

**Supplementary Table 1. Strains used in this study.**

Strains	Relevant characteristics	References
<b><i>E. coli</i> strain</b>		
XL1 blue	$\Delta(\text{ara-leu})$ 7697 araD139 fhuA $\Delta\text{lacX74}$ galK16 galE15 e14- $\phi$ 80dlacZ $\Delta$ M15 recA1 relA1 endA1 nupG rpsL (StrR) rph spoT1 $\Delta(\text{mrr-hsdRMS-mcrBC})$	
<b><i>S. aureus</i> strains</b>		
RN4220	Restriction-defective derivative of 8325-4	Kreiswirth <i>et al.</i> , 1983
N315	Methicillin-resistant <i>S aureus</i> (MRSA) strain isolated in 1982 from the pharyngeal smear of a Japanese patient	Okonogi <i>et al.</i> , 1989
N315_ $\Delta\text{sprG1/sprF1}$	N315 strain deleted for <i>sprG1</i> and <i>sprF1</i>	Pinel-Marie <i>et al.</i> , 2014

**Supplementary Table 2. Plasmids used in this study.**

Plasmids	Relevant characteristics	References
pALC2073	Multicopy shuttle vector with the tetracycline-inducible P <sub>xyI/tetO</sub> promoter and with Cat <sup>R</sup> in <i>S. aureus</i>	Bateman <i>et al.</i> , 2001
pALC2073Ω <i>sprG1</i> <sub>312</sub> / <i>sprF1</i>	pALC2073 with <i>sprG1</i> <sub>312</sub> under the control of tetracycline-inducible P <sub>xyI/tetO</sub> promoter and with <i>sprF1</i> under the control of its endogenous promoter	This study
pALC2073Ω <i>sprG1</i> <sub>312</sub> - <i>STOP</i> <sub>1</sub> / <i>sprF1</i>	pALC2073Ω <i>sprG1</i> <sub>312</sub> / <i>sprF1</i> with the first AUG codon, encoding to the methionine M <sub>1</sub> , mutated to the UAA codon	This study
pALC2073Ω <i>sprG1</i> <sub>312</sub> - <i>M14A/sprF1</i>	pALC2073Ω <i>sprG1</i> <sub>312</sub> / <i>sprF1</i> with the second AUG codon, encoding to the methionine M <sub>14</sub> , mutated to the GCC alanine codon	This study
pALC2073Ω <i>sprG1</i> <sub>312</sub> - <i>M14A</i>	pALC2073 with <i>sprG1</i> <sub>312</sub> with the second AUG codon, encoding to the methionine M <sub>14</sub> , mutated to the GCC alanine codon	This study
pALC2073Ω <i>sprG1</i> <sub>312</sub> - <i>STOP</i> <sub>1,2,14</sub> / <i>sprF1</i>	pALC2073Ω <i>sprG1</i> <sub>312</sub> / <i>sprF1</i> with -the first and the second AUG codon, encoding to the methionine M <sub>1</sub> and M <sub>14</sub> , mutated to the UAA stop codon - the first GUG codon, encoding to the valine V2, mutated to the UAA stop codon	This study
pALC2073Ω <i>sprG1</i> <sub>312</sub> - <i>STOP</i> <sub>1,2,14,17</sub> / <i>sprF1</i>	pALC2073Ω <i>sprG1</i> <sub>312</sub> / <i>sprF1</i> with -the first and the second AUG codon, encoding to the methionine M <sub>1</sub> and M <sub>14</sub> , mutated to the UAA stop codon - the first GUG codon, encoding to the valine V2, mutated to the UAA stop codon -the second ATT codon, encoding to the isoleucine I <sub>17</sub> , mutated to the UAA stop codon	This study
pALC2073Ω <i>sprG1</i> <sub>312</sub> - <i>3XFlag-Ct/sprF1</i>	pALC2073Ω <i>sprG1</i> <sub>312</sub> / <i>sprF1</i> with a 3XFLAG sequence before the termination codon of the internal coding sequence of SprG1 <sub>312</sub>	This study
pALC2073Ω <i>sprG1</i> <sub>312</sub> - <i>3XFlag-Ct-STOP</i> <sub>1</sub> / <i>sprF1</i>	pALC2073Ω <i>sprG1</i> <sub>312</sub> - <i>3XFlag-Ct/sprF1</i> with the first AUG codon, encoding to the methionine M <sub>1</sub> , mutated to the UAA codon	This study

