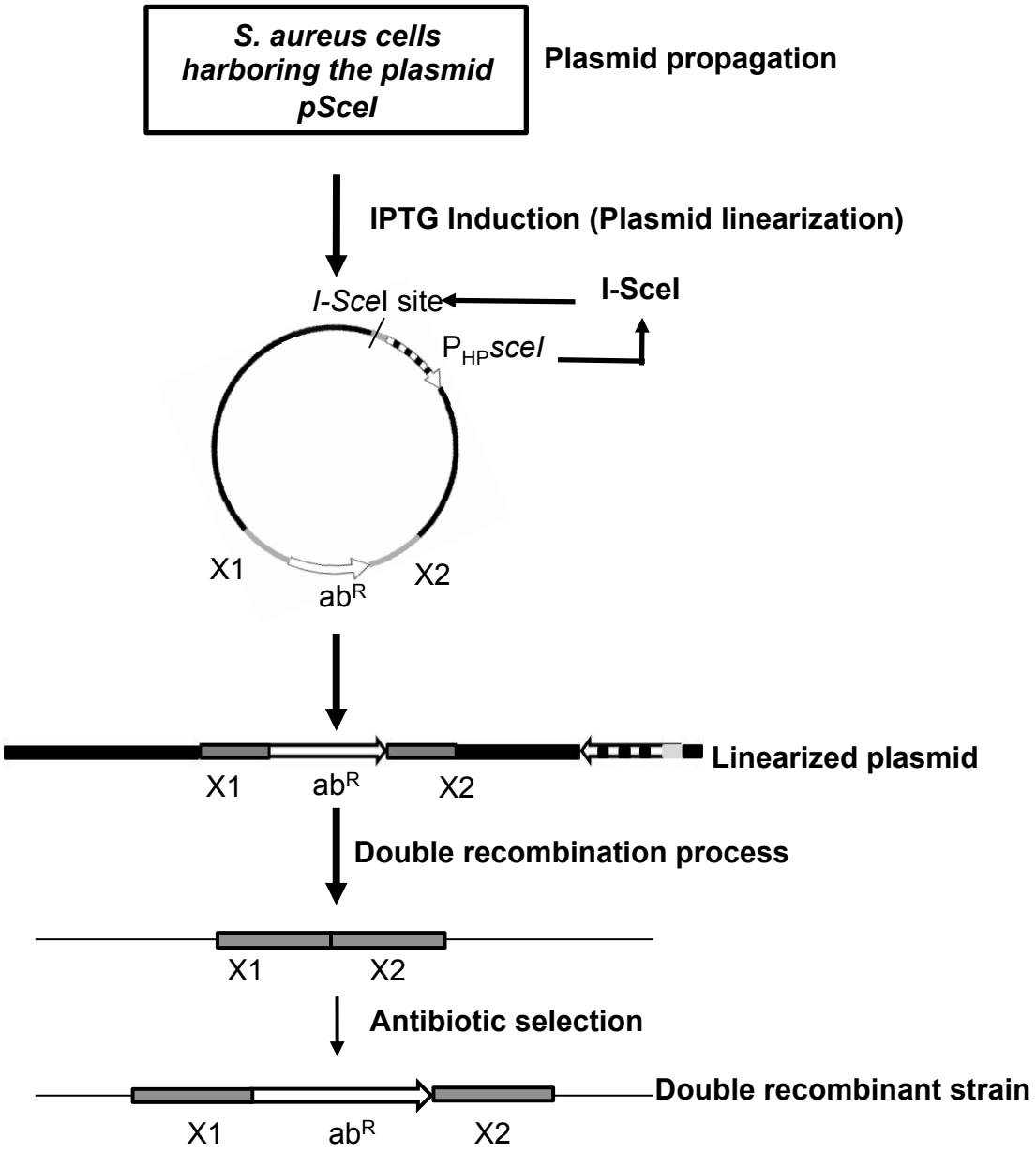


Figure S1: Generation of deletion mutants in *S. aureus* in a single step, double-recombination event. The plasmid pSceI carrying the deletion cassette is propagated in *S. aureus* and linearized by adding IPTG to the culture. The addition of IPTG triggers the expression of I-*SceI* enzyme, which recognizes a unique restriction site I-*Sce*I in the backbone of the plasmid. The double recombination event delete the gene of interest and confers to the resultant strain the antibiotic resistance necessary to grow in selective media. False positives colonies harboring circular plasmid can be easily distinguished from the knock-out mutants because the plasmid harbors the gene *β-gaB* gene thus colonies turn blue in the presence of X-gal.

