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Transketolase of *Staphylococcus aureus* in the Control of Master Regulators of Stress Response During Infection

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1 **Transketolase of *Staphylococcus aureus* is involved in the control**
2 **of master regulators of stress response during infection**

3

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29 **Abstract**

30

31 *Staphylococcus aureus* is a leading cause of both acute and chronic infections in
32 humans. The importance of the pentose phosphate pathway (PPP) during *S. aureus* infection
33 is currently largely unexplored. Here, we focused on one key PPP enzyme, transketolase. We
34 showed that inactivation of the unique gene encoding transketolase activity in *S. aureus*
35 USA300 (Δtkt) led to drastic metabolomic changes. Using time-lapse video imaging and mice
36 infection, we observed a major defect of the Δtkt strain compared to wild-type strain in early
37 intracellular proliferation and in the ability to colonise kidneys. Transcriptional activity of the
38 two master regulators Sigma B and RpiRc was drastically reduced in the Δtkt mutant during
39 host cells invasion. The concomitant increased RNAlII transcription, suggests that TKT -or a
40 functional PPP- strongly influences the ability of *S. aureus* to proliferate within host cells by
41 modulating key transcriptional regulators.

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53 **Background**

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55 *Staphylococcus aureus* is a leading cause of severe skin and soft tissue infections,
56 pneumonia, endocarditis and bone and joint infections [1, 2]. *S. aureus* invades and survives
57 within eukaryotic host cells [3-9]. This is likely to play an important role in chronicity and
58 treatment failures [10-12]. To evade host defences and silently persist within human cells [13,
59 14], *S. aureus* can dynamically switch phenotypes from a highly aggressive and cytotoxic
60 phenotype to a metabolically inactive phenotype associated with decrease TCA activity [15-
61 18]. Reprogramming of the TCA cycle notably involves the glycolysis/gluconeogenesis and
62 pentose phosphate pathway (PPP) [19].

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64 The contribution of glycolysis/gluconeogenesis to intracellular persistence of *S. aureus*
65 has been recently studied [20-23]. In contrast, the precise role of the PPP is unexplored. The
66 PPP is important for producing nucleotide precursors for DNA synthesis and repair as well as
67 precursors for aromatic amino acids or peptidoglycan synthesis. In addition, the oxidative
68 branch of the PPP contributes to bacterial tolerance to oxidative stress by generating the redox
69 molecule NADPH/H⁺, which is the substrate for other reducing agents such as glutathione. The
70 activity of the PPP has been shown to be frequently increased in Gram-positive pathogens in
71 response to environmental stresses and in infection models [24-26] or in some hospital-
72 acquired isolates [27]. While it has been suggested that the transcriptional regulator RpiRc
73 might control the PPP [28-30], no gene regulation has been yet associated with any change in
74 its activity in *S. aureus*.

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76 Here, we study the role of PPP during the intracellular persistence of *S. aureus* by focusing on
77 one of the key enzyme of this pathway, transketolase (TKT). *tkt* genes are found in a wide
78 range of organisms including bacteria, plants and mammals, underlining the ubiquitous role of
79 this enzyme in biology. We show here that *tkt* inactivation in *S. aureus* USA300 leads to
80 deregulation of whole cell metabolism. This correlates with impaired proliferation of intracellular
81 staphylococci *in vitro* and kidney colonisation defect in infected mice. Unexpectedly, we found

82 that the *tkl* defective mutant was unable to regulate the two master regulators of virulence
83 RpirC and SigB, leading to a dramatic increase of RNAIII during intracellular invasion of human
84 cells. Altogether our data suggest a major role of the PPP in the control of *S. aureus* virulence.

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109 **Methods**

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111 **Strains, Cells, Culture conditions and Infection.**

112 *S. aureus* USA300-LAC (designated USA300-WT) was provided by the Biodefense and
113 Emerging Infections Research Resources (BEI). The GFP-expressing strain, the Δtkt mutant
114 strain and the complemented strain were constructed and cultured as described in
115 **supplementary methods**. Strains and plasmids used are summarized in **supplementary**
116 **methods**.

117 *S. aureus* were grown in brain heart infusion (BHI) or chemically defined medium (CDM)
118 supplemented with ribose or fructose or glycerol when it was required [31].

119 EA.hy926 endothelial cells (ATCC CRL-2922) were grown in DMEM 10 % FBS (5 % CO₂ at
120 37 °C). When indicated, cells were incubated with no glucose DMEM supplemented with
121 glucose, ribose or fructose at a concentration of 25mM. EA.hy926 expressing nuclear mKate2
122 were transduced with the IncuCyte® NucLight Red Lentivirus.

123 Cells were infected at a multiplicity of infection (MOI) of 1 for 1 h and washed three times with
124 100 μ L of phosphate buffer saline (PBS) containing 300 μ g/ml gentamicin to remove
125 extracellular bacteria. Cells were then incubated in PBS-gentamicin (50 μ g/ml). Gentamicin is
126 an antibiotic with a high bactericidal effect on *S. aureus* USA300 (CMI=2 μ g/ml) and a very
127 poor penetration inside eukaryotic cells. Its use was necessary to abolish extracellular
128 proliferation.

129 LAMP-1 colocalization assay in EA.hy926 infected cells is described in the **supplementary**
130 **methods**.

131

132 **Time lapse microscopy**

133 EA.hy926 cells expressing nuclear mKate2 were seeded in ImageLock 96-well plates (Essen
134 BioScience Inc). Cells were infected with WT and Δtkt mutant of the GFP-expressing USA300
135 strain. Plates were incubated and monitored at 5% CO₂ and 37°C for 24 hours in the fully
136 automated microscope Incucyte® S3 (Essen BioScience). The detailed analysis is further
137 described in the **supplementary methods**.

138

139 **Transcriptional analyses**

140 The bacteria were recovered from lysed cells or planktonic overnight culture. Nucleic acids
141 were released by resuspending bacteria in TE buffer containing lysostaphin. RNAs recovery,
142 reverse transcription and PCR were performed as described in the **supplementary methods**.
143 We used the “The Relative Standard Curve Method” for analyzing the qRT-PCR data.

144

145 **Metabolomic analyses**

146 Metabolite profiling of *S. aureus* isolates grown to stationary phase in BHI Broth was performed
147 by liquid chromatography–mass spectrometry (LC-MS) as described in the **supplementary**
148 **methods** [9, 32, 33].

149

150 **Transketolase activity assay**

151 TKT activity was analysed on bacteria grown to stationary phase in BHI as described in the
152 **supplementary methods** [34]. TKT activity was expressed as units per microgram of total
153 protein. One unit of enzyme was defined as the amount of enzyme that oxidized 1 μmol of
154 NADH per minute.

