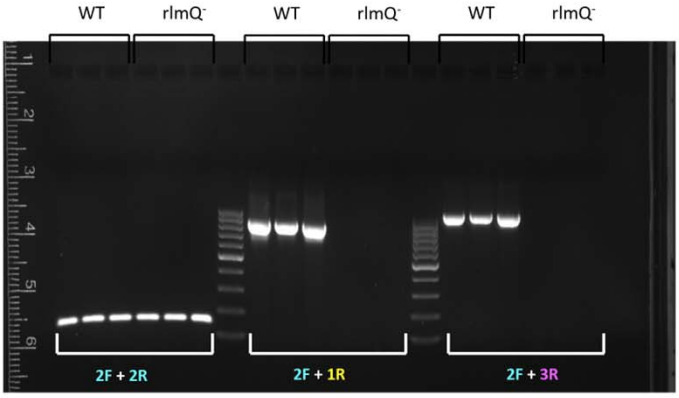
Table S1. DNA oligonucleotides used for this study. Restriction sites for cloning are underlined.

Regulatory sequences used in *rlmQ* complementation plasmid	
Modified *blaZ* promoter	AGCTTACTATGCCATTATTAATAACTTAGCCATTTCAACACCTTCTTTCAAAT ATTTATAATAAACTATTGACACCGATATTACAATTGTAATATCGGAGGGTTTA TT
Native *blaZ* transcription terminator	AGGCGCGCCTATTCTAAATGCATAATAAATACTGATAACATCTTATATTTTGT ATTATATTTTGTATTATCGTTGACATGTATAATTTTGATATCAAAAACTGATTT TCCCTCTATTATTTTCGAGATTTATTTTCTTAATTCTCTTTAACAAACTAGAA ATATTGTATATACAAAAAATTATAAATAATAGATGAATAGTTTAATTATAGGT GTTCATCAATCGAAAAAGCAACGTATCTTATTTAAAGTGCGTTGCTTTTTTC TCATTTATAAGGTTAAATAATTCTCATATATCAAGCAAAGTGACAGGCGGTA CCGAGCTC
DNA primers used for construction of *rlmQ* complementation plasmid	
rlmQ-F	taagcaggatccCTACAACGAAACACAGAAAGGAAG
rlmQ-R	tgcttagaattcTTAATGTCTAATATTTACAAAAACAGC
PblaZ-F	taagcagcatgcAGCTTACTATGCCATTATTAATAA
TT-rev	tgcttaggtaccGCCTGTCACTTTGCTTGATATATGAG
DNA primer used for detection of m^7G2575 by primer extension	
H90	AATTTCCTACGCCCACGA
RNase H isolation of *S. aureus* 23S rRNA fragment containing m^7G2601	
H90-1	GTGATGAGCCGACATCGAGGTGCC
H90-2	TTCTGAACCCAGCTCGCGTA
RNase H isolation of *S. aureus* 23S rRNA fragment containing C1989	
H70-1	TTTCGCTACCTTAGGACCGTTATAGTT
H70-2	GTCTTTCCGTCCTGTCGCGGGT
RNase H isolation of *E. coli* 23S rRNA fragment containing m^5C1962	
H70-1	TTTCGCTACCTTAGGACCGTTATAGTT
H70-3	GTCTTTCCGTCTTGCCGCGGGT