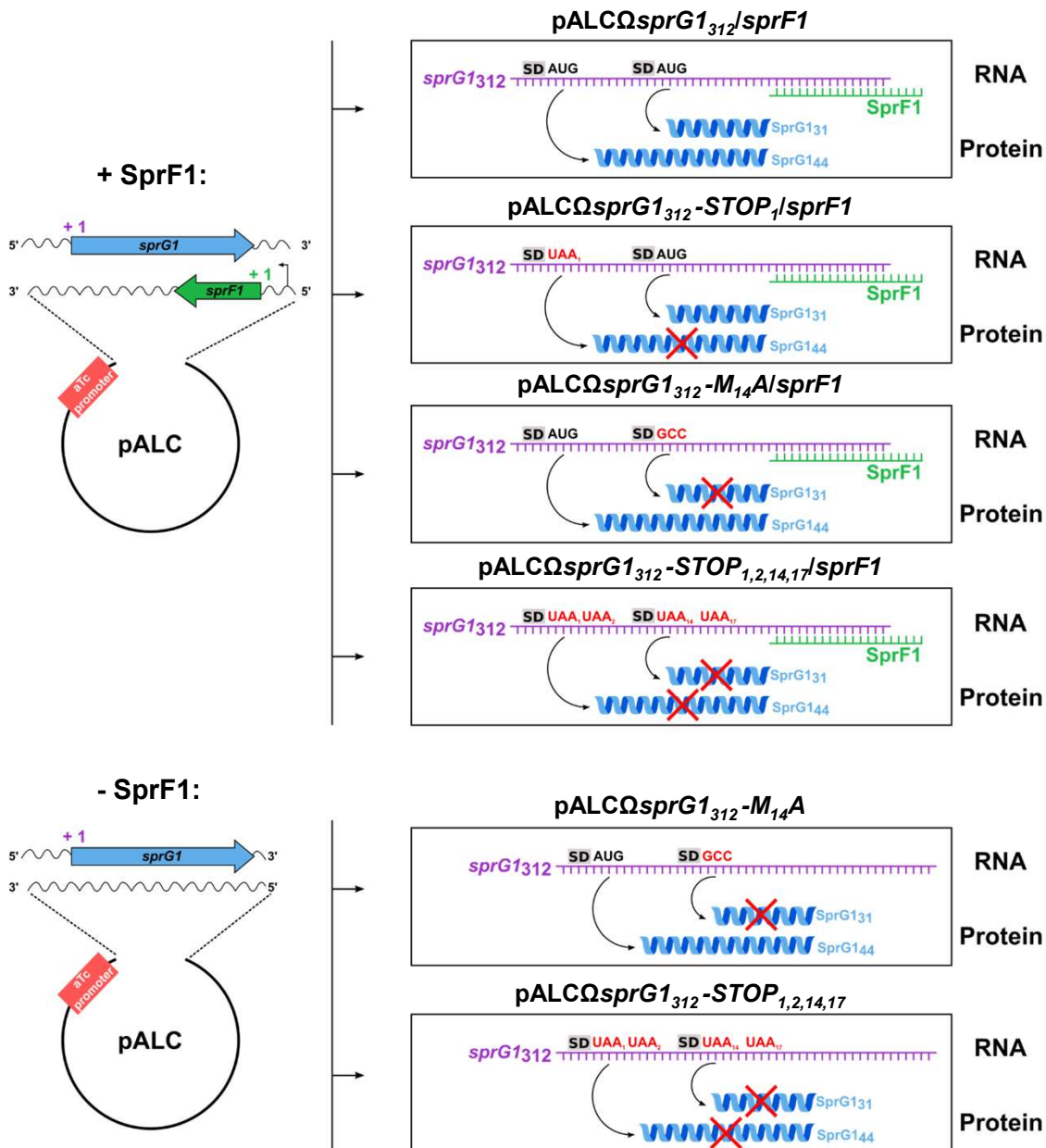
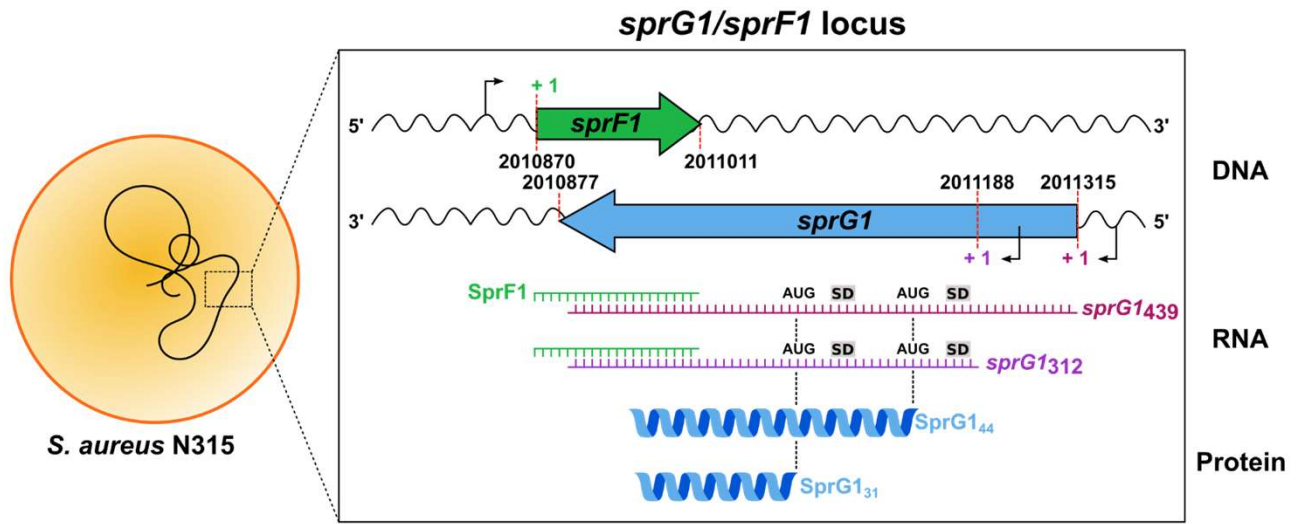
pALC2073 $\Omega$ sprG1 <sub>312</sub> - 3XFlag-Ct-M14A/sprF1	pALC2073 $\Omega$ sprG1 <sub>312</sub> -3XFlag-Ct/sprF1 with the second AUG codon, encoding to the methionine M <sub>14</sub> , mutated to the GCC alanine codon	This study
pALC2073 $\Omega$ sprG1 <sub>312</sub> - 3XFlag-Ct-STOP <sub>1,2,14</sub> /sprF1	pALC2073 $\Omega$ sprG1 <sub>312</sub> -3XFlag-Ct/sprF1 with -the first and the second AUG codon, encoding to the methionine M <sub>1</sub> and M <sub>14</sub> , mutated to the UAA stop codon - the first GUG codon, encoding to the valine V2, mutated to the UAA stop codon	This study
pALC2073 $\Omega$ sprG1 <sub>312</sub> - 3XFlag-Ct- STOP <sub>1,2,14,17</sub> /sprF1	pALC2073 $\Omega$ sprG1 <sub>312</sub> -3XFlag-Ct/sprF1 with -the first and the second AUG codon, encoding to the methionine M <sub>1</sub> and M <sub>14</sub> , mutated to the UAA stop codon - the first GUG codon, encoding to the valine V2, mutated to the UAA stop codon -the second ATT codon, encoding to the isoleucine I <sub>17</sub> , mutated to the UAA stop codon	This study
pALC2073 $\Omega$ sprG1 <sub>312</sub> - STOP <sub>1</sub> - $\Delta$ 9/sprF1	pALC2073 $\Omega$ sprG1 <sub>312</sub> -STOP <sub>1</sub> /sprF1 with the AAG codon, encoding to the lysine K23, mutated to the UAA stop codon	This study
pALC2073 $\Omega$ sprG1 <sub>312</sub> - STOP <sub>1</sub> - $\Delta$ 2/sprF1	pALC2073 $\Omega$ sprG1 <sub>312</sub> -STOP <sub>1</sub> /sprF1 with the AAA codon, encoding to the lysine K30, mutated to the UAA stop codon	This study
pALC2073 $\Omega$ sprG1 <sub>312</sub> - STOP <sub>1</sub> -K2K3/sprF1	pALC2073 $\Omega$ sprG1 <sub>312</sub> -STOP <sub>1</sub> - $\Delta$ 2/sprF1 with two AAA codons, encoding to two lysine, added after the first AUG codon of SprG1 <sub>31</sub> peptide	This study
pALC2073 $\Omega$ sprG1 <sub>312</sub> - STOP <sub>1</sub> -F10A-F13A/sprF1	pALC2073 $\Omega$ sprG1 <sub>312</sub> -STOP <sub>1</sub> /sprF1 with the two codons UUC, encoding to the two phenylalanine F10 and F13, mutated to the GCA alanine codon	This study
pALC2073 $\Omega$ sprG1 <sub>312</sub> - STOP <sub>1</sub> -F10E-F13E/sprF1	pALC2073 $\Omega$ sprG1 <sub>312</sub> -STOP <sub>1</sub> /sprF1 with the two codons UUC, encoding to the two phenylalanine F10 and F13, mutated to the GAA glutamic acid codon	This study
pALC2073 $\Omega$ sprG1 <sub>312</sub> -STOP <sub>1</sub> - 1XFlag-Nt/sprF1	pALC2073 $\Omega$ sprG1 <sub>312</sub> -STOP <sub>1</sub> /sprF1 with a 1XFLAG sequence after the first AUG codon of SprG1 <sub>31</sub> peptide	This study
pALC2073 $\Omega$ sprG1 <sub>312</sub> -STOP <sub>1</sub> - 1XFlag-Ct/sprF1	pALC2073 $\Omega$ sprG1 <sub>312</sub> -STOP <sub>1</sub> /sprF1 with a 1XFLAG sequence before the termination codon of the internal coding sequence of SprG1 <sub>312</sub>	This study

**Supplementary Table 3. DNA primers used in this study.**

<b>Genetic constructions</b>		
<i>DNA</i>	<i>Sequences</i>	<i>Purposes</i>
SprG1 <sub>311</sub> <b>KpnI</b> _forw SprG1 <b>EcoRI</b> _rev	ATCGGGTACCCTTTGAGCAAGTTGGATAGATGGTGGCTAT ATCGGAATTCGAAAAAGGGCAACATGCGCAAACAT	pALCΩsprG1 <sub>312</sub>
SprG1311pALCKpnI_forw SprG1SprF1EcoRI_rev	ATCGGGTACCCTTTGAGCAAGTTGGATAGATGGTGGCTAT ATCGGAATTCAAAAGACGACTAATAAGCCGTCTAT	pALCΩsprG1 <sub>312</sub> /sprF1
SprG1- <b>STOP1</b> _forw SprG1- <b>STOP1</b> _rev	AAGGAGGTGGTGCCTT <b>TA</b> AGTGGCATTACTGAAA TTTCAGTAATGCCACT <b>TA</b> AGGCACCACCTCCTT	pALCΩsprG1 <sub>312</sub> - STOP <sub>1</sub> /sprF1
SprG1- <b>M14A</b> _forw SprG1- <b>M14A</b> _rev	TAGAAAGGAGACGCCTA <b>GCC</b> ATTACAATTAGTACCATG CATGGTACTAATTGTAAT <b>GGC</b> TAGGCGTCTCCTTTCTA	pALCΩsprG1 <sub>312</sub> - M14A/sprF1
SprG1- <b>STOP1,2,14</b> _forw SprG1- <b>STOP1,2,14</b> _rev	AAGGAGGTGGTGCCT <b>TAATA</b> AGCATTACTGAAATCTTTAGA- AGGAGACGCCTA <b>TAA</b> ATTACAATTAGTACCATG CATGGTACTAATTGTAAT <b>TTA</b> TAGGCGTCTCCTTTCTAAAGAT T-TCAGTAATGCT <b>TTATTA</b> AGGCACCACCTCCTT	pALCΩsprG1 <sub>312</sub> - STOP <sub>1,2,14</sub> /sprF1
SprG1- <b>STOP17</b> _forw SprG1- <b>STOP17</b> _rev	GCCTATAAATTACAT <b>TAA</b> AGTACCATGTTGC GCAACATGGTACT <b>TTA</b> TGTAATTTATAGGC	pALCΩsprG1 <sub>312</sub> - STOP <sub>1,2,14,17</sub> /sprF1
SprG1-Flag3XCter_forw SprG1-Flag3XCter_rev	<u>GACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGAC</u> <u>TACAAGGATGACGATGACAAGTAACCATCGCTAACTTTGGCTG</u> GTTT <u>CTTGTCATCGTCATCCTTGTAGTCGATGTCATGATCTTTATAATCA</u> <u>CCGTCATGGTCTTTGTAGTC</u> TTTTTTATTGCTTAATTCAAT	pALCΩsprG1 <sub>312</sub> - 3XFlag-Ct/sprF1 pALC2073ΩsprG1 <sub>312</sub> - 3XFlag-Ct- STOP <sub>1</sub> /sprF1 pALC2073ΩsprG1 <sub>312</sub> - 3XFlag-Ct- M14A/sprF1 pALC2073ΩsprG1 <sub>312</sub> - 3XFlag-Ct- STOP <sub>1,2,14</sub> /sprF1 pALC2073ΩsprG1 <sub>312</sub> - 3XFlag-Ct- STOP <sub>1,2,14,17</sub> /sprF1