155

156 ***In vivo* infection**

157 Mice were infected intravenously (iv) in the tail vein as described in the **supplementary**
158 **methods**. For bacterial burden determination, mice were euthanized after 4, 24, 48 and 72
159 hours. Sequential dilutions of homogenized preparations from spleen and kidney were spread
160 onto BHI agar plates.

161

162 **Statistics**

163 Statistical significances were assessed using one-way analysis of variance (ANOVA) with
164 Dunnett’s correction, unpaired two-tail Student’s t-test, or Kruskal Wallis test. P values of
165 $p < 0.05$ were considered to indicate statistical significance.

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167 **Ethics statement**

168 All experimental procedures involving animals were conducted in accordance with guidelines
169 established by the French and European regulations for the care and use of laboratory animals
170 (Decree 87–848, 2001–464, 2001–486 and 2001–131 and European Directive 2010/63/UE)
171 and approved by the INSERM Ethics Committee (Authorization Number: 75-906).

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195 **Results**

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197 **Transketolase inactivation in *S. aureus* USA300**

198 *S. aureus* genomes possess a unique transketolase-encoding gene, designated *tkt*,
199 which is highly conserved within species. Transketolase (TKT) is an enzyme of the non-
200 oxidative branch of the PPP involved in two main reversible enzymatic reactions: i) Fructose-
201 6-P + Glyceraldehyde-3-P \leftrightarrow Erythrose-4-P + Xylulose-5-P; and ii) Sedoheptulose-7-P +
202 Glyceraldehyde-3-P \leftrightarrow Ribose-5-P + Xylulose-5-P (**Supplementary Figure S2A**). Several
203 TKT structures have been solved [35] and allow the modelisation of the *S. aureus* TKT
204 (**Supplementary Figure S1B**). The protein of *S. aureus* is 662 amino acid long and harbours
205 three domains (**Supplementary Figure S1A**). Of note, although ubiquitously expressed in
206 Eukaryotes and Bacteria, *S. aureus* and *Homo sapiens* TKT proteins share only 22.4% amino
207 acid identity (**Supplementary Figure S1C**). We constructed a chromosomal deletion of the *tkt*
208 gene in *S. aureus* strain USA300 (Δtkt strain), and generated a complemented strain
209 (designated Cp-*tkt*) by expressing the wild-type *tkt* allele preceded by its own promoter in the
210 Δtkt strain. Gene inactivation was confirmed by quantification of the transketolase activity
211 (**Supplementary Figure S2**).

212 The Δtkt mutant showed only a slight growth decrease compared to the WT strain in
213 complete BHI Broth or in Dulbecco's Modified Eagle Medium (DMEM) (**Figure 1A**). In contrast,
214 the Δtkt mutant strain was unable to grow in chemically defined medium [31] supplemented
215 with ribose (which can be converted to Ribose-5-phosphate by ribokinase) (**Supplementary**
216 **Figure S2C**). In all assays, functional complementation was observed in the Cp-*tkt* strain.

217 We next performed a whole cell metabolomic analysis on WT and Δtkt strains grown for
218 24h in BHI Broth (*i.e.*, when identical numbers of cells were recorded) to evaluate the global
219 impact of *tkt* deletion on bacterial metabolism (**Figure 1B and Supplementary Figure S3 and**
220 **Table S1**). Remarkably, we observed a massive accumulation of ribose-5-phosphate (R5P;
221 56-fold increase) concomitant with a decrease in ribose content (4-fold decrease) in the Δtkt
222 mutant compared to WT. This accumulation was associated with an increased amount of
223 metabolites derived from Ribose-5P, such as inosine-5-monophosphate (IMP), xanthosine-5-
224 monophosphate (XMP) and hypoxanthine. We also observed a 2.5-fold decrease in D-sedo-

225 heptulose-7P amount in the Δtkt mutant compared to WT accompanied by a significant
226 decrease in tyrosine, histidine and tryptophan amounts (5, 10 and 12 fold respectively), three
227 aromatic amino-acids produced from the PPP. The glutamate pathway seemed to be
228 significantly more active in the Δtkt strain compared to WT as the amounts of glutamate, proline
229 and ornithine were all increased. The higher amounts of glutamate, possibly fuelling the TCA
230 cycle at the level of α -ketoglutarate, could be responsible for the increased expression of some
231 TCA cycle metabolites (*i.e.*, fumarate, malate, succinate and α -ketoglutarate) in the Δtkt strain.
232 Of note, ATP content was not altered in Δtkt strain. These results demonstrate that
233 deregulation of the PPP by *tkt* inactivation has direct effects on the whole cell metabolism,
234 including aromatic amino acid production, TCA activity and pathways involved in adenine,
235 guanine or peptidoglycan synthesis.

236

237 ***Tkt* is essential for intracellular proliferation of *S. aureus***

238 Intracellular staphylococci exist as two different populations: bacteria that actively
239 proliferate during the first 24 hrs after invasion, leading to their release in extracellular milieu
240 as a consequence of host cell death, and bacteria that do not replicate. These latter
241 staphylococci can persist within host-cell cytoplasm for several days [3-9, 36]. Here we
242 addressed the role of TKT in these two phenotypes in EA.hy926 human endothelial cells by
243 using: i) time lapse-microscopy, to evaluate early intracellular proliferation of *S. aureus* and ii)
244 colony forming units counting, to monitor the survival kinetics of internalized bacteria over a
245 ten days period.

246 *Time-lapse video microscopy.* EA.hy926 cells expressing mKate2 nuclear fluorescent
247 protein were infected by GFP-expressing bacteria. Gentamicin was used throughout the
248 experiment to eliminate extracellular bacteria and prevent re-infection (see Methods), allowing
249 us to solely focus on the fate of intracellular persistent bacteria. Cell infection was monitored
250 using an IncuCyte[®] S3 imaging system and images were captured every 30 minutes. Bacterial
251 entry into endothelial cells was similar for WT and Δtkt strains, showing 0.3~0.5 GFP
252 expressing particles recorded per cell (**Figure 2A-B**). Active proliferation of WT *S. aureus*
253 inside EA.hy926 cells was confirmed after 24 hours of infection (**Figure 2A-B and Movie 1**)

254 with an average number of GFP expressing particles rising from 0.5 to 2 per cells. At the single
255 cell level, WT *S. aureus* showed active intracellular multiplication, ultimately leading to cell
256 death and bacterial release in the extracellular medium (**Figure 2A-B and Movie 1**). Thanks
257 to the permanent presence of gentamicin in the medium bacterial release did not lead to
258 proliferation in the extracellular medium. In contrast, the Δtkt mutant strain showed limited
259 proliferation during the first 24 hours (**Figure 2A-B and Movie 2**). Finally, using LAMP-1
260 colocalization assay (Lysosome-associated membrane protein 1), we confirmed that the
261 intracellular Δtkt strain growth defect was not due to a decreased phagosomal escape
262 (**Supplementary Figure S4**).