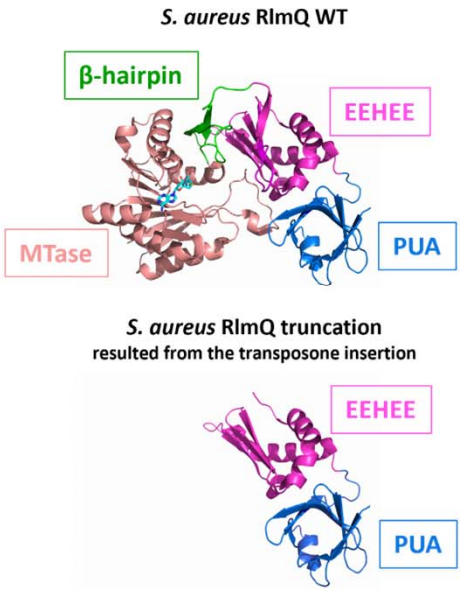
Supplementary Figure 1

A

B



D



C

ATGAAAATAGCAACTCTGAACAAAGGCAAAGAAACAAAATATTTTAATGGATATCCTTTAATTGAAGAAGAGGATATCTATTCACAAGATCATTTAAAAGAAGGAGATATTT
TTCAAATTGTGACTGATAAATCACAATATGTCGCAACGGCTTATGTTGGTCGCCAACATAAAGGATTAGGTTGGGTTCTAACATACGATAAAGCTCAACAAATCAACACAGC
TTTCTTTGTAAAATTGTTTAATACTGCATTAGCAGAACGTGATTATTATTTTAATATAGATGGAACAAATGCTTTTAGATTATTTAATGCTGAAGGTGATGGTGTTGGGGGA
TTAACAATCGACAATTACGATGGTCATTTGTTGATTCAATGGTACTCAAAAGGTATTTATAAATTTAAATATGCCATTCTTGAAGCGGTTAGAAAAGTATTTGATTATAAAT
CTA*ACAGGTTGGCTGATAAGTCCCCGGTCTCTAGACGCGTATAACTATATAGGAACTATGAAAAAGGAACGGATTTTGGTACTTGATTTACTTAAAAAACATTTAGAAAAA
GCAGGAAAATGAAATGGTACCATAAACGGTCGTTTATGGTACCATTCAAATTTATCCTTATTGTACAAAATAACAGCGAAATTTTTAAATCTATTCCTTATCGATACAAATT
CCCCGTAGGCGCTAGGGACCTCTTTAGCTCCTTGGAAGCTGTCAGTAGTATACCTAATAATTTATCTACATTCCCTTTAGTAACGTGTAACTTTCCAAATTTACAAAAGCGA
CTCATAGAATTATTTCCTCCCGTTAAATAATAGATAACTATTAAAAATAGACAATACTTGCTCATAAGTAACGGTACTTAAATTGTTTACTTTGGCGTGTTTCATTGCTTGA
TGAAACTGATTTTTAGTAAACAGTTGACGATATTCTCGATTGACCCATTTTGAAACAAAGTACGTATATAGCTTCCAATATTTATCTGGAACATCTGTGGTATGGCGGGTAA
GTTTTATTAAGACACTGTTTACTTTTGGTTTAGGATGAAAGCATTCCGCTGGCAGCTTAAGCAATTGCTGAAT.... **The transposon sequence extends for an additional 2564 nucleotides from this point.**

E

WT

MKIATLNKGKETKYFNGYPLIEEEDIYSQDHL
KEGDIFQIVTDK**SQYVATAYVGR**QHK**GLGWVL**
TYDKAQQINTAFFVKLFNTALAERDYYFNIDG
TNAFRLFNAEGDGVGGLTIDNYDGHLLIQWYS
KGIYKFK**YAILEAVR**KVFDYKSIYEKVRFKDS
EYSGGFVEGDAPEFPIVIEENFTFYNVDLEDG
LMTGIFLDQKEVRKKLRDQYAKERHVLNLFSY
TGAFSAIAASEASSTTSVDLANRSR**SLTEENF**
GLNAIDPKSQYIYVMDTFDFYKYAARHGHSYD
TIVIDPPSFARNKKRTFSVQKDYDKLINGALN
ILSSEGTLLLCTNASVYPLKQFKNTIKK**TLEE**
SGVDYELTEVMGLPKDFKTHPHYKPSKYLK**AV**
FVNIRH

Number of spectra	
WT_1	11
WT_2	9

***rlmQ*⁻**

MKIATLNKGKETKYFNGYPLIEEEDIYSQDHL
KEGDIFQIVTDKSQYVATAYVGRQHKGLGWVL
TYDKAQQINTAFFVKLFNTALAERDYYFNIDG
TNAFRLFNAEGDGVGGLTIDNYDGHLLIQWYS
KGIYKFKYAILEAVRKVFDYKSIYEKVRFKDS
EYSGGFVEGDAPEFPIVIEENFTFYNVDLEDG
LMTGIFLDQKEVRKKLRDQYAKERHVLNLFSY
TGAFSAIAASEASSTTSVDLANRSRSLTEENF
GLNAIDPKSQYIYVMDTFDFYKYAARHGHSYD
TIVIDPPSFARNKKRTFSVQKDYDKLINGALN
ILSSEGTLLLCTNASVYPLKQFKNTIKKTLEE
SGVDYELTEVMGLPKDFKTHPHYKPSKYLKAV
FVNIRH