SprG1- $\Delta$ 9_forw	GGTCTAGTAATCT <u>TA</u> ACTTATTGAATT	pALC2073 $\Omega$ sprG1 <sub>312</sub> -
SprG1- $\Delta$ 9_rev	AATTCATAAAGTTAGAT <u>TT</u> ACTAGACC	STOP <sub>1</sub> - $\Delta$ 9/sprF1
SprG1- $\Delta$ 2_forw	GAATTAAGCAAT <u>TAAAA</u> ATAACCATCGC	pALC2073 $\Omega$ sprG1 <sub>312</sub> -
SprG1- $\Delta$ 2_rev	GCGATGGTTATTT <u>TTA</u> ATTGCTTAATTC	STOP <sub>1</sub> - $\Delta$ 2/sprF1
SprG1-F10A-F13A_forw	GTTGCAG <u>GC</u> AGGTTTAG <u>CA</u> CTTATTG	pALC2073 $\Omega$ sprG1 <sub>312</sub> -
SprG1-F10A-F13A_rev	CAATAAG <u>TG</u> CTAAACCT <u>GC</u> CTGCAAC	STOP <sub>1</sub> -F10A- F13A/sprF1
SprG1-F10E-F10E_forw	GTTGCAG <u>GA</u> AGGTTTAG <u>AA</u> CTTATTGC	pALC2073 $\Omega$ sprG1 <sub>312</sub> -
SprG1-F10E-F10E_rev	GCAATAAG <u>TT</u> CTAAACCT <u>TT</u> CCTGCAAC	STOP <sub>1</sub> -F10E- F13E/sprF1
SprG1-K2K3_forw	GGAGACGCCTAATG <u>AAAAAA</u> ATTACAATTAG	pALC2073 $\Omega$ sprG1 <sub>312</sub> -
SprG1-K2K3_rev	CTAATTGTAAT <u>TTTTTT</u> CATTAGGCGTCTCC	STOP <sub>1</sub> -K <sub>2</sub> K <sub>3</sub> /sprF1
SprG1-Flag1XNter_forw	CGCCTAATG <u>GACTACAAAGACGATGACGACAAG</u> ATT	pALC2073 $\Omega$ sprG1 <sub>312</sub> -
SprG1-Flag1XNter_rev	ACAATTAG CTAATTGTAAT <u>CTTGTCGTCATCGTCTTTGTAGTCCA</u> TTAGGCG	STOP <sub>1</sub> 1XFlag- Nt/sprF1
SprG1-Flag1XCter_forw	GCAATAAAAAA <u>GACTACAAAGACGATGACGACAAG</u>	pALC2073 $\Omega$ sprG1 <sub>312</sub> -
SprG1-Flag1XCter_rev	TAACCATCGC GCGATGGTTA <u>CTTGTCGTCATCGTCTTTGTAGTCTT</u> TTTTATTGC	STOP <sub>1</sub> 1XFlag- Ct/sprF1

<b>Northern blot</b>		
<i>DNA</i>	<i>Sequences</i>	<i>Purposes</i>
SprG1-NB	CAGCCAAAGTTAGCGATGGT	Detection of sprG1 <sub>312</sub>
SprF1-NB	TAACTTTGGCTGGTTTCGATGGTT	Detection of SprF1
ARN5S - NB	CGTAAGTTCGACTACATCG	Detection of 5S rRNA
FLAG-NB	ATCCTTGTAGTCGATGTCATGATCTT	Detection of flagged RNAs

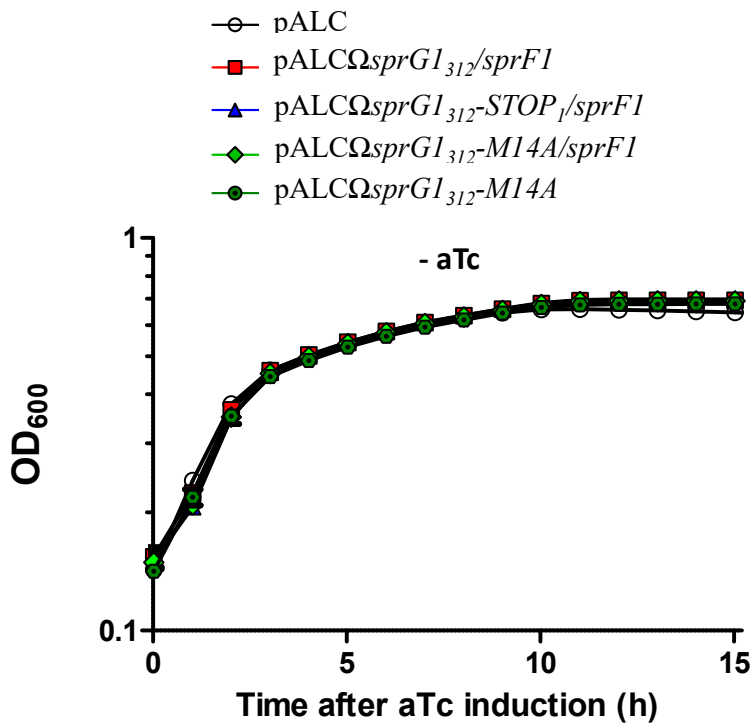


**Supplementary Figure 1: Genetic map of the wild-type *sprG1/sprF1* locus and the various constructs in the pALC vector used in this work.** The AUG54, GUG57 and AUG93 initiation codons and the AUU102 isoleucine codon were replaced with a UAA termination codon that refers to STOP<sub>1</sub>, STOP<sub>2</sub>, STOP<sub>14</sub> and STOP<sub>17</sub> mutations respectively. The AUG93 initiation codon was replaced with a GCC alanine codon that refers to M14A mutation.