263 *Long-term intracellular survival.* Using the same infection conditions as for time-lapse
264 experiment, we monitored survival of internalised Δtkt mutant compared to that of the WT strain
265 at three, seven or ten days after infection by CFU counting. The Δtkt strain behaved like the
266 WT strain throughout the infection period, ultimately leading to its complete elimination after
267 10 days (**Figure 2C**).

268 Thus, the role of TKT during *S. aureus* host cell infection seems to be essential for
269 bacteria proliferation and restricted to early time points.

270

271 ***Tkt* inactivation is associated with an altered *rpiRc* and *sigB* expression.**

272 *S. aureus* is known to sense its environment and to regulate virulence factors
273 depending on the availability of carbon sources. For example, recent studies have shown that
274 the transcriptional regulator RpiRc could sense metabolic shifts, especially in the PPP, and
275 control expression of RNAIII [29, 30]. RNAIII is a key effector in the quorum-sensing system
276 that regulates the expression of a large number of virulence factors [37]. RpiRc is also linked
277 to the alternative Sigma B factor (SigB) that has pleiotropic roles in gene regulation and is a
278 master regulator of intracellular survival [29, 38, 39].

279 These data prompted us to follow the expression profile of the master regulator SigB
280 and RpiRc in the WT and the Δtkt mutant strains during endothelial cells invasion (**Figure 3**).
281 EA.hy296 cells were infected as described above to solely focus on intracellular bacteria.
282 Between day 1 and day 7, *sigB* and *rpiRc* transcription increased in the WT strain (by 4-fold

283 and 3-fold, respectively) (**Figure 3**). In sharp contrast, as early as one day after infection, both
284 genes were significantly down-regulated in the Δtkt mutant strain compared to WT (8-fold and
285 7-fold decrease for *sigB* and *rpiRC* expression, respectively). Consistent with reports
286 demonstrating inhibition of RNAIII expression by RpiRC and SigB [18], we observed a dramatic
287 increase in the expression of *RNAIII* in the Δtkt mutant strain (**Figure 3**), suggesting that the
288 Δtkt mutant strain is unable to control *rpiRC* and *sigB* expression during intracellular survival.
289 Functional complementation always restored the wild type phenotype (**Supplementary Figure**
290 **S5**). To further confirm these data, we monitored the expression of two major regulators of
291 SigB activity, RsbU and RsbW [40]. RsbW, which is co-expressed with *sigB*, is known to
292 sequester and inhibit SigB. RsbU, whose expression is under the control of SigA, is a
293 phosphatase involved in the dephosphorylation of RsbV. In turn, dephosphorylated RsbV
294 (dRsbV) is able to sequester RsbW, allowing the release of active SigB. RsbU is thus
295 considered as an activator of SigB, allowing a fine-tuning of SigB activity.

296 We found that the transcription of both *rsbW* and *rsbU* genes was down-regulated in
297 the Δtkt mutant during infection of endothelial cells. The down-regulation of *rsbW* is consistent
298 with the down-regulation of its co-expressed gene *sigB* (**Figure 3**). The concomitant down-
299 regulation of *rsbU* suggests a global inhibition of the *sigB* pathway in the Δtkt mutant strain.
300 Finally, we followed expression of *asp23*, a *sigB*-dependent locus [41]. The recorded down-
301 regulation of *asp23* transcription in the Δtkt mutant strain confirmed the inhibition of SigB-
302 dependent transcription. All together these transcriptional analyses suggest that the metabolic
303 deregulation observed in Δtkt strain is sufficient to promote a dramatic reprogramming of the
304 RpiRC and SigB pathways when bacteria invade human cells.

305

306 ***Tkt* inactivation impairs proliferation of *S. aureus* in the kidney of infected mice.**

307 The ability of internalized *S. aureus* to readily proliferate inside host cell cytoplasm may
308 be seen as an effective mechanism to avoid host innate immunity, ultimately allowing an
309 increased colonization of the infected site. In this respect, it has been previously shown in the
310 mouse model that after intravenous (IV) injection, although rapidly cleared from the

311 bloodstream, bacteria were able to proliferate in the kidney, leading to abscesses formation
312 within the first days of infection [42].

313 Here, we monitored the kinetics of *in vivo* proliferation of WT, Δtkt and Cp-*tkt* strains in
314 kidneys and spleens of BalB/c mice (**Figure 4**) after IV infection with 10^7 bacteria per mouse.
315 As previously reported, *S. aureus* poorly proliferates in the spleen of infected mice. We
316 therefore used the spleen as a non proliferative control. The bacterial burden in kidneys and
317 spleens was quantified by plating CFUs at 4 h, 24 h, 48 h and 72 h after infection. We observed
318 no significant differences between the bacterial counts in the spleens of mice infected either
319 with WT, the Δtkt mutant or the Cp-*tkt* strain. In each case, a progressive reduction of the
320 bacterial burden was recorded (ranging from 2.5×10^5 , 1.1×10^5 and 1.5×10^5 at 4 h; down to
321 3×10^3 , 7.3×10^2 and 1.2×10^2 at 72 h, for WT, Δtkt and Cp-*tkt* respectively). In contrast, in kidneys,
322 whereas the WT and the Cp-*tkt* strains readily proliferated (from $3.9 \times 10^4 \pm 8.6 \times 10^3$ WT and
323 $2.1 \times 10^4 \pm 4.6 \times 10^2$ Cp-*tkt* *S. aureus* per organ at 4 hrs to $7.7 \times 10^7 \pm 3.3 \times 10^7$ WT and $3.6 \times 10^7 \pm$
324 1.3×10^7 Cp-*tkt* *S. aureus* per organ at 72 hrs), multiplication of the Δtkt mutant was severely
325 impaired at all time-points tested, with a 10^4 -fold reduction of Δtkt counts at 24h compared to
326 WT counts (**Figure 4**). This result confirms the importance of TKT -and most likely of the PPP-
327 in bacterial proliferation *in vivo* and especially during the first 24 hours after infection. This
328 result is consistent with the previously described role of SigB in intracellular growth *in vivo* [43].
329

330 **The SigB pathway may be controlled by modulating carbon source availability.**

331 Inside the cytoplasm of host cells, the Δtkt mutant is likely to deal simultaneously with
332 PPP blocking and altered levels of carbon-based nutrients compared to the outside
333 compartment. The above results suggested that the PPP might take control of *rpiRc* and *sigB*
334 expression when bacteria invade endothelial cells. We thus hypothesized that PPP-associated
335 carbon sources may possibly influence *rpiRc* and/or *sigB* expression *in vitro*. We therefore
336 grew WT USA300 in CDM supplemented either with glucose (CDM-glucose), ribose (CDM-
337 ribose) or fructose (CDM-fructose) a carbon source possibly fuelling the PPP) and quantified
338 by qRT-PCR the transcription of *sigB*, *rpiRc*, *rsbU/W* and *asp23* genes. Remarkably, we found
339 that the regulation of the whole *sigB* operon was impacted by the carbon source used in CDM

340 **(Figure 5)**. Indeed, expression of *sigB*, *rsbUW* and *rpiRc* decreased in CDM-fructose or CDM-
341 ribose, compared to CDM-glucose. In contrast, *asp23* expression was only decreased by 2-
342 fold in CDM-fructose and increased by 3-fold in CDM-ribose, suggesting that in this growth
343 conditions, *asp23* expression might be regulated by other factor than *sigB* **(Figure 5)**. Hence,
344 the availability of carbon sources, and especially those related to PPP, may be sensed by the
345 bacteria and affect the regulation of *rpiRc* and *sigB* expression.