Yepes *et al*. Figure S1

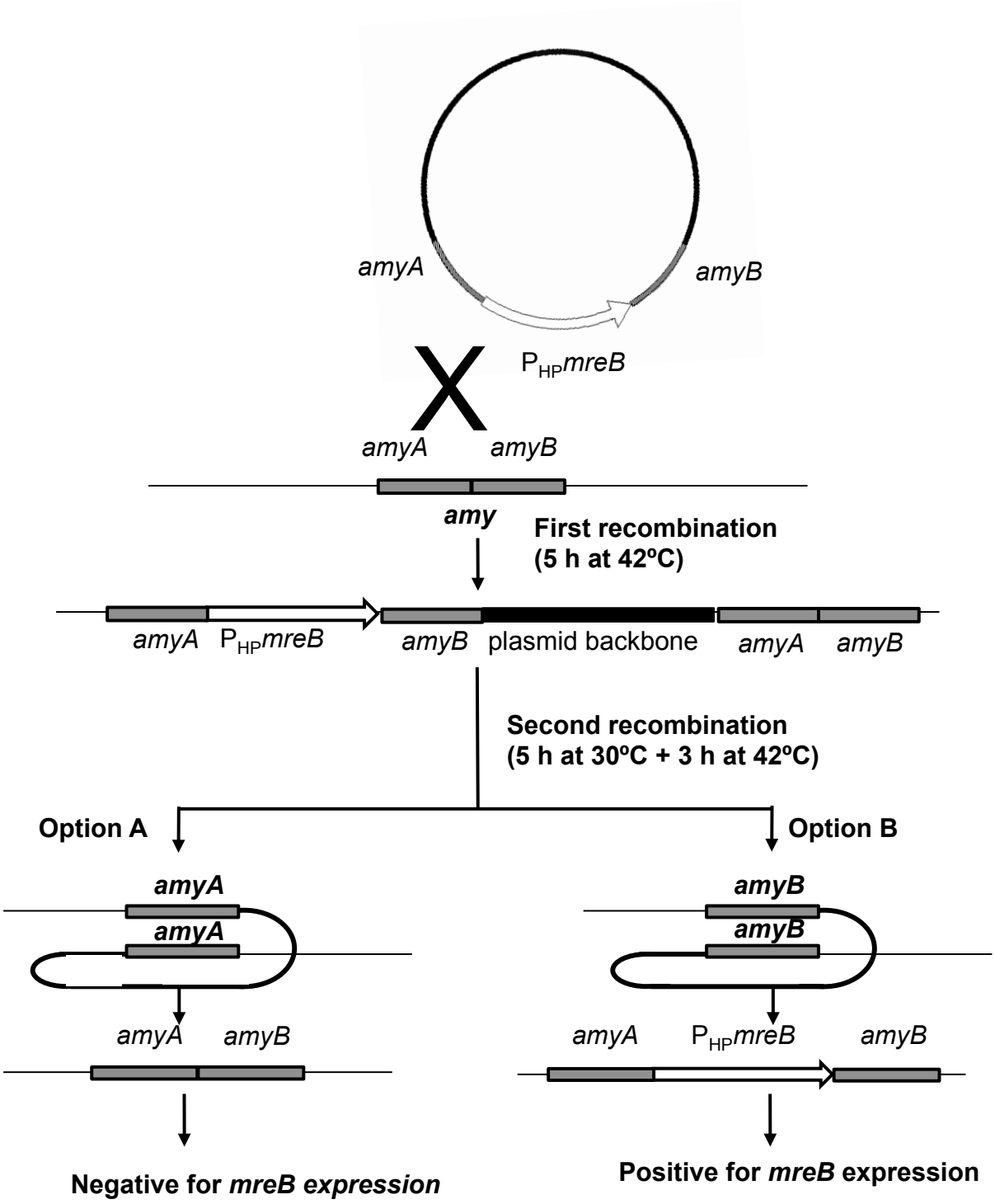


Figure S2: Insertion of *mreB* gene of *B. subtilis* into the neutral locus *amy* of *S. aureus*. pAmy plasmid inserted its cargo into the neutral locus *amy* via two sequential processes of recombination. The resultant colonies that went through two recombination steps did not express the *β-gaB* and *ermC* genes placed in the backbone of the plasmid. Those colonies were sensitive to erythromycin and not blue in the presence of X-gal. The process of sequential recombination generated two different genetic backgrounds. One type of colonies had restored the native *amy* locus (option A) while another type of colonies had disrupted the *amy* locus and incorporated the cargo (option B). PCR validation of the resultant colonies is necessary to discard option A.