Number of spectra	
rlmQ⁻_1	0
rlmQ⁻_2	0

Comp

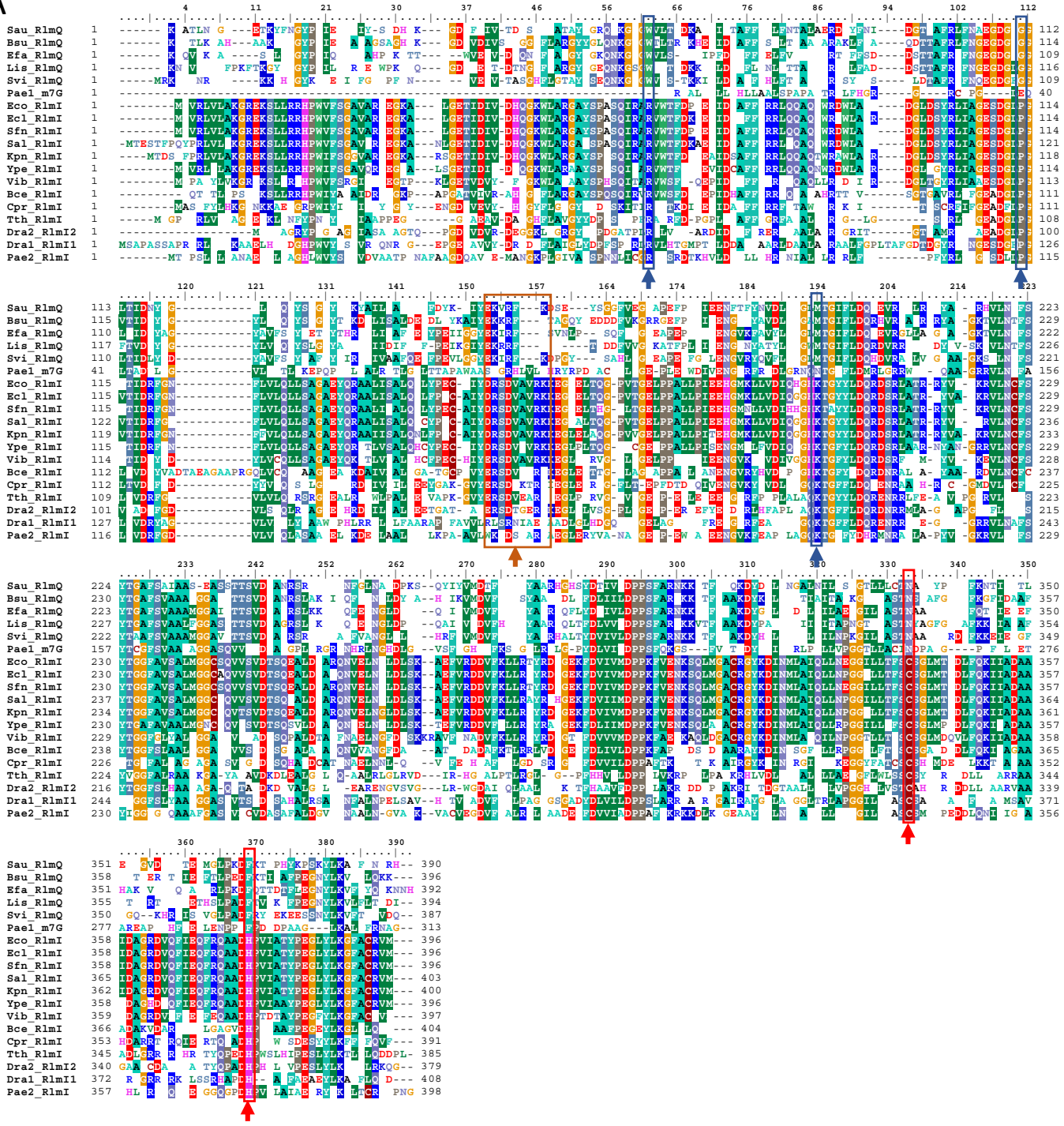
MKIATLNKGKETKYFNGYPLIEEEDIYSQDHL
KEGDIFQIVTDK**SQYVATAYVGR**QHK**GLGWVL**
TYDKAQQINTAFFVKLFNTALAERDYYFNIDG
TNAFRLFNAEGDGVGGLTIDNYDGHLLIQWYS
KGIYKFKYAILEAVRKVFDYKSIYEKVRFKDS
EYSGGFVEGDAPEFPIVIEENFTFYNVDLEDG
LMTGIFLDQKEVRKKLRDQYAKERHVLNLFSY
TGAFSAIAASEASSTTSVDLANR**SRSLTEENF**
GLNAIDPKSQYIYVMDTFDFYKYAARHG**HSYD**
TIVIDPPSFARNKKRTFSVQKDYDKLINGALN
ILSSEGTLLLCTNASVYPLKQFKNTIKK**TLEE**
SGVDYELTEVMGLPKDFKTHPHYKPSKYLK**AV**
FVNIRH