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366 **Discussion**

367 Inactivation of the *tkt* gene, encoding the unique transketolase of *S. aureus* USA300,
368 led to a major decrease in early intracellular proliferation and impaired *in vivo* the multiplication

369 in a kidney colonization model. Our results suggest that the intracellular proliferation defect of
370 the Δtkt mutant occurs through the inhibition of *sigB* and *rpiRc* expression and the concomitant
371 increased *RNAIII* transcription, thus highlighting a novel connection between the PPP and
372 these master regulators during cell invasion.

373 Our whole cell metabolomic analysis of planktonic-grown bacteria (**Figure 1**,
374 **Supplementary Figure S2 and Table S1**) showed a huge increase in R5P intermediates in
375 the Δtkt mutant compared to WT that could be explained by the impaired entry of R5P into
376 glycolysis and a concomitant significant decrease in SH7P relative concentration. In *S. aureus*,
377 ribose is imported by the RbsU transporter and converted to R5P by the RbsD/RbsK pathway.
378 RbsR, the repressor of *rbsUDK* operon, is itself highly regulated by SigB [44]. Interestingly, *S.*
379 *aureus* grown in CDM-ribose showed a decreased expression of *sigB*. Hence, stress signals
380 that modulate SigB activity are likely to be important effectors for controlling the quantity of
381 RbsR, thereby affecting ribose uptake.

382 Significant changes in the relative concentrations of TCA cycle intermediates were also
383 recorded in the Δtkt mutant compared to WT (**Supplementary Figure S3**). The TCA cycle
384 plays a central role in maintaining the bacterial metabolic status and has been repeatedly
385 implicated in the regulation of staphylococcal virulence [45, 46]. Hence, changes in TCA cycle
386 activity are likely to induce alterations of the overall metabolome of the bacterium that, in turn,
387 may modulate the activity of metabolite-responsive global regulators such as SigB or RpiR.

388 RpiRc, a RpiR family-member controlling enzymes involved in sugar catabolism in *S.*
389 *aureus*, has been shown to repress *RNAIII* [28], supporting the notion of a direct connection
390 between the PPP and the regulation of *S. aureus* virulence. The expression of *sigB* has also
391 been shown to play a crucial role during the intracellular life of bacteria possibly by down-
392 regulating pro-inflammatory virulence factors and increasing the expression of factors
393 promoting persistence [18, 39].

394 We propose (**Figure 6**) that in wild-type *S. aureus*, transketolase activity results in a
395 positive regulation of *rpiRc* and *sigB* transcription and allows the repression of *RNAIII*.
396 Consequently, repression of toxins production favours intracellular growth. In contrast, in a Δtkt
397 mutant, *rpiRc* and *sigB* transcriptions are not activated, possibly due to metabolic changes

398 (such as R5P accumulation) and RNAIII is not repressed. At this stage, it cannot be excluded
399 that the enzyme itself might also directly affect transcription of these regulators.

400 TKT inhibitors are currently actively tested in cancer therapy [47] and TKT could
401 constitute an efficient target against tuberculosis [35] and malaria [48]. The present work,
402 highlighting an unprecedentedly reported role of transketolase in *S. aureus* intracellular
403 survival, suggests that modulation of the pentose phosphate pathway activity may also
404 represent an interesting mean to fight *S. aureus* infections.

405

406 Legends

407

408 **Figure 1. Phenotypic characterization of the Δtkt mutant**

409 **A. Growth of USA300 wild type (WT), Δtkt mutant (Δtkt) and its complemented derivative**

410 **(Cp-*tkt*).** USA300 WT, Δtkt and Cp-*tkt* strains were grown in BHI Broth or DMEM for 21 hours
411 at 37°C. Optic densities were measured every hour from 0 to 8 hours and then at 21 hours.
412 Each panel is a representative experiment.

413 **B. Metabolomic analysis of the Δtkt mutant strain.** Quantitative metabolomic analysis was
414 performed by ion chromatography and tandem mass spectrometry (IC-MS/MS) after overnight
415 cultures, in BHI Broth, from WT USA300 and Δtkt mutant strains (see **Table S1**). The heatmap,
416 obtained using MetaboAnalyst (<https://www.metaboanalyst.ca>), illustrates the metabolic shift
417 between the two strains.

418

419 **Figure 2. Intracellular proliferation and persistence of *S. aureus*.**

420 **A, B. Intracellular proliferation of USA300 wild type (WT) and Δtkt mutant (Δtkt) strains.**

421 Endothelial EA.hy926 cells expressing mKate2 nuclear restricted red fluorescent protein were
422 infected with GFP expressing WT and Δtkt strains. One hour after infection, cells were washed
423 several times with gentamicin containing medium to eliminate extracellular bacteria.
424 Gentamicin concentration of 50µg/ml was maintained throughout the experiment. Images were
425 acquired every 30 minutes using Incucyte[®] S3 live cell imaging system. A. Representative
426 images (see also Movie 1 and 2). Red: cell nuclei. Green: GFP expressing bacteria. For each
427 line, a white circle was centred on a unique infected cell. Bar = 100µm. B. Quantitative analysis
428 representing the number of green particles divided by the number of red nuclei during the first
429 day after infection. Blue line: WT strain. Red line: Δtkt strain. Black line: non infected cells.

430 **C. Persistence of intracellular WT and Δtkt strains.**

431 Endothelial EA.hy926 cells were
432 infected with WT and Δtkt strains as described above. Gentamicin was maintained throughout
433 the experiment. Survival of *S. aureus* was assessed by plating and counting CFUs three, seven
434 and ten days after infection. Results are shown as mean \pm SD of triplicate measurements.