Yepes *et al*. Figure S2

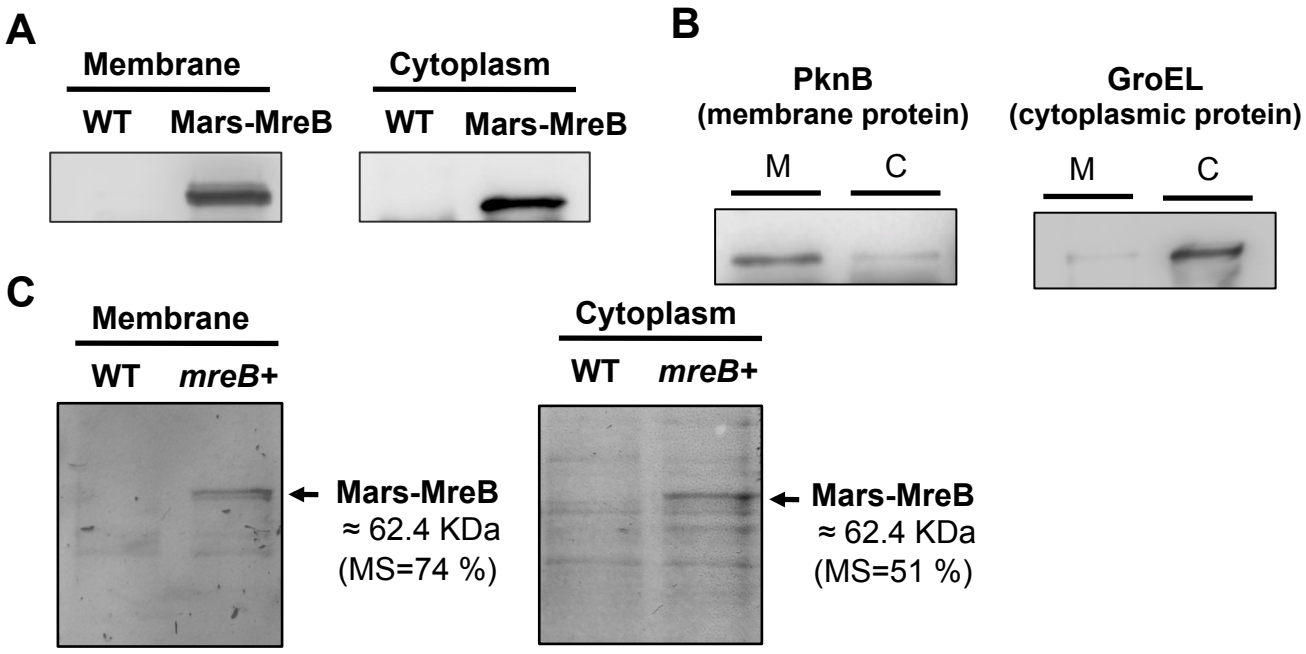


Figure S3: Subcellular localization of MreB (A) Detection of Mars-MreB in membrane (left) and cytoplasmic (right) fractions by western blot analysis, using antibodies against Mars protein. **(B)** Controls of cross contamination of the membrane fraction (M) and the cytoplasmic fraction (C). The Detection of the membrane-bound histidine-kinase PknB was used as membrane marker and the cytoplasmic-associated chaperone GroEL was used as cytoplasmic marker. **(C)** Purification of MreB from the membrane and cytoplasmic associated protein fraction and identification by mass spectrometry analysis. Identification coverage is shown in brackets (MS).

