin                       | 2      | 2        |
| Clindamycin                      | 0.94   | 0.94     |
| Chloramphenicol                  | 4      | 3        |
| Tetracycline                     | 0.19   | 0.25     |
| Gentamicin                       | 0.75   | 1        |
| Erythromycin                     | 0.25   | 0.25     |
| Novobiocin                       | 0.0313 | 0.0313   |
| **Fatty acids**                  |        |          |
| Capric acid                      | 512    | 512      |
| Linoleic acid                    | 16     | 16       |
| Cis-6-hexadecenoic acid          | 64     | 64       |
### Supplementary Table S2. Strains and plasmids used in this study.

| Strains    | Relevant genotype and phenotype                                                                 | Reference or source |
|------------|--------------------------------------------------------------------------------------------------|---------------------|
| **S. aureus** |                                                                                                    |                     |
| Newman     | Clinical isolate (ATCC 25904), rsbU<sup>r</sup>                                                    | [5]                 |
| RN4220     | NCTC 8325-4 r<sup>m</sup>                                                                          | [6]                 |
| CQ33       | Newman Δsa2056                                                                                     | [1]                 |
| CQ38       | Newman Δsa2056 pME2, Te<sup>c</sup>, Mc<sup>e</sup>                                                | [1]                 |
| CQ39       | Newman pME2, Te<sup>c</sup>, Mc<sup>e</sup>                                                        | [1]                 |
| CQ48       | RN4220 sa2056::pCQ44, SA2056-GFP, Em<sup>i</sup>                                                   | This study          |
| MS146      | Newman femB::Tn551, Em<sup>i</sup>, Lss<sup>i</sup>                                                 | This study          |
| MS147      | Newman Δsa2056 femB::Tn551, Em<sup>i</sup>, Lss<sup>i</sup>                                        | This study          |
| UT34-2     | NCTC 8325 mec Ω2006(femB::Tn551), Em<sup>i</sup>, Lss<sup>i</sup>                                 | [7]                 |
| **E. coli** |                                                                                                    |                     |
| BL21       | Expression strain, DE3 (E. coli B F<sup>−</sup> ompT hsdS<sub>B</sub> gal dcm)<sup>λ</sup> prophage carrying T7 polymerase | Novagen             |
| CE43       | Membrane protein overproducer selected from BL21                                                  | [8]                 |
| CC118      | Reporter strain for PhoA fusion, Δ(ara-leu)7697 ΔlacZ47 ΔphoA20 galE galK                       | [9]                 |
| CQ44       | DH5α pCQ44, Ap<sup>i</sup>                                                                        | This study          |
| DH5α       | Cloning strain (F− Φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rk−, mk<sup>+</sup>) phoA supE44 thi-1 gyrA96 relA1 λ−] | Invitrogen          |
| DHM1       | BACTH reporter strain, cyu                                                                        | [10]                |
| **Plasmids** |                                                                                                    |                     |
| pCQ44      | Suicide vector, SA2056<sub>sal</sub>-GFP fusion at C-terminus, Ap<sup>i</sup>, Em<sup>i</sup>      | This study          |
| pET24b(+)  | Expression vector, N-terminal T7-Tag or C-terminal His<sub>N</sub>-Tag, T7 promoter, Km<sup>i</sup> | Novagen             |
| pET24b(+)-femA | Expression vector, femA with His<sub>N</sub>-Tag at the C-terminus, Km<sup>i</sup>        | [11]                |
| pET24b(+)-femB | Expression vector, femB with His<sub>N</sub>-Tag at the C-terminus, Km<sup>i</sup>       | [11]                |
| pET24b(+)-femX | Expression vector, femX with His<sub>N</sub>-Tag at the C-terminus, Km<sup>i</sup>      | This study          |
| pET24b(+)-sa2056 | Expression vector, sa2056 with His<sub>N</sub>-Tag at the C-terminus, Km<sup>i</sup>   | This study          |
| pGEX-2T    | Expression vector, N-terminal GST-Tag, Ap<sup>i</sup>                                             | GE                  |
| pGEX-2T-femA | Expression vector, femA with GST-Tag at the N-terminus, Ap<sup>i</sup>                      | This study          |
| pGEX-2T-femB | Expression vector, femB with GST-Tag at the N-terminus, Ap<sup>i</sup>                       | This study          |
| pGEX-2T-femX | Expression vector, femX with GST-Tag at the N-terminus, Ap<sup>i</sup>                    | This study          |
| pGEX-2T-sa2056 | Expression vector, sa2056 with GST-Tag at the N-terminus, Ap<sup>i</sup> | This study          |
| pHA-1(yedZ) | PhoA fusion expression plasmid containing yedZ (XhoI-KpnI) with phoA fused to the 3′-end, araB promoter | [12]                |
| pHA-F1-F14 | PhoA fusion vectors, sa2056 fragments encoding F1-F14 fused to the 5′-end of phoA               | This study          |
| pKT25      | BACTH vector, MCS at the C-terminus of the CyaA domain T25, Km<sup>i</sup>                       | [10]                |
| pKNT       | BACTH vector, MCS at the N-terminus of the CyaA domain T25, Km<sup>i</sup>                       | [13]                |
| pKT25-femA | BACTH vector, femA fused to the C-terminus of T25, Km<sup>i</sup>                                 | [11]                |
| pKT25-femB | BACTH vector, femB fused to the C-terminus of T25, Km<sup>i</sup>                                 | [11]                |
### Table S2. Cont.