434

435 **Figure 3. Transcription profile of intracellular *S. aureus*.**

436 Endothelial EA.hy926 cells were infected with USA300 WT and Δtkt strain as described in
437 figure 2. One, three or seven days after infection, cells were washed and collected. RNA from
438 intracellular *S. aureus* were prepared and gene expression of *RNAIII*, *rpiRc*, *sigB*, *rsbU*, *rsbW*
439 and *asp23* was analysed by quantitative RT-PCR. Gene expression level was normalized by
440 that of *gyrA* at days 1, 3 and 7 after infection of EA-hy296 cells. Data are shown as mean
441 normalized expression \pm SD of triplicate measurements from two independent experiments.
442 (ANOVA, ns: not statistically different; * $p < 0.01$, ** $p < 0.001$). Functional complementation was
443 assessed on two other independent experiments and presented in **Supplementary Figure S5**.

444
445 **Figure 4. Proliferation of *S. aureus* in the spleen and the kidney of infected mice.**

446 Bacterial loads in the spleen and the kidney of BalB/c mice infected intravenously with 10^7
447 bacteria of the WT, Δtkt and Cp-*tkt* strain. Bacterial counts are expressed in CFU/organ (WT
448 and Δtkt n=10; Cp-*tkt* n=6). Data are shown as mean \pm SD. Statistical significance was
449 determined using the Kruskal Wallis test with Dunn's correction (ns: not statistically different,
450 *** $p < 0.001$, **** $p < 0.0001$). No statistical significance between strains was observed in the
451 spleen of infected mice.

452
453 **Figure 5. Carbon sources influence the transcription profile of the SigB pathway.**

454 *S. aureus* USA300 WT strain was grown to stationary phase in CDM supplemented with
455 glucose or ribose or fructose and gene expression profile of *rpiRc*, *sigB*, *rsbU*, *rsbW* and *asp23*
456 was analysed by quantitative RT-PCR. Gene expression level was normalized by that of *gyrA*.
457 Data are shown as mean normalized expression \pm SD of triplicate measurements from two
458 independent experiments. Statistical significance was assessed in comparison to bacteria
459 grown in CDM glucose (ANOVA, * $p < 0.01$, ** $p < 0.001$).

460
461 **Figure 6. Schematic depiction of the impact of TKT inactivation on *sigB* and *rpiRc***
462 **regulation.**

463 *SigB* dependent regulation of *RNAIII* in Δtkt context.

464

465 **Movie 1: Intracellular proliferation of USA300 wild type strain.** Endothelial EA.hy926 cells
466 expressing a nuclear restricted Red Fluorescent Protein were infected with GFP expressing
467 wild type strains. One hour after infection cells were washed several times with gentamicin
468 containing medium to eliminate extracellular bacteria (see materials and methods section).
469 Gentamicin concentration of 50µg/ml was maintained throughout the experiment. Images were
470 acquired every 30 minutes using Incucyte® S3 live cell imaging system.

471

472 **Movie 2: Intracellular proliferation of USA300 Δtkt mutant strain.** Same protocol as above
473 in movie 1.

474

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479 *Staphylococcus aureus* (NARSA) for distribution by BEI Resources, NIAID, NIH:
480 *Staphylococcus aureus*, Strain USA300-0114, NR-46070; *Escherichia coli* – *Staphylococcus*
481 *aureus* Shuttle Vector pNR-46158, Recombinant in *Staphylococcus aureus*, NR-46158.

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Figure 1

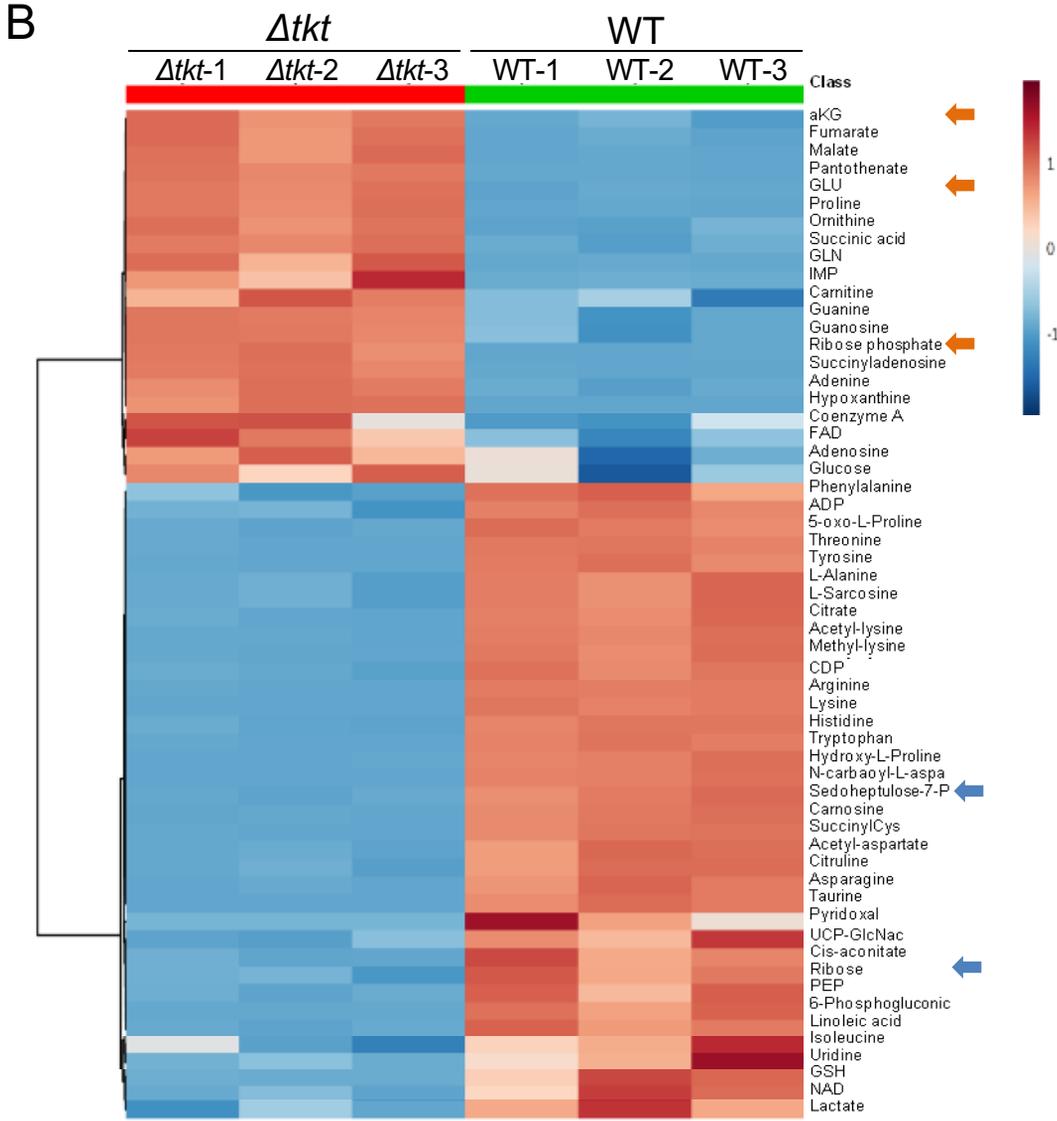
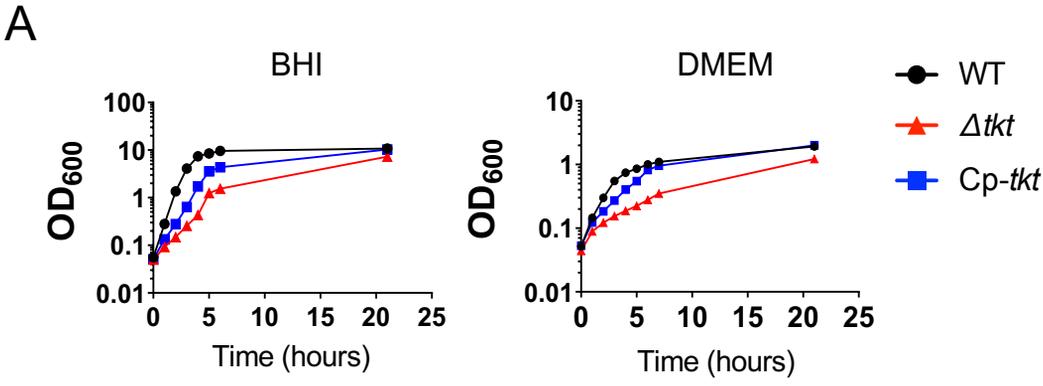