Number of spectra	
Comp_1	17
Comp_2	14

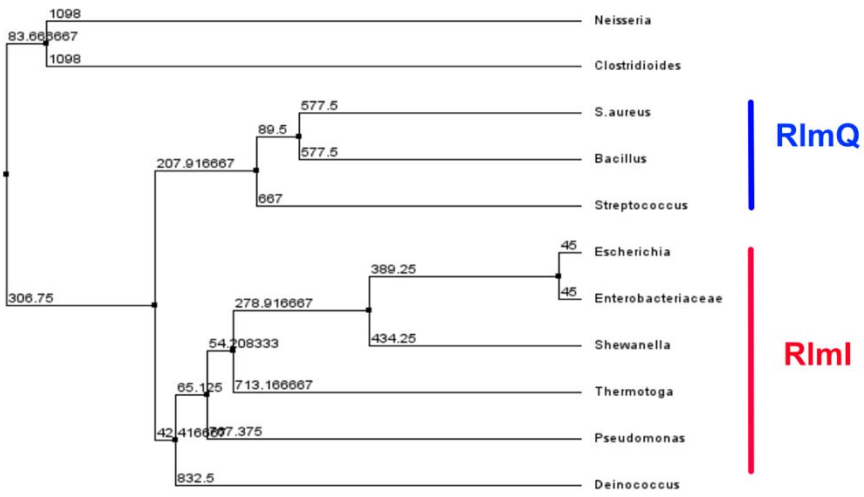
Supplementary Figure 1. Verification of SAUSA300_0981 (*rlm*Q) gene disruption by transposon insertion. A) Schematic representation of the SAUSA300_0981 (*rlm*Q) gene. In red is indicated the transposon insertion site in the *rlm*Q$^-$ mutant at position 451 from the beginning of the ORF. Blue, yellow and pink indicate the positions and orientations of the primers used for RT-PCR. B) Analysis of RT-PCR products on gel. RNA was extracted from an 8 h culture in BHI medium. RT-PCR was performed on 1 µg of RNA and 10 µl of reaction were analyzed on a 1% agarose gel. C) Beginning of the gene sequence of *rlm*Q gene interrupted by the 3193 nucleotides long transposon. The insertion at position 451 of the transposon unit creates a stop codon, shown in red. The last 10 nucleotides of the interrupted *rlm*Q sequence are underlined and an asterisk marks the transition with the transposon sequence. The sequences corresponding to the two first domains of RlmQ (EEHEE and PUA) are colored in blue and violet, respectively. The first 629 nucleotides of the transposon sequence is also shown. D) Structure models for RlmQ WT and the expected truncated protein of the *rlm*Q$^-$ strain. Based on the sequence of the *rlm*Q$^-$ locus, we determined that the truncated protein retains 150 amino acids of the original sequence, covering the PUA domain and part of the EEHEE domains. E) Mass spectrometry analysis of RlmQ protein in the protein extracts prepared from WT, *rlm*Q$^-$ mutant and the complemented mutant strains. The analysis was performed using a TIMS-TOF Pro2 (Bruker) instrument. Briefly, 10 µg of each lysate was methanol precipitated with 0.1 M NH_4OAc, reduced with 5 mM dithiothreitol, alkylated with 10 mM iodoacetamide, and digested with sequencing grade trypsin (Promega). Generated peptides were injected on a nano-Elute 2 nano-LC coupled with a TIMS-TOF Pro 2 (Bruker) and analyzed with 70 min gradients. Identification of peptides was done on *S. aureus* (Uniprot v2023_05, USA300 strain, 5581 sequences) with mascot (v2.8, Matrix Science) and the data were validated with an 1% FDR for both peptide spectrum matches (PSM score) and protein sets (Protein Set score). RlmQ is identified only in the WT strain and complemented mutant strain samples. No spectra could be detected from the *rlm*Q$^-$ strain, confirming the absence of active RlmQ in the mutant strain.