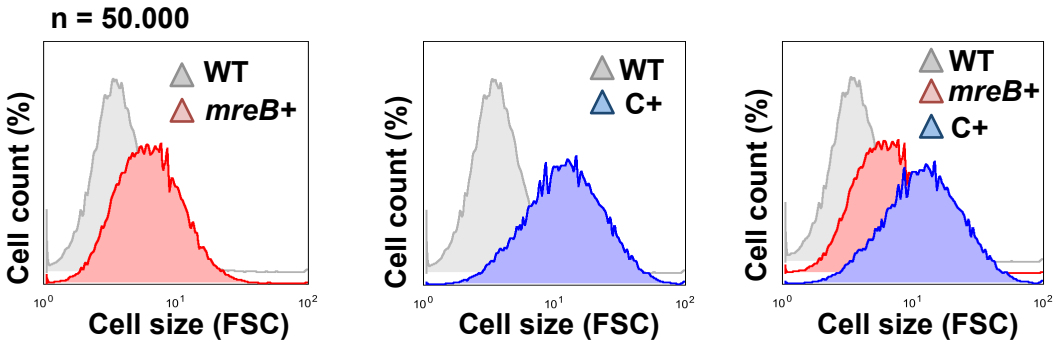
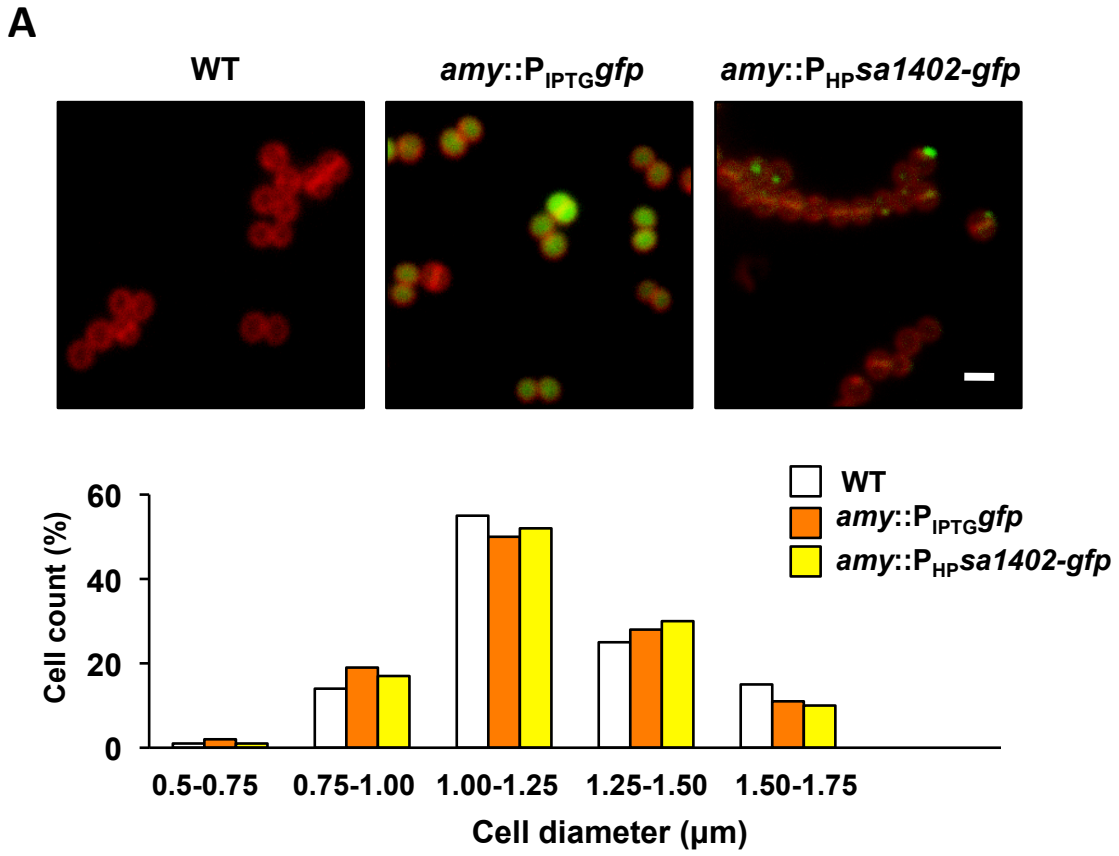


Figure S4: Flow cytometry analysis to monitor the diameter of 50,000 cells selected from wild type (grey profile) and *mreB*+ strains (red profile). Positive control of cell size is *B. subtilis* cells (blue profile). Cell size was monitored using forward light scatter detection (FSC). Notice that FSC values are in logarithmic scale.