Figure 2

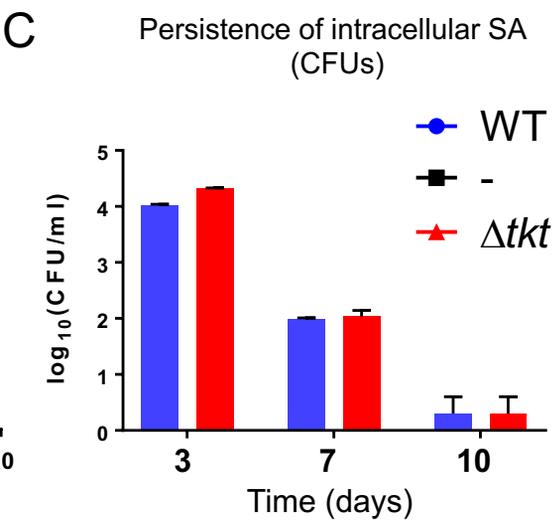
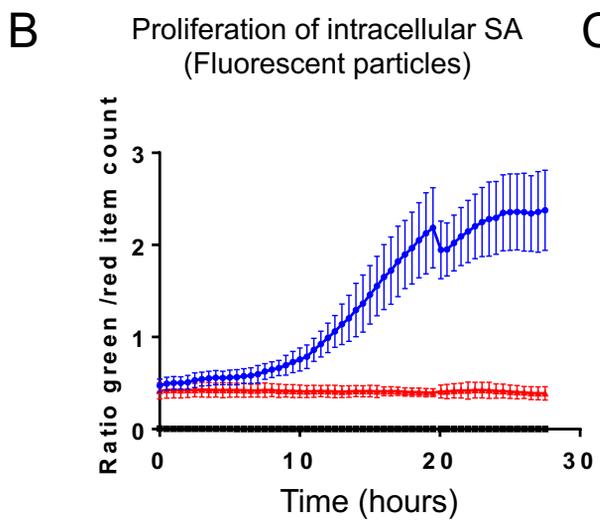
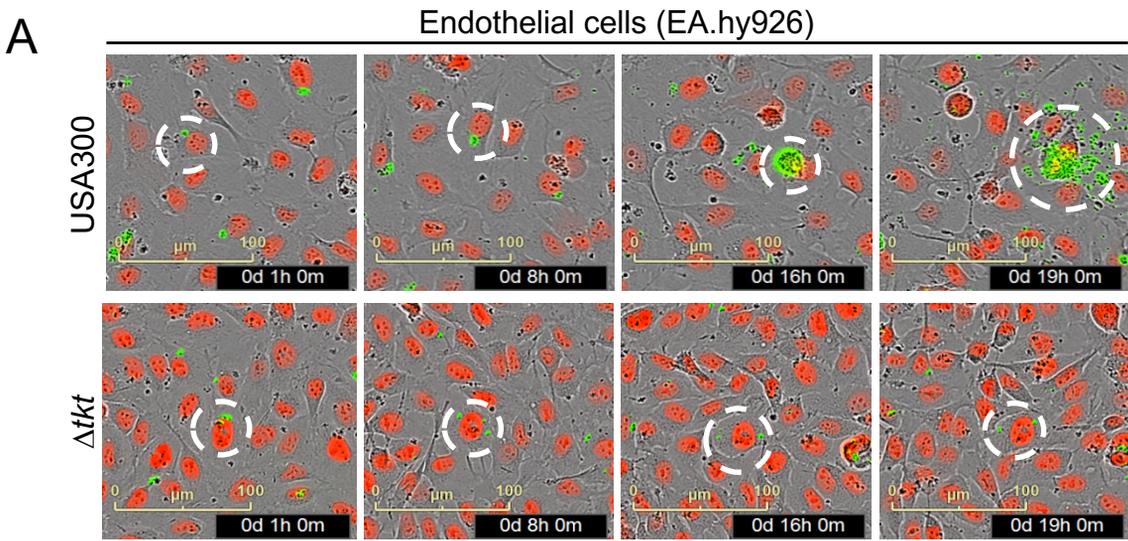