| Strains          | Relevant genotype and phenotype | Reference or source |
|------------------|---------------------------------|---------------------|
| pKT25-femX       | BACTH vector, *femX* fused to the C-terminus of T25, Km<sup>i</sup> | This study          |
| pKT25-php1       | BACTH vector, *php1* fused to the C-terminus of T25, Km<sup>i</sup> | [14]                |
| pKT25-php2       | BACTH vector, *php2* fused to the C-terminus of T25, Km<sup>i</sup> | [14]                |
| pKT25-php3       | BACTH vector, *php3* fused to the C-terminus of T25, Km<sup>i</sup> | This study          |
| pKNT25-php4      | BACTH vector, *php4* fused to the N-terminus of T25, Km<sup>i</sup> | [14]                |
| pKT25-php2a      | BACTH vector, *php2a* fused to the C-terminus of T25, Km<sup>i</sup> | This study          |
| pUT18            | BACTH vector, MCS at the N-terminus of the CyaA domain T18, Ap<sup>i</sup> | [10]                |
| pUT18C           | BACTH vector, MCS at the C-terminus of the CyaA domain T18, Ap<sup>i</sup> | [10]                |
| pUT18C-femA      | BACTH vector, *femA* fused to the C-terminus of T18, Ap<sup>i</sup> | [11]                |
| pUT18C-femB      | BACTH vector, *femB* fused to the C-terminus of T18, Ap<sup>i</sup> | This study          |
| pUT18C-femX      | BACTH vector, *femX* fused to the C-terminus of T18, Ap<sup>i</sup> | This study          |
| pUT18C-php1      | BACTH vector, *php1* fused to the C-terminus of T18, Ap<sup>i</sup> | [14]                |
| pUT18C-php2      | BACTH vector, *php2* fused to the C-terminus of T18, Ap<sup>i</sup> | This study          |
| pUT18C-php3      | BACTH vector, *php3* fused to the C-terminus of T18, Ap<sup>i</sup> | [14]                |
| pUT18C-php4      | BACTH vector, *php4* fused to the N-terminus of T18, Ap<sup>i</sup> | [14]                |
| pUT18C-php2a     | BACTH vector, *php2a* fused to the C-terminus of T18, Ap<sup>i</sup> | This study          |
| pUT18C-sa2056    | BACTH vector, *sa2056* fused to the C-terminus of T18, Ap<sup>i</sup> | This study          |

MCS, multiple cloning site; Ap<sup>i</sup>, ampicillin resistant; Cm<sup>i</sup>, chloramphenicol resistant; Em<sup>i</sup>, erythromycin resistant; Lss<sup>i</sup>, lysostaphin resistant; Mc<sup>i</sup>, methicillin resistant; Tc<sup>i</sup>, tetracycline resistant.
**Supplementary Table S3.** Primers used in this study.