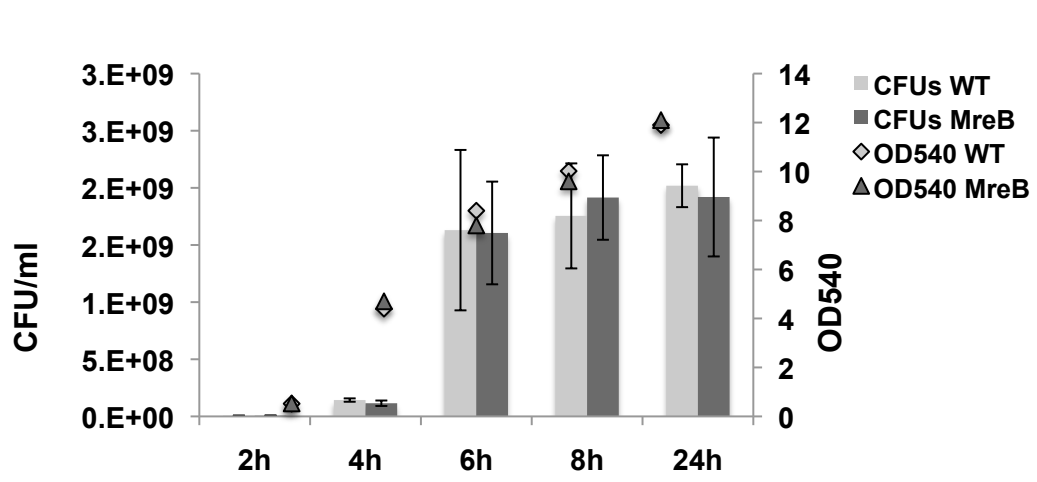


Figure S5: (A) Production of proteins different from MreB does not affect the cell size of *S. aureus*. Fluorescence microscopy pictures of wild-type cells (left panel), cells producing a cytoplasmic GFP (center panel) and a GFP fused to a membrane-associated protein that oligomerizes in the membrane of *S. aureus* cells (SA1402 protein) (right panel). Cells membrane was stained with Nile Red (false coloured in red). Scale bar is 2 µm. Quantitative cell size assay in wild-type and strains expressing a cytoplasmic GFP (orange bars) and GFP fused to a membrane-associated protein (yellow bars) are shown below. N=500. **(B) *mreB*-expressing cells exhibit similar growth rate than wild-type cells**. Quantification of growth rate of different *S. aureus* strains. Cell growth was monitored by counting colony forming units (CFU) (left Y axis) simultaneously to measuring OD_{540} (right Y axis). Cells were grown in TSB medium at 37°C during 24h (X axis).