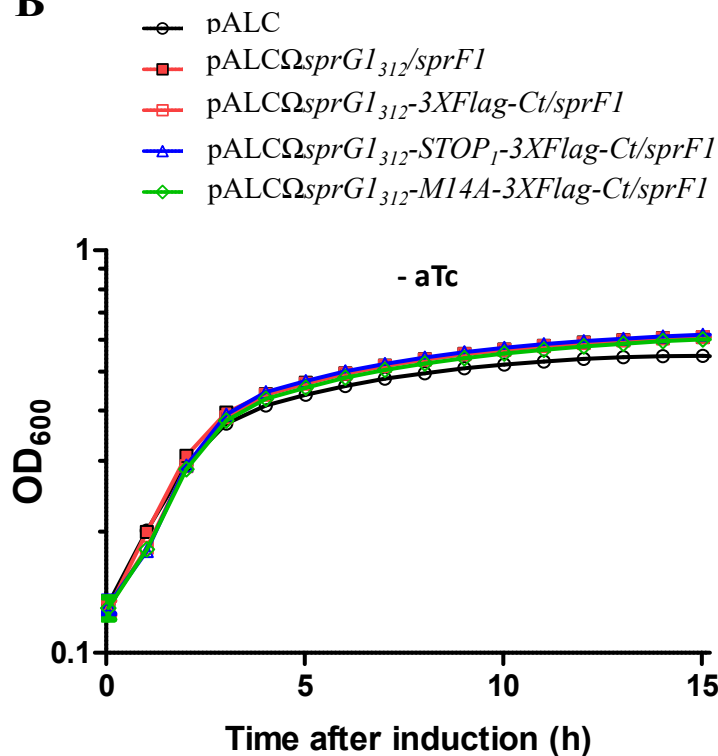
CUUUGAGCAAGUUGGAUAGAUGGGUGGCUAUCU  
 20  
 GAGUAUAAGGAGGGUGGCCU **AUGGUGGCAU**  
 40 60  
 STOP<sub>1</sub> STOP<sub>2</sub>  
 UACUGAAAUCUUUAGAAAGGAGACGCCUA **AUG**  
 80 M14A/STOP<sub>14</sub>  
 AUUACA **AUU** AGUACCAUGUUGCAGUUUGGUUU  
 100 120  
 STOP<sub>17</sub>  
 AUUCCUUAUUGCAUUGAUAGGUCUAGUAAUCA  
 140  
 AGCUUAUUGAAUUAAGCAAUAAAAAAUAACCA  
 160 180  
 UCGCUAACUUUGGCUGGUUUCGAUGGUUAAAU  
 200 220  
 GGUUAUUAAUUUAAUCUUUAAUCUAAAAUAGC  
 240  
 CACCGUCUUUUUAAACGGGCUCACUAGGGUAAC  
 260 280  
 AUGUUUGCGCAUGUUGCCCUUUUUC  
 300

**Supplementary Figure 2: The *sprG1*<sub>312</sub> mRNA sequence.** The open reading frame is highlighted in grey. The AUG54, GUG57 and AUG93 initiation codons and the AUU102 isoleucine codon were replaced with a UAA termination codon that refers to STOP<sub>1</sub>, STOP<sub>2</sub>, STOP<sub>14</sub> and STOP<sub>17</sub> mutations respectively. The AUG93 initiation codon was replaced with a GCC alanine codon that refers to M14A mutation.