| Primer | Sequence 5'-3' | Use | Reference |
|--------|----------------|-----|-----------|
| CQ10  | TCCACCTCTCCACTGACAGA | Confirmation pCQ44 integration | This study |
| CQ31  | AGTGTGGGGAGATCTAAGTG | Confirmation pCQ44 integration | [3] |
| CQ33  | ATGGACGAGCTGACACTA | Sequencing CQ48 | This study |
| CQ72  | TATAGCTGTCCTAGTTGATATACTG-TTTT | Construction of pCQ44 | This study |
| CQ73  | AAATCGAGCAAGAAACAGGGATTATTGC | Construction of pCQ44 | This study |
| CQ74  | CTAATCTGTCCTGTTTGTGC | Sequencing CQ48 | This study |
| EH4   | CTGCCACTTATGTAATATCTGTTAATCCCAC | Construction of pU18C-php2 | This study |
| EH34  | CGGTCTCACGATGATAAATATCTGATTAC | Construction of pHAI-F1-14 | This study |
| EH35  | CCGGTACCCGTTAGTAAATCTAAATTCTCCA | Construction of pHAI-F1 | This study |
| EH36  | ATAGGTACCGAGTACCCCATATTCTT | Construction of pHAI-F2 | This study |
| EH37  | ACCTGACCGGATTTTGAATTTGAC | Construction of pHAI-F3 | This study |
| EH38  | GATGTTACCACATACCTCAATTTCAGAG | Construction of pHAI-F4 | This study |
| EH39  | CCGGTACCCGATATATTTTCAACAA | Construction of pHAI-F5 | This study |
| EH40  | TATGGTACCCTAATATCTGCTACTG | Construction of pHAI-F6 | This study |
| EH41  | ATCCTGTAACATCTCTTACTGATGTGT | Construction of pHAI-F7 | This study |
| EH42  | ATGGTACCTTCTGCTGCTGCT | Construction of pHAI-F8 | This study |
| EH43  | TATGGTACCCTGAGAATATCTGACAGTGAC | Construction of pHAI-F9 | This study |
| EH44  | CCGGTACCCCATTCATTTAATTTGATAA | Construction of pHAI-F10 | This study |
| EH45  | TAGGTACCCGTTCTCCGATTAATAG | Construction of pHAI-F11 | This study |
| EH46  | TATGGTACCCTCATCTCCATGCC | Construction of pHAI-F12 | This study |
| EH47  | GTGGTGACGAAAAATGATTACGCACTAT | Construction of pHAI-F13 | This study |
| EH48  | CATGGTACCCGCTCTAGTGAATCTG | Construction of pHAI-F14 | This study |
| EH50  | AACCTGACGGAGAAACAAAAAGAGATCTTCA | Construction of pU18C-php2 | This study |
| MS79  | ATGGATCTCCGGAAGCAGAAAAATTTAATTTA | Construction of pET24b-php1 | This study |
| MS80  | TTAATCGAGGGATCTCCTTAACTG | Construction of pET24b-php1 | This study |
| MS81  | TTGGGATCCCTAAAAGACTAAAAAGATTTCAAAT | Construction of pET24b-php3 | This study |
| MS82  | TTAATCGAGTTTGCTCTTGTCTTTATTTTATC | Construction of pET24b-php3 | This study |
| MS83  | ATGGGATCCCCAAATTATATTTATATTATCATCAGTTT | Construction of pET24b-php4 | This study |
| MS84  | TTAATCGAGTTTCTTTTTCTTAAATATACGATTT | Construction of pET24b-php4 | This study |
| MS85  | ATGGGATCCCAAAATGAAAAATATTTCTTCACT | Construction of pET24b-mecA | This study |
| MS86  | TTAATCGAGTCTTATATCTGTTACTTTTATT | Construction of pET24b-mecA | This study |
| MS106 | CTAAGATCTTCTTTTCTTTTATTTTACCGATATTT | Construction of pGEX-2T-sa2056 | This study |
| MS107 | CGTGAATCCATAAAAAGCATTTAATTTTTC | Construction of pGEX-2T-sa2056 | This study |
| MS108 | CTAGAATCTTCTTTTATTTTACCTG | Construction of pGEX-2T-sa2056 | This study |
| MS109 | GTAAGATCTGAAAAATGATCTATGCTACAATAC | Construction of pGEX-2T-femX | This study |
| MS116 | GCAGGTACCCTTATTCTTATTTTACG | Construction of pKT25-femB and pU18C-femB | This study |
| MS117 | GTTGAATCCTTTCTTCTTTTATTTTTAG | Construction of pGEX-2T-femB | This study |
| MS118 | CTAGAATCTTATTCTTTTGTTTATTTACGAG | Construction of pGEX-2T-femX | This study |
| MS155 | ATGGGATCCGTACGTTTCTATTAAACAG | Construction of pHAI-F3a | This study |
| MS156 | GAAGGTACCTGTAAGATTGTCTTTTCAAAAC | Construction of pHAI-F3b | This study |
| MS157 | ATGGGATCCCGTCTGCTACGAATGTTTTC | Construction of pHAI-F3c | This study |
| MS158 | GATGGTACCCCAATTGGGCTGCTAAGATGTTTTC | Construction of pHAI-F3d | This study |
Table S3. Cont.

| Primer | Sequence 5′-3′ | Use | Reference |
|--------|----------------|-----|-----------|
| MS159  | GATGGTACCGAATTGCGCGTCGTACGAATG | Construction of pHAI-F3e | This study |
| SR2    | CGAGCTACGCGAAAAATGCTATACATAAATT | Construction of pET24b-femX | [15] |
| SR3    | GCACTCGAGTTTGGTTTAAATTCAG | Construction of pET24b-femX | [15] |
| SR71   | CGTCTCGAGTGTGTTAGGATGAACTGGTTT | Construction of pET24b-sa2056 | This study |
| SR73   | GCAGCTACGATTAAAAAGCTATACATAATTTTCTTTT | Construction of pET24b-sa2056 | This study |
| SR100  | GCACGTCAAGGAATTTACAGAGTTAATCTG | Construction of pUT18C-femB | [11] |
| SR101  | GCACGTCAAGGAATTTACAGAGTTAATCTG | Construction of pKT25-femB | [11] |
| SR103  | CATCTCGAGGAAAAAGATGCATATCAG | Construction of pKT25-femX | [11] |
| SR104  | CATCTCGAGGAAAAAGATGCATATCAG | Construction of pUT18C-femX | [11] |
| SR105  | GCAGGTACCTATTTTGCATTAAAAATTCAG | Construction of pKT25-femX | [11] |
| SR106  | GTTGGATCCAAGTTTACAAATTTACAGCTA | Construction of pGEX-2T-femA | [11] |
| SR107  | GTTGGATCCAAGTTTACAAATTTACAGCTA | Construction of pGEX-2T-femA | [11] |
| SR108  | CAAGGATCCAAATTTACAGAGTTAATCTGTTAC | Construction of pGEX-2T-femB | [11] |

Restriction sites are underlined.

**References and Notes**

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