Yepes *et al*. Figure S5

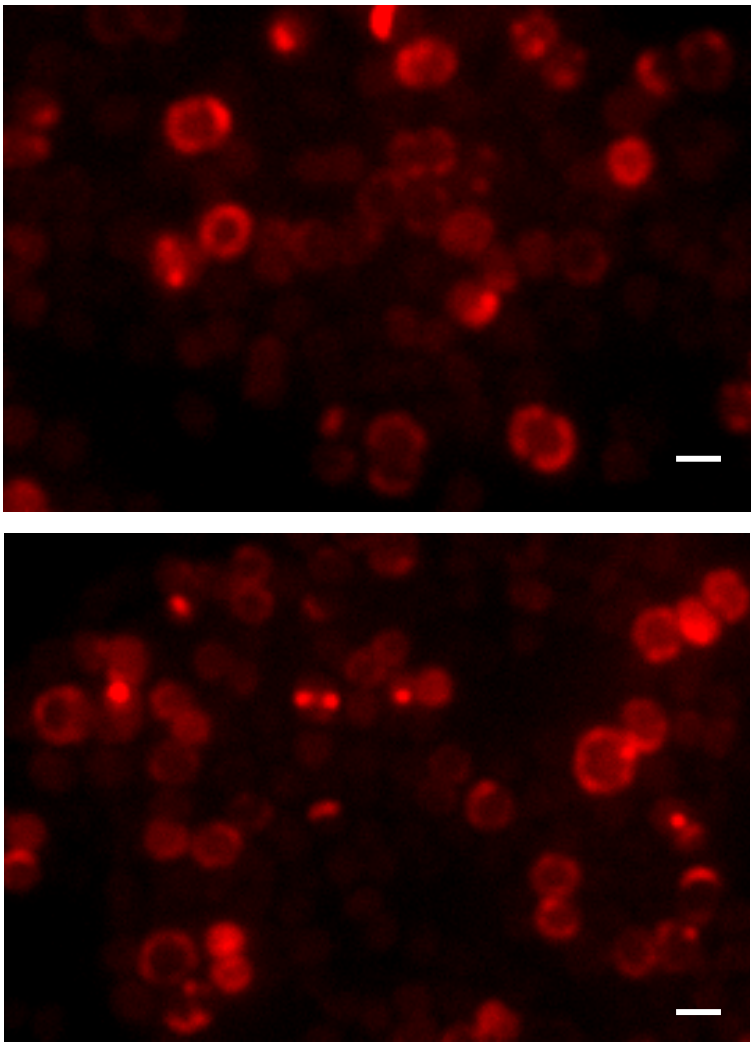


Figure S6: Microscopic fields of *S. aureus* cells expressing *mars-mreB*. Fluorescence micrographs of a field of exponentially growing cells labeled with the translational fusion Mars-MreB (false colored in red). The fluorescence signal is mainly localized beneath membranes. *S. aureus* cells divide in sequential orthogonal planes and generates fields of cells positioned at different planes. Scale bar is 1 μm.

Supplementary tables

Table S1

Strain	*amy* locus	*lac* locus
RF122	SAB2338c	SAB2075c
USA300	SAUSA300_2400	SAUSA300_2154
COL	SACOL2463	SACOL2185
JH1	SaurJH1_2531	SaurJH1_2264
JH9	SaurJH9_2482	SaurJH9_2225
MRSA252	SAR2545	SAR2285
MSSA476	SAS2347	SAS2095
MW2	MW2379	MW2120
Mu3	SAHV_2439	SAHV_2178
Mu50	SAV2455	SAV2194
N315	SA2244	SA1996
NCTC8325	SAOUSHC_02755	SAOUSHC_02454
Newman	NWMN_2354	NWMN_2098

Table S1: Identification of *amy* and *lac* loci in the distinct strains of *S. aureus*.

The numeration of the distinct chromosomal genes differs between *S. aureus* strains due to strain-specific insertion of genetic mobile elements whose genes are subsequently included in the gene numeration of each particular strain. For better clarification, we have decided to name our neutral loci as *amy* and *lac* throughout the body of the paper.

Table S2: List of strains used in this study.

Strain	Genotype	Reference
S. aureus		
Newman	wild type	(1)
Newman mreB+	*amy::* $P_{hyper\text{-}spank}$-*mreB*	This study
Newman marsmreB	*amy::* P_{XYL}-*marsmreB*	This study
Newman gfp	*amy::* $P_{hyper\text{-}spank}$-*gfp*	This study
Newman sa1402-gfp	*amy::* $P_{hyper\text{-}spank}$-*sa1402-gfp*	This study
E. coli		
BTH101	pKT25-mreB; pUT18C-ftsZ	This study
BTH101	pKT25-mreB; pUT18C-pbp2	This study
BTH101	pKT25-mreB; pUT18C-pbp3	This study
BTH101	pKT25-mreB; pUT18C-mreB	This study
BTH101	pKT25-mreB; pUT18C-mreC	This study
BTH101	pKT25-mreB; pUT18C-mreD	This study
BTH101	pKT25-mreB; pUT18-ezrA	This study
BTH101	pKT25-mreB; pUT18-ftsA	This study
BTH101	pUT18C-mreB; pKT25-pbp2	This study
BTH101	pUT18C-mreB; pKT25-pbp3	This study
BTH101	pUT18C-mreB; pKT25-mreC	This study
BTH101	pUT18C-mreB; pKT25-mreD	This study

BTH101	pUT18C-mreB; pKT25-ftsZ	This study
BTH101	pUT18C-mreB; pKNT25-ezrA	This study
BTH101	pUT18C-mreB; pKNT25-ftsA	This study
B. subtilis		
Wild type 168		(2)

Table S2: List of strains used in this study.