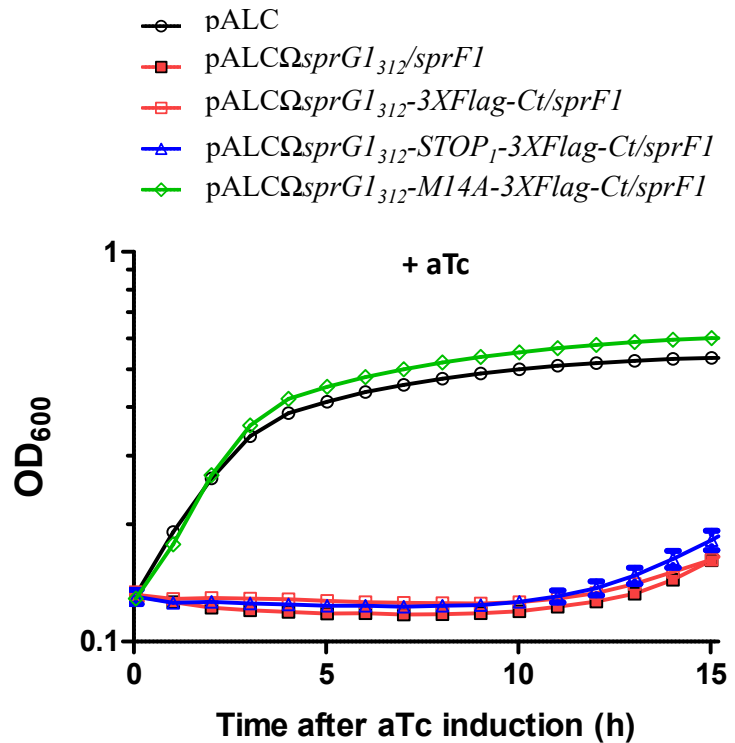
A



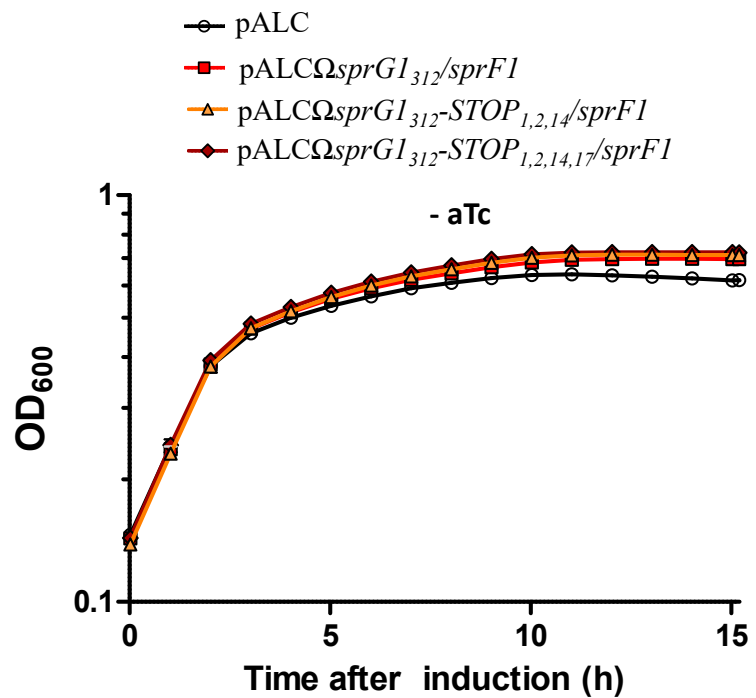
B



C



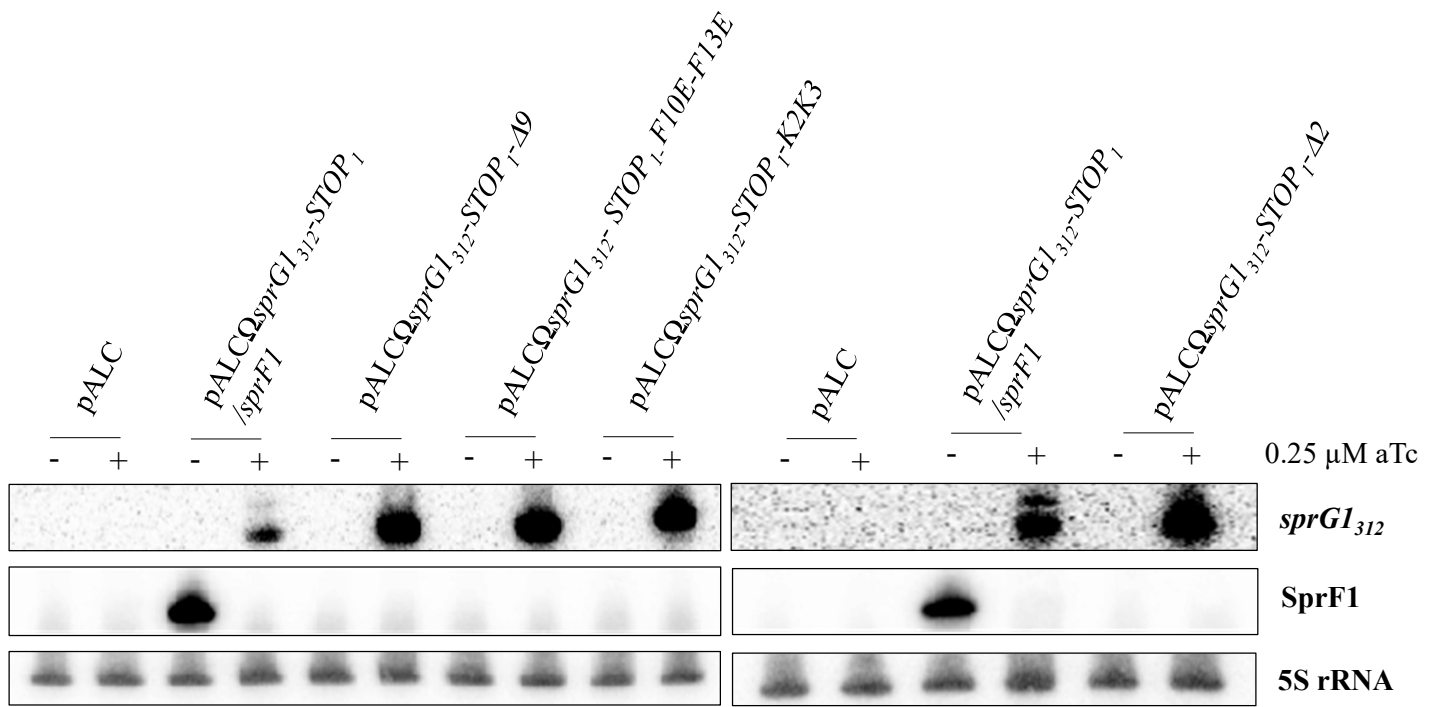
D



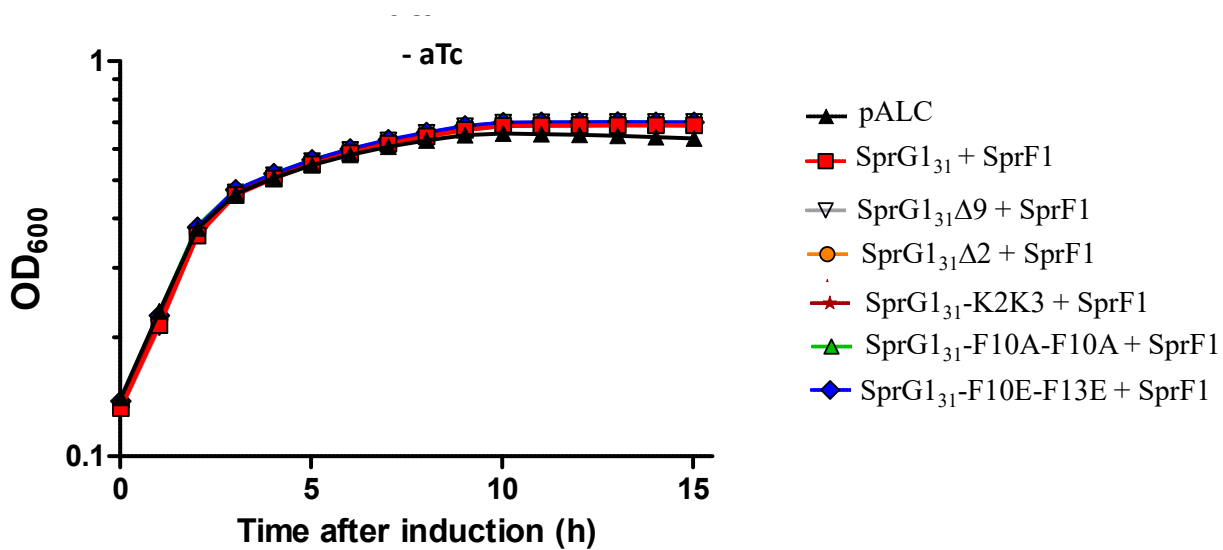
**Supplementary Figure 3: Growth kinetics of *Staphylococcus aureus* strains.** (A) Growth kinetics of *S. aureus* N315Δ*sprG1/sprF1* strains carrying pALC, pALCΩ*sprG1*<sub>312</sub>/*sprF1*, pALCΩ*sprG1*<sub>312</sub>-*STOP*<sub>1</sub>/*sprF1*, pALCΩ*sprG1*<sub>312</sub>-*M14A*/*sprF1* or pALCΩ*sprG1*<sub>312</sub>-*M14A* cultivated in MH medium in the absence of aTc. (B) Growth kinetics of *S. aureus* N315Δ*sprG1/sprF1* strains carrying pALC, pALCΩ*sprG1*<sub>312</sub>/*sprF1*, pALCΩ*sprG1*<sub>312</sub>-3*XFlag*-*Ct*/*sprF1*, pALCΩ*sprG1*<sub>312</sub>-*STOP*<sub>1</sub>-3*XFlag*-*Ct* /*sprF1* or pALCΩ*sprG1*<sub>312</sub>-*M14A*-3*XFlag*-*Ct*/*sprF1* cultivated in MH medium until the exponential growth phase and incubated in the presence of 0.25 μM aTc. (C) Growth kinetics of *S. aureus* N315Δ*sprG1/sprF1* strains carrying pALC, pALCΩ*sprG1*<sub>312</sub>/*sprF1*, pALCΩ*sprG1*<sub>312</sub>-*STOP*<sub>1,2,14</sub>/*sprF1* or pALCΩ*sprG1*<sub>312</sub>-*STOP*<sub>1,2,14,17</sub>/*sprF1* cultivated in MH medium in the absence of aTc. (D) Growth kinetics of *S. aureus* N315Δ*sprG1/sprF1* strains carrying pALC, pALCΩ*sprG1*<sub>312</sub>/*sprF1*, pALCΩ*sprG1*<sub>312</sub>-3*XFlag*-*Ct*/*sprF1*, pALCΩ*sprG1*<sub>312</sub>-*STOP*<sub>1</sub>-3*XFlag*-*Ct*/*sprF1* or pALCΩ*sprG1*<sub>312</sub>-*M14A*-3*XFlag*-*Ct*/*sprF1* cultivated in MH medium in the absence of aTc. Error bars show the means and standard deviations of three biological replicates (n=3).