Figure 3

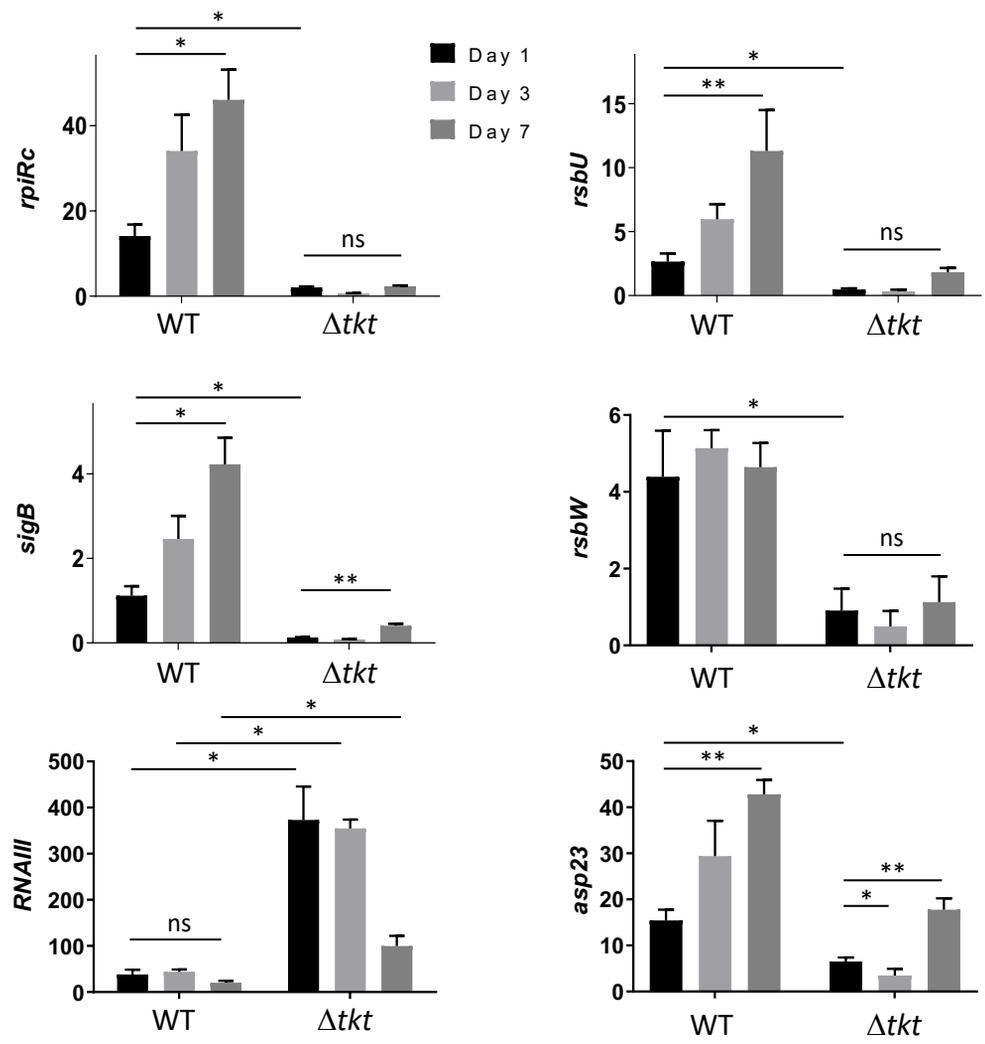


Figure 4

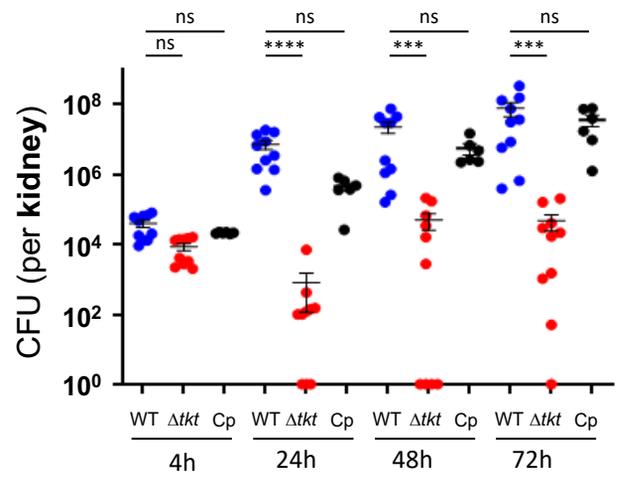
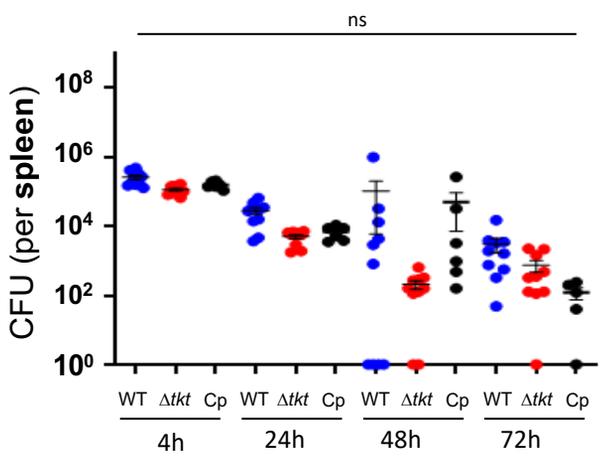