Supplementary Figure 2

A

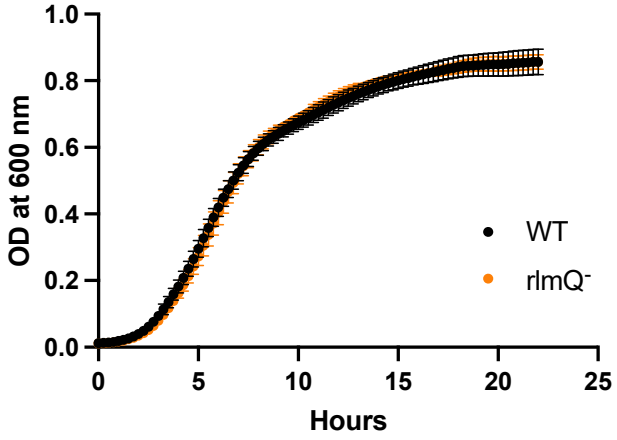


B



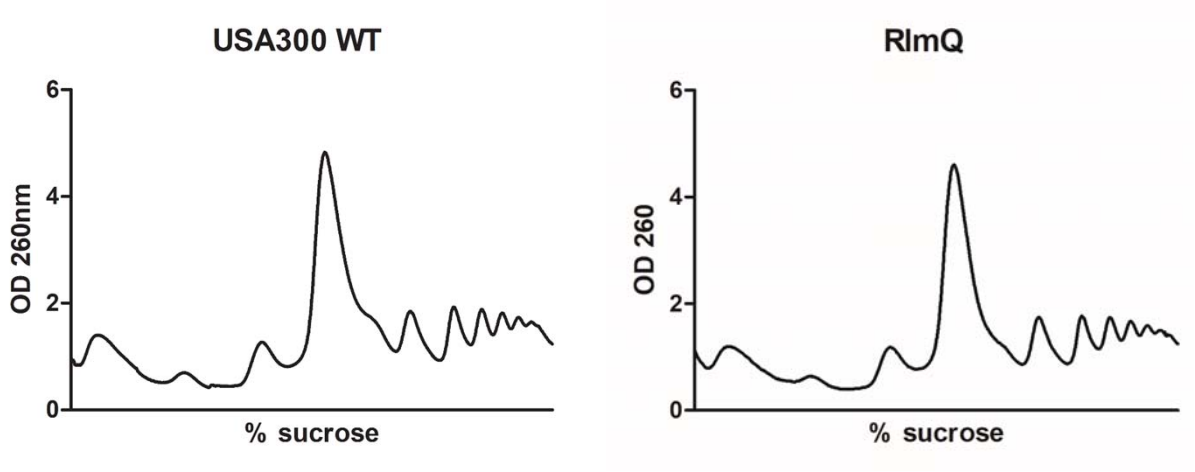
Supplementary Figure 2. RlmQ and RlmI distinctive motifs. A) Multi sequence alignment of 19 homologous protein sequences taken from different taxa allowed the identification of co-variation motifs using BIS2 software (Dib et al., 2012). γ-Proteobacteria: Eco: *Escherichia coli* (strain K12) (Uniprot RLMI_ECOLI); Vib: *Vibrio cholerae* serotype O1 (Uniprot RLMI_VIBCH); Sal: *Salmonella typhimurium* (Uniprot RLMI_SALTY); Ecl: *Enterobacter cloacae* (Uniprot A0A6S5JT50_ENTCL gene *rlmI*); Sfn: *Shigella flexneri* (Uniprot RLMI_SHIFL); Kpn: *Klebsiella pneumoniae* (Uniprot RLMI_KLEP3); Ype: *Yersinia pestis* (Uniprot RLMI_YERPE). Deinococci: Tth: *Thermus thermophilus* (Uniprot RLM0_THET8 gene rlmO); Dra1: *Deinococcus radiodurans* (Uniprot MTR49_DEIRA gene DR_0049); Dra2: *Deinococcus radiodurans* (Uniprot Q9RTR2_DEIRA gene DR_1694). β-Proteobacteria: Bce: *Burkholderia cepacia* (Uniprot A0A118FYW8_BURCE gene rlmI); Pae2: *Pseudomonas aeruginosa* (Uniprot A0A0C7D2Z7_PSEAI PA0354); Pae1: *Pseudomonas aeruginosa* (Uniprot A0A072ZM57_PSEAI gene PA1407). Firmicutes: Sau: *Staphylococcus aureus* (Uniprot Q2FZH8_STAA8 gene SAOUHSC_01026); Bsu: *Bacillus subtilis* (Uniprot YWBD_BACSU gene rlmI); Efa: *Enterococcus faecium* (Streptococcus faecium) (Uniprot A0A6N3CVE6_ENTFC); Cpr: *Clostridium perfringens* (Uniprot A0A336Q3Q1_CLOPF); Svi: *Streptococcus viridans* (Uniprot A0A447Z526_9STRE); Lis: *Listeria monocytogenes* (Uniprot A0A5L2UGJ9_LISMN). The sequences have been selected among the set of RlmQ (*S. aureus*) and RlmI (*E. coli*) homologues retrieved from the Uniprot database using PSIBLAST (Altschul et al., 1997). Colored boxes highlight the most