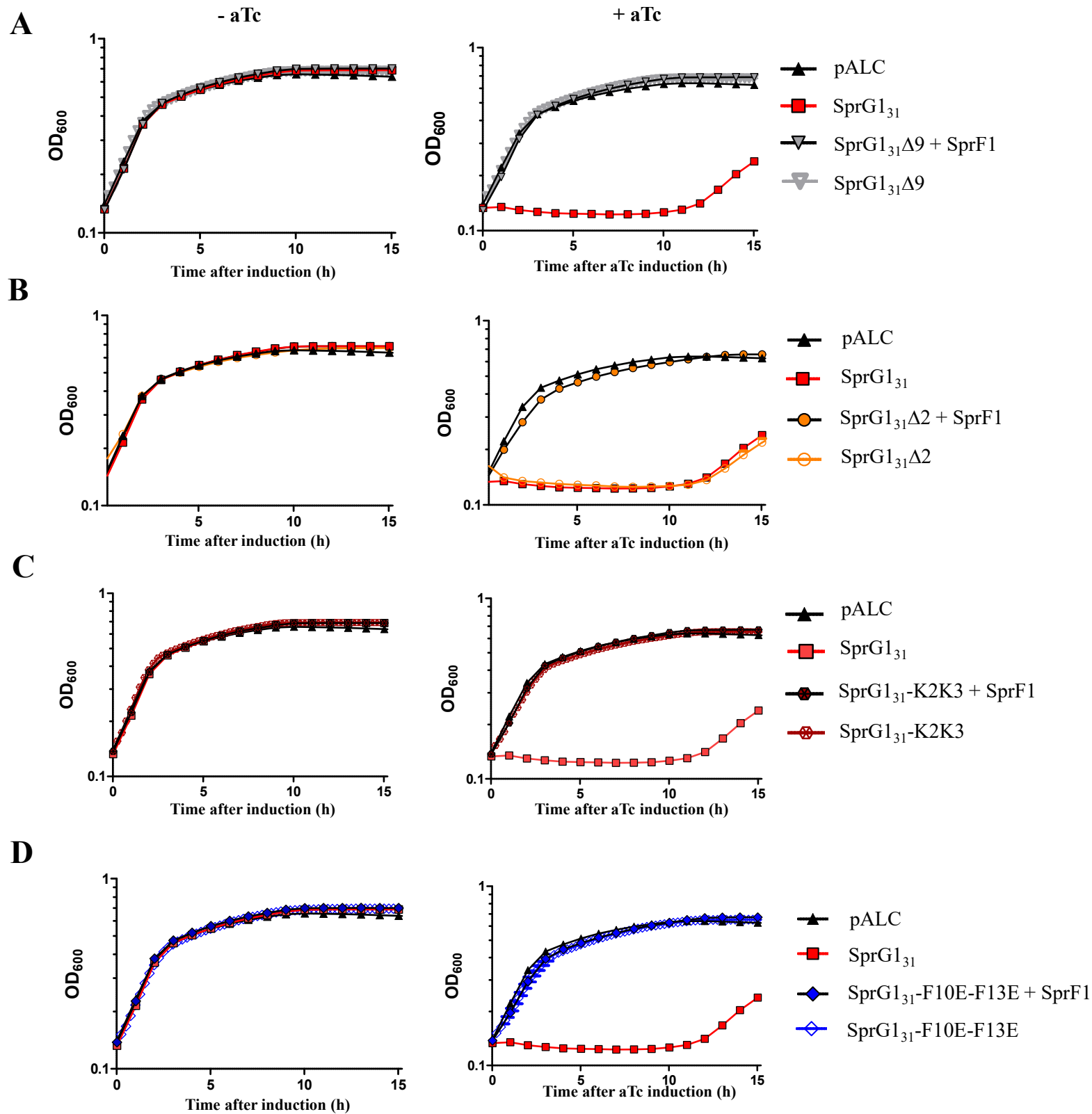
**A**



**B**

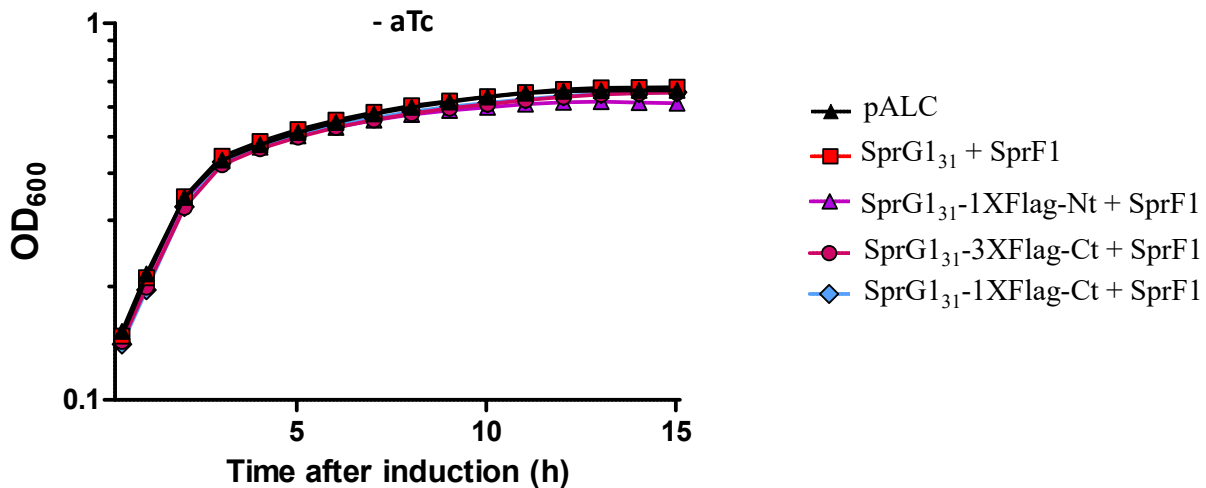


**Supplementary Figure 4: Northern blot analysis of *sprG1*<sub>312</sub> mRNA expression and growth kinetics in the absence of aTc for SprG1<sub>31</sub> mutants.** (A) *S. aureus* N315Δ*sprG1/sprF1* strains carrying pALC, pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>/*sprF1*, pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>-Δ9, pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>-Δ2, pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>-K2K3 or pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>-F10E-F13E were cultivated in MH medium until exponential growth phase and incubated in the absence (-) or presence (+) of 0.25  $\mu$ M aTc. After RNA extraction, northern blot analysis was done on *sprG1*<sub>312</sub>, *sprG1*<sub>312</sub> mutants and SprF1 expression with 5S rRNA used as the loading control. (B) Growth kinetics of *S. aureus* N315Δ*sprG1/sprF1* strains carrying pALC, pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>/*sprF1* (SprG1<sub>31</sub> + SprF1), pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>-Δ9/*sprF1* (SprG1<sub>31</sub>Δ9 + SprF1), pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>-Δ2/*sprF1* (SprG1<sub>31</sub>Δ2 + SprF1), pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>-K2K3/*sprF1* (SprG1<sub>31</sub>-K2K3 + SprF1), pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>-F10A-F13A/*sprF1* (SprG1<sub>31</sub>-F10A-F13A + SprF1) or pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>-F10E-F13E/*sprF1* (SprG1<sub>31</sub>-F10E-F13E + SprF1) cultivated in MH medium in the absence of aTc. Error bars show the means and standard deviations of three biological replicates (n=3).

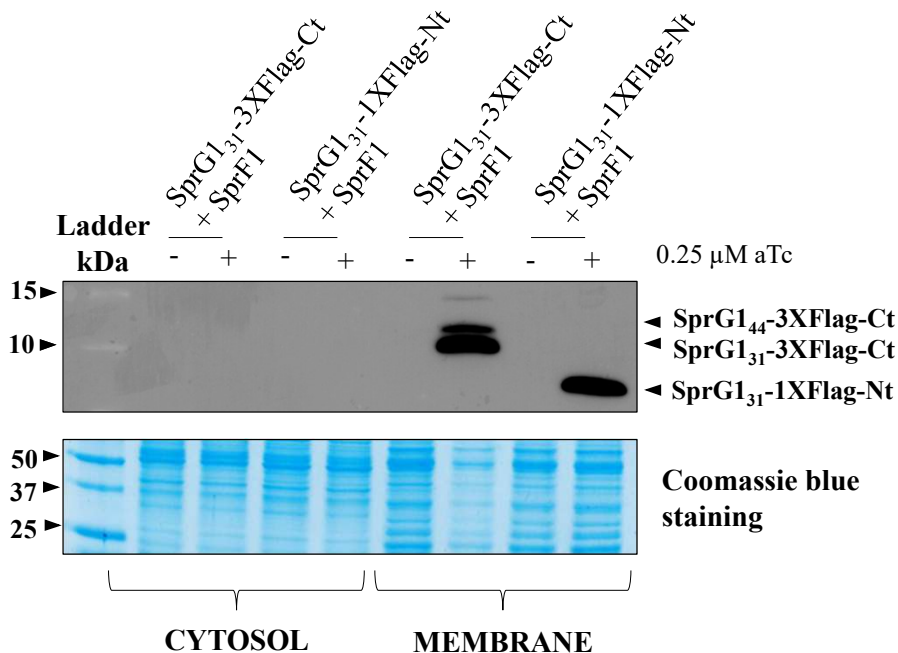


**Supplementary Figure 5: Growth kinetics of SprG<sub>131</sub> mutants in the absence or presence of SprF1 antitoxin.** (A) Growth kinetics of *S. aureus* N315Δ*sprG1/sprF1* strains carrying pALC, pALCΩ*sprG1<sub>312</sub>-STOP<sub>1</sub>/sprF1* (SprG<sub>131</sub>), pALCΩ*sprG1<sub>312</sub>-STOP<sub>1</sub>-Δ9/sprF1* (SprG<sub>131</sub>Δ9 + SprF1) or pALCΩ*sprG1<sub>312</sub>-STOP<sub>1</sub>-Δ9* (SprG<sub>131</sub>Δ9) cultivated in MH medium in the absence (-) or presence (+) of 0.25 μM aTc. (B) Growth kinetics of *S. aureus* N315Δ*sprG1/sprF1* strains carrying pALC, pALCΩ*sprG1<sub>312</sub>-STOP<sub>1</sub>/sprF1* (SprG<sub>131</sub>), pALCΩ*sprG1<sub>312</sub>-STOP<sub>1</sub>-Δ2/sprF1* (SprG<sub>131</sub>Δ2 + SprF1) or pALCΩ*sprG1<sub>312</sub>-STOP<sub>1</sub>-Δ2* (SprG<sub>131</sub>Δ2) cultivated in MH medium in the absence (-) or presence (+) of 0.25 μM aTc. (C) Growth kinetics of *S. aureus* N315Δ*sprG1/sprF1* strains carrying pALC, pALCΩ*sprG1<sub>312</sub>-STOP<sub>1</sub>/sprF1* (SprG<sub>131</sub>), pALCΩ*sprG1<sub>312</sub>-STOP<sub>1</sub>-K2K3/sprF1* (SprG<sub>131</sub>-K2K3 + SprF1) or pALCΩ*sprG1<sub>312</sub>-STOP<sub>1</sub>-K2K3* (SprG<sub>131</sub>-K2K3) cultivated in MH medium in the absence (-) or presence (+) of 0.25 μM aTc. (D) Growth kinetics of *S. aureus* N315Δ*sprG1/sprF1* strains carrying pALC, pALCΩ*sprG1<sub>312</sub>-STOP<sub>1</sub>/sprF1* (SprG<sub>131</sub>), pALCΩ*sprG1<sub>312</sub>-STOP<sub>1</sub>-F10E-F13E/sprF1* (SprG<sub>131</sub>-F10E-F13E + SprF1) or pALCΩ*sprG1<sub>312</sub>-STOP<sub>1</sub>-F10E-F13E* (SprG<sub>131</sub>-F10E-F13E) cultivated in MH medium in the absence (-) or presence (+) of 0.25 μM aTc. Error bars show the means and standard deviations of three biological replicates (n=3).