Table S3

Plasmid	**Features**	**Reference**
pDR111	*amyE::* $P_{hyper\text{-}spank}$ *-spc*	(3)
pSG1154	*bla amyE:: spc* P_{XYL} *gfpmut1*	(4)
pSW-1	*bla*, *ori*RK2, *xylS*, *Pm→I-sce*I	(5)
pMAD	*bla*, Ori pBR325, *ermC*, *bgaB*, *pclp*B	(6)
pCell	*lacB* (Nwmn 2098)::MCS	This study
pLac	*cell* (Nwmn 2354)::MCS	This study
pSce	$P_{hyper\text{-}spank}$ *–I-sce*I	This study
$pAmy_{IPTG}$	*amy::* $P_{hyper\text{-}spank}$	This study
$pAmy_{XYL}$	*amy::* P_{XYL}	This study
$pAmy_{YFP}$	*amy::* YFP	This study
$pAmy_{MARS}$	*amy::* Mars	This study
$pAmy_{HIS}$	*amy::* His(6x)	This study
$pLac_{IPTG}$	*lac::* $P_{hyper\text{-}spank}$	This study
$pLac_{XYL}$	*lac::* P_{XYL}	This study
$pLac_{YFP}$	*lac::* YFP	This study
$pLac_{MARS}$	*lac::* MARS	This study
$pLac_{HIS}$	*lac::* His(6x)	This study
$pCell_{IPTG}$MreB	*amy::* $P_{hyper\text{-}spank}$-*mreB*	This study
pCellMARSMreB	*amy::* P_{Xyl}-*Mars-mreB*	This study
pKT25-mreB	*km, T25 domain in frame with mreB in C-terminal*	This study
pKT25-pbp2	*km, T25 domain in frame with pbp2 in C-terminal*	This study
pKT25-pbp3	*km, T25 domain in frame with pbp3 in C-terminal*	This study
pKT25-mreC	*km, T25 domain in frame with mreC in C-terminal*	This study
pKT25-mreD	*km, T25 domain in frame with mreD in C-terminal*	This study
pKT25-ftsZ	*km, T25 domain in frame with ftsZ in C-terminal*	This study
pKNT25-ezrA	*km, T25 domain in frame with ezrA in N-terminal*	This study
pKNT25-ftsA	*km, T25 domain in frame with ftsA in N-terminal*	This study
pUT18-ezrA	*Amp, T18 domain in frame with ezrA in N-terminal*	This study
pUT18-ftsA	*Amp, T18 domain in frame with ftsA in N-terminal*	This study
pUT18C-mreB	*Amp, T18 domain in frame with mreB in C-terminal*	This study
pUT18C-pbp2	*Amp, T18 domain in frame with pbp2 in C-terminal*	This study
pUT18C-pbp3	*Amp, T18 domain in frame with pbp3 in C-terminal*	This study
pUT18C-mreC	*Amp, T18 domain in frame with mreC in C-terminal*	This study
pUT18C-mreD	*Amp, T18 domain in frame with mreD in C-terminal*	This study
pUT18C- ftsZ	*Amp, T18 domain in frame with ftsZ in C-terminal*	This study

Table S3: List of plasmids used in this study.

