Supplemental Methods

Construction of bacterial mutants

For deletion of *esxA* and *esxB* a 2-kbp DNA fragments flanking the *esx* genes were amplified by PCR and cloned into the *Escherichia coli*/*S. aureus* shuttle/suicide vector pKOR1 (1) with abutted XhoI restriction sites and *att* site using primers (*esxA* flanking region 1 FW: GGGGACAAGTTTGTA CAAAAAAGCAGGCTTAAAATTGATCCTCGTGTTG - *esxA* flanking region 1 RW: GAGAGAC TCGAGAACTAGAAACCTCCTGAAT; *esxA* flanking region 2 FW: GAGAGACTCGAGGC ATTCTGAAATTGGCAAAG – *esxA* flanking region 2 RW: GGGGACCACTTTGTACAA GAAAGCTGGGTGTATC TTGCATACTTGATTG and *esxB* flanking region 1 FW: GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGCAATGATTTCATCAGAA - *esxB* flanking region 1 RW: GAGAGACTCGAGCATATCTTCACCTCAATATTAAT; *esxB* flanking region 2 FW: GAGAGACTCGAGTGAAAGATGTTAAGCGAAT - *esxB* flanking region 2 RW: GAGAG AATGCTCGAGAACCCATGATGAAAGATGTTAAGCGAAT). Constructs containing flanking genomic regions of *esxA* and *esxB* were cloned first in *E. coli* then into RN4220 (restriction negative, methylation positive strain). Plasmid DNA extracted was used to transform *S. aureus* USA300 (lac). Integration of plasmid into the chromosome was obtained at non permissive conditions for pKOR1 replication (incubation twice at 43°C in TSB) and the selection for homologous recombination and pKOR1 integration into the bacterial chromosome was induced by growing bacteria at permissive temperature (incubation twice 2 at 30°C in TSB). Anhydrotetracycline-mediated induction of pKOR1-encoded *secY* antisense RNA which inhibits growth was used for selecting for chromosomal excision and loss of plasmid at 30°C.

For complementation of mutant strains, the plasmid pOS1CK was generated by cloning the P1 constitutive promoter of the *sarA* gene into the pOS1 plasmid (primers used: P_{sarA}_FW_SwaI:

GCGATTTAAATCCAAATGCTAACCCA, P_{sarA}_RW_PstI: GCGCTGCAGTTTGATGCATCTTG CTCG (2). The full length coding sequences of *esxA* and *esxB* genes were amplified (primers used: *esxA* pOS1_PstI_FW: GCGCTGCAGTTGAGAGGAGAGAAAATGGCAATGATTAAGATG *esxA* pOS1_smaI_RW: GCGCCCGGGTTATTGCAAACCGAAATTAT, *esxB* pOS1_PstI_FW**:** GCGCTGCAGTTGAGAGGAGAGAAAATGGGTGGATATAAAG, *esxB* pOS1_SmaI_RW: GCGCCCGGGTCATGGGTTCACCC) and cloned downstream of the promoter to generate the plasmids pOS1_*esxA*, pOS1_*esxB* and pOS1_*esxAB*. For complementing with both genes, *esxB* was cloned downstream of *esxA* in the pOS1CK*esxA* construct. Expression of proteins was confirmed by immunoblotting.

Site directed mutagenesis of esxA

The Leucine, Serine double mutations were introduced into the *esxA* gene cloned onto a pET vector by PIPE (3) To create the mutations in glycine95 we used *esxA*LSGFW: GCGCTGCAGTT GAGAGGAGAGAAAATGGCAATGATTAAGATG *esxA*LSGRW: GCGCCCGGTTATTGCAAAC CGAAATTAT primers carrying the mutated amino acid sequence. *esxA* truncated gene was amplified using primers *esxA*truncFW: GCGCTGCAGCCGCTCGAGATGGCAATGATTAAG and *esxA*truncRW: TCCCCCGGGTTGTTGGTCTTGTTC. All mutants were cloned into the episomal plasmid pOS1CK.

qRT-PCR

Specific primers for *esxA* gene qRT*esxA*FW: AGGTGAAATTGCAGCGAACT qRT*esxA*RW: CTTGTTCTTGAACGGCATCA and for *esxB* gene qRT*esxB*FW: AGCGGCAAAAACAGCTAAAG and qRT*esxB*RW: TATTGGCGAACTGTCCTTCC were used. A relative quantification was performed using 16S rRNA amplification (qRT16SFW: TAACGGCTTACCAAGGCAAC and qRT16SRW: CGGAAGATTCCCTACTGCTG) as internal control.

Preparation and analysis of bacterial fractions

Bacterial strains were grown O/N from glycerol stocks in TSB at 37°C with shaking. Cultures were diluted 1:100 in fresh broth and shaken at 37°C until they reached an OD600 of 1. For whole culture lysates, 5ml of cultures were centrifuged, washed once with PBS and suspended 1 ml di Tris-HCl pH 6.8 with protease inhibitors and incubated in the presence of lysostaphin (Sigma) at a final concentration of 100 μg/mL for 1h at 37°C at 750 rpm, followed by freezing/thawing thrice in a dry ice/thermomixer at 37°C. The bacterial lysates were centrifuged at 14,000 rpm for 10 min, the supernatants were filtered and the proteins in the lysates were precipitated with TCA at final concentration of 10%. The blots were probed with polyclonal anti-EsxA (1:500), anti-hla (1:1000) or anti RNAPβ (1:1000) (Abcam).

Cloning of esxA for transfections

For cloning *esxA* into mammalian pEYFP-N1 (4.7Kb) vector, primers *esxA* pEYFP_XhoI5: CCGCTCGAGATGGCAATGATTAAG; *esxA*_pEYFP_SmaI3: TCCCCCGGGTTTGCAAACCG AAA were used.

References:

1. **Bae, T., and O. Schneewind.** 2006. Allelic replacement in Staphylococcus aureus with inducible counter-selection. Plasmid **55:**58-63.
2. **Schneewind, O., D. Mihaylova-Petkov, and P. Model.** 1993. Cell wall sorting signals in surface proteins of gram-positive bacteria. EMBO J **12:**4803-11.

3. **Klock, H. E., and S. A. Lesley.** 2009. The Polymerase Incomplete Primer Extension (PIPE) method applied to high-throughput cloning and site-directed mutagenesis. Methods Mol Biol **498:**91-103.