**A**

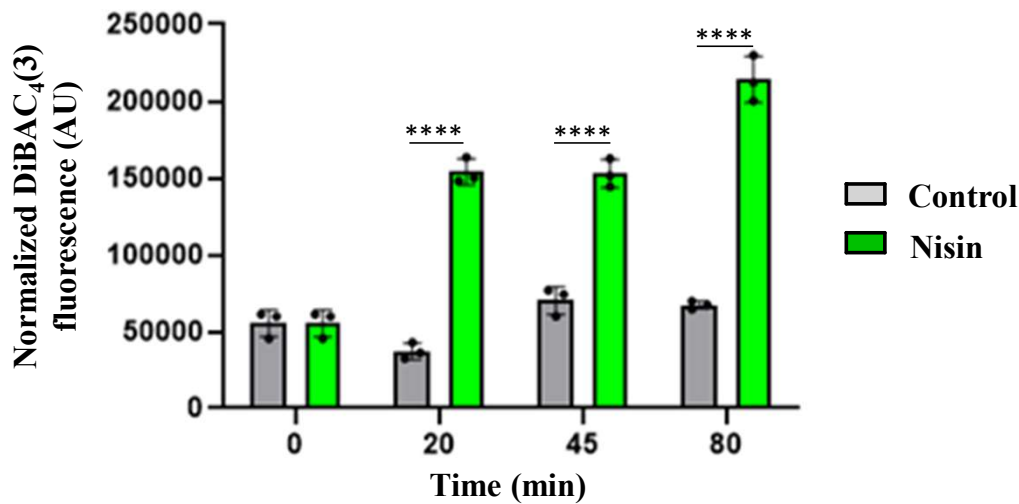


**B**

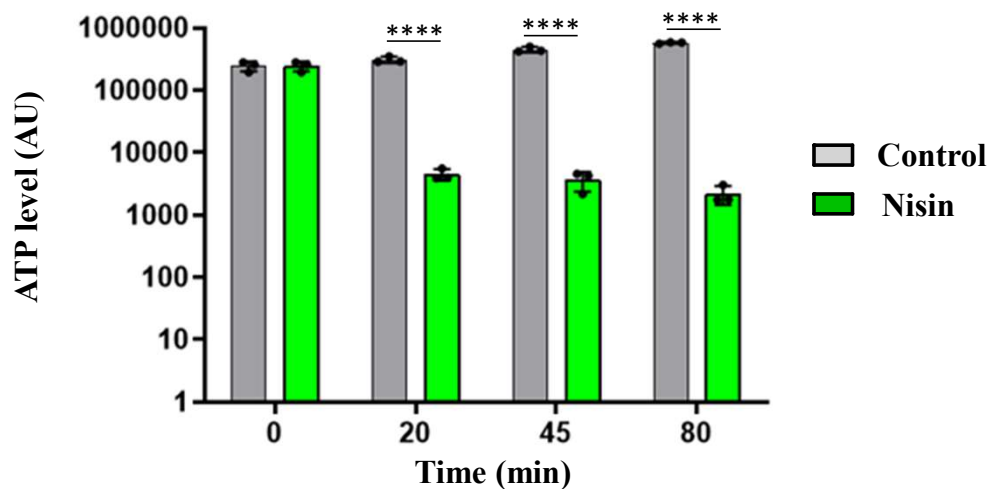


**Supplementary Figure 6: Growth kinetics and western blot analysis of flagged SprG1<sub>31</sub> mutants.** (A) Growth kinetics of *S. aureus* N315Δ*sprG1*/*sprF1* strains carrying pALC, pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>/*sprF1* (SprG1<sub>31</sub> + SprF1), pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>-1XFlag-Nt/*sprF1* (SprG1<sub>31</sub>-1XFlag-Nt + SprF1), pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>-1XFlag-Ct/*sprF1* (SprG1<sub>31</sub>-1XFlag-Ct + SprF1) or pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>-3XFlag-Ct/*sprF1* (SprG1<sub>31</sub>-3XFlag-Ct + SprF1) cultivated in MH medium in the absence of aTc. Error bars show the means and standard deviations of three biological replicates (n=3). (B) *S. aureus* N315Δ*sprG1*/*sprF1* strains carrying pALCΩ*sprG1*<sub>312</sub>-3XFlag-Ct-STOP<sub>1</sub>/*sprF1* (SprG1<sub>31</sub>-3XFlag-Ct + SprF1) or pALCΩ*sprG1*<sub>312</sub>-STOP<sub>1</sub>-1XFlag-Nt/*sprF1* (SprG1<sub>31</sub>-1XFlag-Nt + SprF1) were cultivated in MH medium until exponential growth phase and incubated in the absence (-) or presence (+) of 0.25 μM aTc. After cell fractionation, the expression of the *sprG1*<sub>312</sub>-encoded flagged peptides, SprG1<sub>44</sub>-3XFlag-Ct, SprG1<sub>31</sub>-3XFlag-Ct and SprG1<sub>31</sub>-1XFlag-Nt, was analyzed by western blot by using anti-FLAG antibodies. Coomassie blue staining was used as the loading control.