21 **Table S4**

Name	Sequence
AYG63	TTTTAGATCTTTTGAAAAATTAGAGCCTAAATATT
AYG104	GAATTCTCGAGTCGACCATGGCTAGCGGATCCAGCTAATGCACAGCCATAG
AYG105	GGATCCGCTAGCCATGGTCGACTCGAGAATTCAGGTATTAAAACGTTTAAAAG
AYG66	TTTTGCCGGCTGCATCAAATAACTGATTCGTTG
AYG59B	TTTTGCCATGGCTTTTCTCAAGATTTTCATAACC
AYG60B	TGAATTCGGATCCGTCGACCCGGGCTCGAGCTAGCGTCATATCACGTACTAGTGC
AYG61B	GCTAGCTCGAGCCCGGGTCGACGGATCCGAATTCAGAAAAATGGGACAGAGGCG
AYG62	TTTTGCCGGCTACACTTATTGTCGGAATATTCAC
AYG147	TTTTTGTCGACAAGGAGGGTGAATGAATCGGAC
AYG148	TTTTTGCTAGCCCTAGCGTTTGCAATGCACC
AYG149	TAGGGATAACAGGGTAATACGTTGCTCGAGGGTAAATG
AYG151N	TTTTGCTCTTCCAGCAACTCACATTAATTGCGTTG
AYG150	TTTTGCTCTTCCGCTTAGGGATAACAGGGTAATACG
cm fwd	CTTGATAATAAGGGTAACTATTGCC
cm rev	GGGTAACTAGCCTCGCCGGTCCACG
lac5 down	CTTGATAATAAGGGTAACTATTGCCGTAATTTTTCCTCCTTAG
lac3 up	GGGTAACTAGCCTCGCCGGTCCACGGACACATATGATTTTAAC
amy5 down	CTTGATAATAAGGGTAACTATTGCCATTCATCCTCCTGTATTTC
amy3 up	GGGTAACTAGCCTCGCCGGTCCACGCAAATCCATAAATAATAAAG
mreBfwd	AAAAGTCGACTAAGGAGGAAGAAAGGAAGATACATACATA
mreBrev	AAAAGCTAGCCTCTTCTATTGAACTCCCG
AYG 207	TTTTTACTAGTCCTCTTCTATTGAACTCCCG
AYG 213	CATTCAACAGGTGCAGGAATGTTTGGAATTGGTGC
AYG 211	TTTTTCTCGAGTAAGGAGGTTTAAATGGCATCATCAGAAGATG
AYG 212	GCACCAATTCCAAACATTCCTGCACCTGTTGAATG
AYG 165	TTTTTGGATCCACGATATTCACGGTTTACCC
AYG 166	TTTTTGCTAGCGATAATTCGCGGCCGCTCTAG
AYG173	TTTTTGCTAGCATATCAAGCTTATCGATACCG
GK142	AAAAGTCGACATGGCATCATCAGAAGATGTT
GK143	TTTTGCTAGCTTATCCTGCACCTGTTGAATG
JC86	TTTTTGGATCCTTATCCTGCACCTGTTGAATG
GK139	AAAAGTCGACATGGACAAAGACTGCG
GK140	TTTTTTGCTAGCTTAGCCTGCAGGACCC
GK141	TTTTTTGGATCCTTAGCCTGCAGGACCC
GK144	CTTAGTGATGATGATGATGATGATGGTCGACGGATCCGAATTC
GK146	GATCCTTAGTGATGATGATGATGATGATGGTCGACTCGAGAATTC
GK145	GACCATCATCATCATCATCATCACTAAGCTAGCGTCATATCACG
AYG100	ATCCGTCGACTACATAAGGAGGAACTACTATGAG
AYG101	CTCAGGATCCTTATTTGTATAGTTC
AYG101b	TTTTTGCTAGCTTATTTGTATAGTTCATCCATGC
GK38	AGCAAAGAATTCCGTATTAAAGATATAGG
GK19	ATTTATAAGCTTCACAATTTCACCTCGC
GK2	AAAAAAAAGCTTATGTTTAGTTTAAG
GK3	CTTCTCCTTTACTCATATGTTCAGGTGACTCA
GK4	TGAGTCACCTGAACA TATGAGTAAAGGAGAAG
GK201	TCTAGAGGATCCATTTGGAATTGGTGC
GK202	ACGGCCGAATTCTTATCTAGTTTTCCCTTTG
GK203	CGCCACTGCAGAACGGAAAACAAAGGATC
GK204	ACCCGGGGATCCTTAGTTGAATATACCTG
GK205	ACGCCACTGCAGATTAAAAAGACTAAAAG
GK206	ACCCGGGGATCCTTATTTGTCTTTGTC

GK207	ACGCCACTGCAGGCTTAAGTTTTTTAAAAATAAC
GK208	ACCCGGGGATCCTTATTTATCCCTGCTTTCATC
GK209	ACGCCACTGCAGACGTACACTGTATTATTTTTTG
GK210	ACCCGGGGATCCTTACCATTGACGACGTTTC
GK211	CTGCAGGTCGACATTAGAATTTGAACAAGG
GK212	ACCCGGGGATCCTTAACGTCTTGTTCTTCTTG
GK213	CGCCAAAGCTTATGGAAGAACATTACTACG
GK214	ACCCGGGGATCCTCAAATAGAGATTTCATTAG
GK215	CGCCAGCATGCATGGTGTTATATATCATTTTGG
GK216	ACCCGGGGATCCTGCTTAATAACTTCTTC
GK235	AAAAAAGCTTATGTTTGGAATTGGTGC
GK236	AAAAGGATCCCTAGTTTTCCCTTTGAAAAG
GK237	AAAACTGCAGATGACGGAAAACAAAGG
GK238	AAAAGGATCCTTGAATATACCTGTTAATC
GK239	AAAAAAGCTTATGCTTAAGTTTTTTAAA
GK240	AAAAGGATCCTTATCCCTGCTTTCATC
GK241	AAAAAAGCTTATGCGTACACTGTATTATTT
GK242	AAAAGGATCCCATTGACGACGTTTCATGTC
GK243	AAAACTGCAGAACGTACACTGTATTAT
GK244	AAAACTGCAGTGCTTAAGTTTTTT
GK245	AAAACTGCAGTGACGGAAAACAAAG

Table S4: List of primers used in this study.

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