significative coevolution signals discriminating RlmQ and RlmI enzymes, as determined by BIS2 analysis (Dib et al., 2012). Red boxes: cluster 1, with a p-value cutoff at $4.3x10^{-6}$, including the catalytic Cys/Asn and the His/Phe co-variations in RlmI/RlmQ. Blue boxes: cluster 2, with a p-value cutoff at $2.6x10^{-5}$, including Lys/Met, Pro/Gly and Arg/Trp co-variations in RlmI/RlmQ. Brown box: cluster 3, with a p-value cutoff at $3.7x10^{-5}$, associating the fragment DRSDVAVRKK/EKVRFK (RlmI/RlmQ) with the covariations of cluster 1. B) Phylogenetic tree of RlmI-related family proteins. Sequences are denoted by their National Center for Biotechnology Information (NCBI) numbers. Node values represent statistical support for branches determined through bootstrap testing.

Supplementary Figure 3



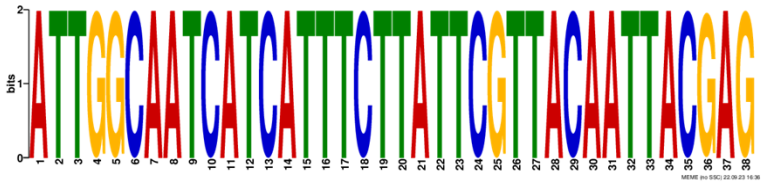
Supplementary Figure 3. Growth at 30°C in BHI. Bacterial growth of the wild-type strain (WT) and the mutant inactivated for *rlmQ* (rlmQ$^-$) was compared in BHI medium at 30°C during 24h.

Supplementary Figure 4



Supplementary Figure 4. **Absence of m^7G2601 does not change polysome distribution.** Sucrose density gradient profile 5-50% from a USA300 (WT) and NE1546 (*rlmQ*$^-$) cultured at 37 °C and harvested at OD_{600nm}=2.

Supplementary Figure 5



Supplementary Figure 5. Sequence and conservation of rlmQ predicted promoter. Promotech v1.0 software (Chevez-Guardado and Pena-Castillo 2021) was used in all 301 clinical strains for promoter detection. The promoter sequence with the highest prediction value (0.83) was detected in all strains and analysis of the conservation of this promoter revealed a sequence of 38 nucleotides common to all strains. DNA motif was generated using MEME v4.11.2 (Bailey et al. 2009).