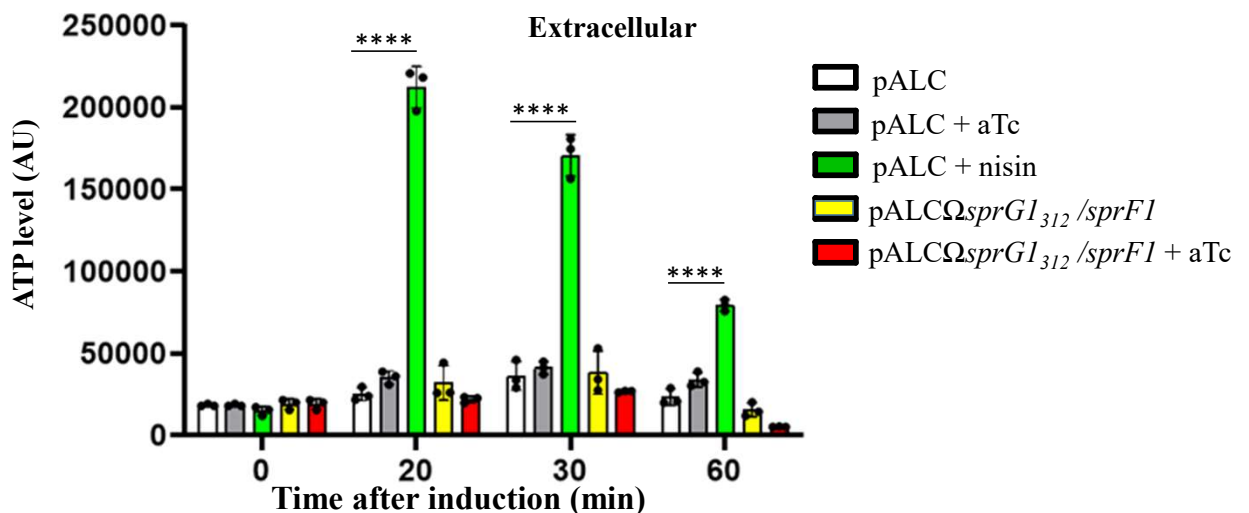
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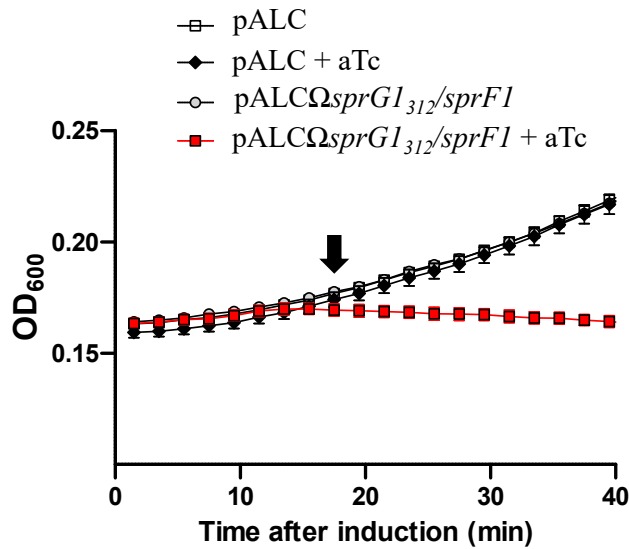
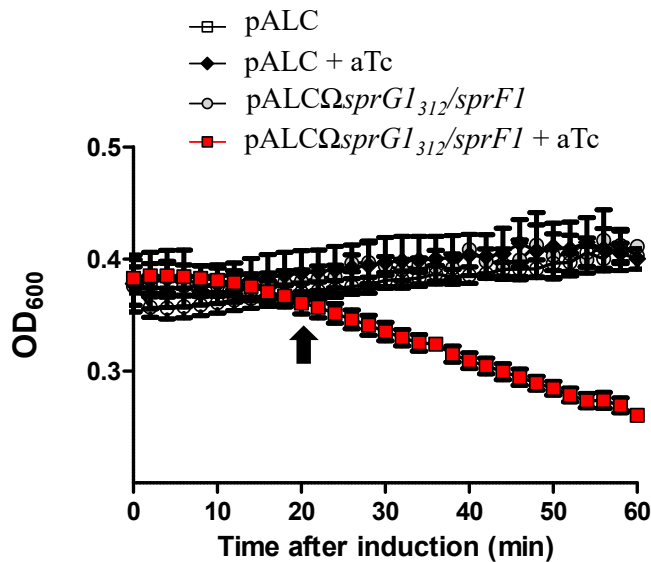
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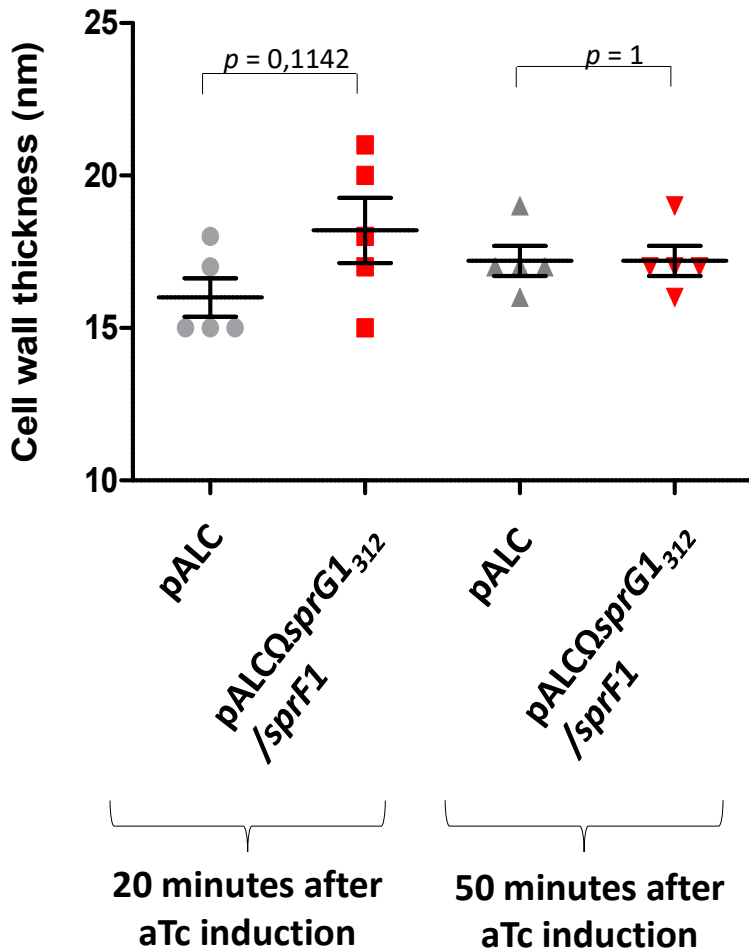
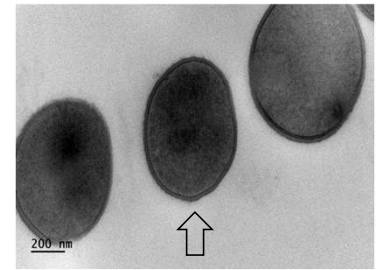
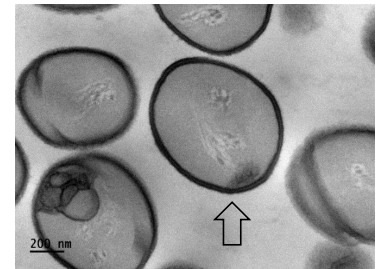
C



**Supplementary Figure 7: The pore-forming peptide nisin causes *Staphylococcus aureus* membrane depolarization followed by an intracellular ATP drop and an increase of ATP leakage into the extracellular medium.** (A-B) *S. aureus* N315Δ*sprG1/sprF1* strains carrying pALC was cultivated in MH medium until the exponential growth phase and incubated in absence (Control) or presence of 12.5 μg/mL nisin. (A) At each time point, bacteria were resuspended in PBS and stained with 0.5 μg/mL DiBAC<sub>4</sub>(3). DiBAC<sub>4</sub>(3) fluorescence was measured in a microplate reader and normalized with OD<sub>600</sub>. (B) At each time point, bacteria were resuspended in MH and incubated with BacTiter-Glo Microbial Cell Viability Assay. Luminescence values were normalized with OD<sub>600</sub>. (C) *S. aureus* N315Δ*sprG1/sprF1* strains carrying pALC and pALCΩ*sprG1*<sub>312</sub>/*sprF1* were cultivated in MH medium until the exponential growth phase and incubated in absence or presence of 0.25 μM aTc or 12.5 μg/mL nisin used as the positive control. At each time point, supernatants (extracellular fraction) were incubated with BacTiter-Glo Microbial Cell Viability Assay. Luminescence values normalized with OD<sub>600</sub>. Error bars show the means and standard deviations of three biological replicates (n=3). Statistical significance was calculated with the two-way ANOVA with Tukey's correction. \*\*\*\**P*<0.0001. AU refers to arbitrary unit.

**A****B**

**Supplementary Figure 8: SprG1<sub>44</sub> and SprG1<sub>31</sub> arrest the growth of *Staphylococcus aureus* within 20 minutes after overexpression.** (A) *S. aureus* N315ΔsprG1/sprF1 strains carrying pALC and pALCΩsprG1<sub>312</sub>/sprF1 were cultivated in MH medium until the exponential growth phase and incubated in absence or presence of 0.25 μM aTc. (B) *S. aureus* N315ΔsprG1/sprF1 strains carrying pALC or pALCΩsprG1<sub>312</sub>/sprF1 were cultivated in MH medium until the exponential growth phase, resuspended in PBS supplemented with 25 mM glucose and incubated in absence or presence of 0.25 μM aTc. (A-B) OD<sub>600</sub> was measured in a microplate reader. The results are expressed as the mean ± SD of three biological replicates (n=3). Arrows indicate the time for which the *S. aureus* growth is inhibited by the overexpression of SprG1<sub>44</sub> and SprG1<sub>31</sub>.

**A****B****C**

**Supplementary Figure 9: Measurement of cell wall thickness by transmission electron microscopy.** *S. aureus* N315 $\Delta$ sprG1/sprF1 strains carrying pALC or pALC $\Delta$ sprG1<sub>312</sub>/sprF1 were cultivated in MH medium until the exponential growth phase and incubated with 0.25  $\mu$ M aTc during 20 or 50 min. (A) Cell wall thickness of *S. aureus* was measured using transmission electron microscopy. The results are expressed as the mean  $\pm$  SD of 5 representative bacteria. *P* values inferred using the Student's *t*-test. (B-C) Captions of representative bacteria (arrow) carrying pALC (B) or pALC $\Delta$ sprG1<sub>312</sub>/sprF1 (C) 20 minutes after aTc induction.

## Supplemental references

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