Figure 5

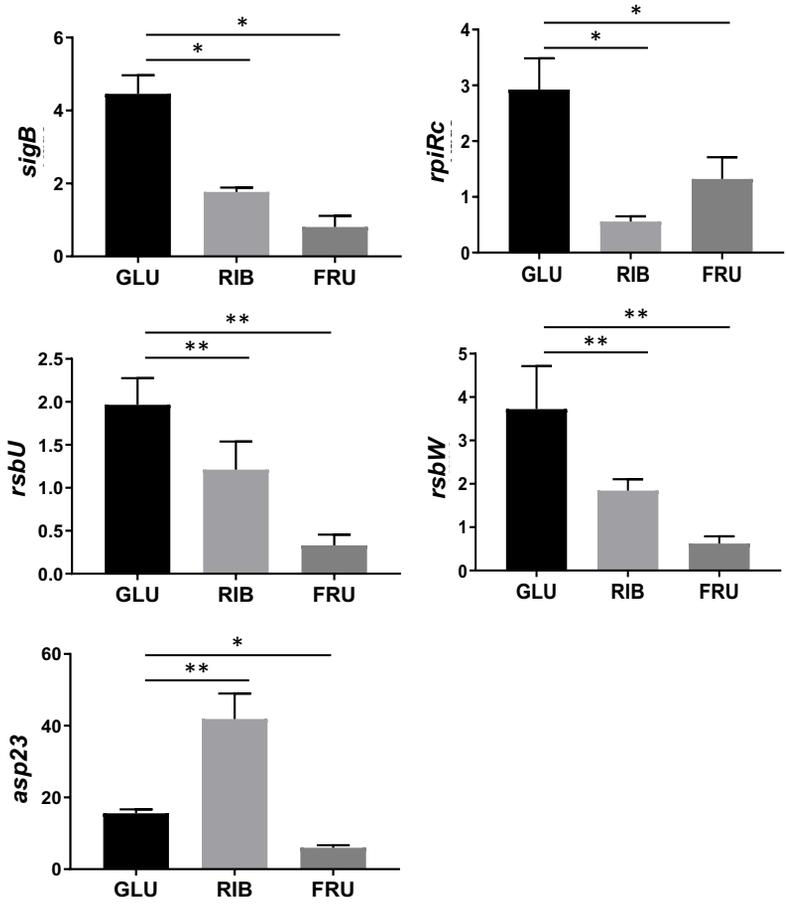


Figure 6

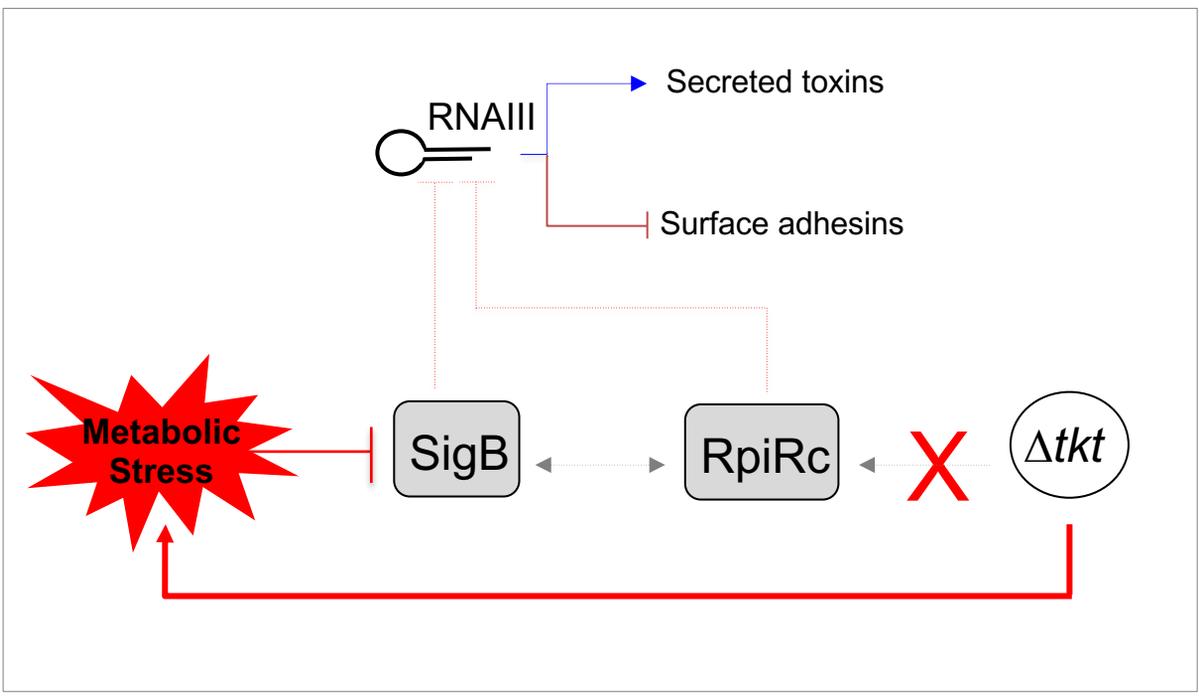
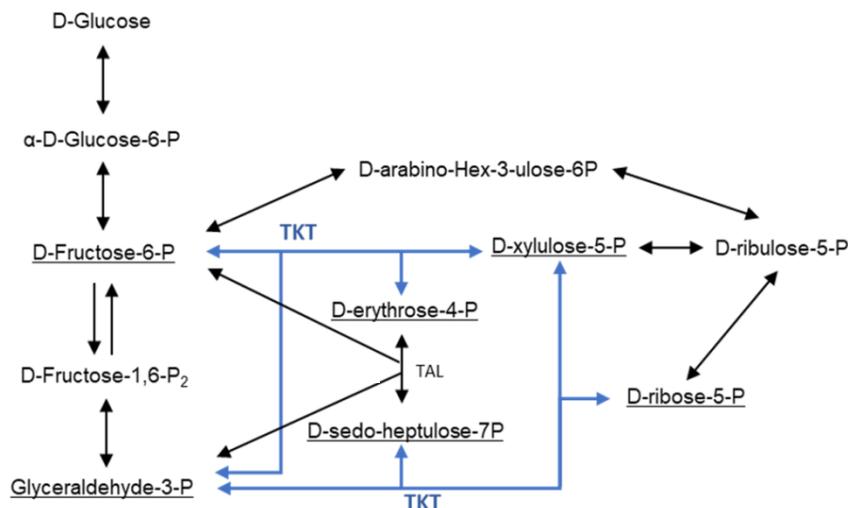
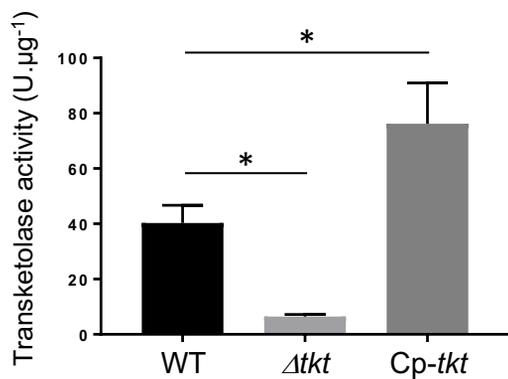


Figure S1

A



B



C

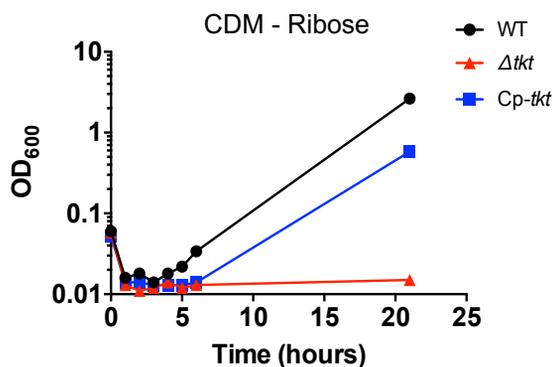


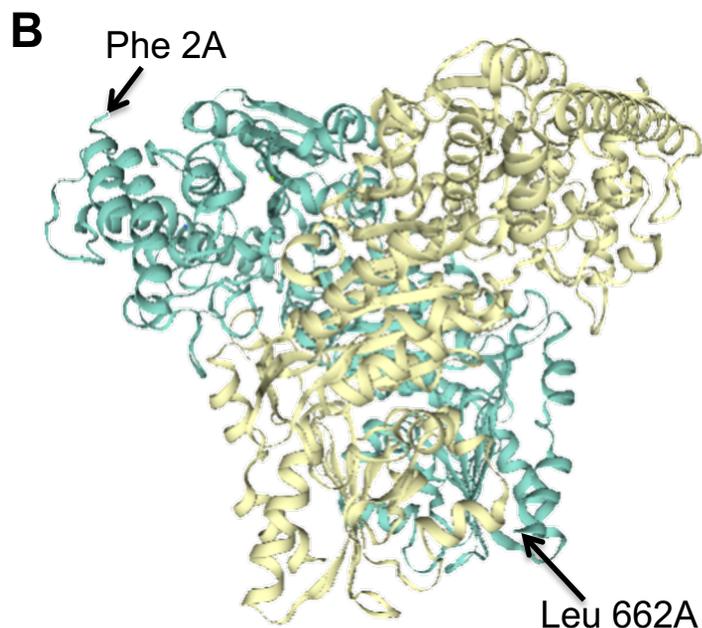
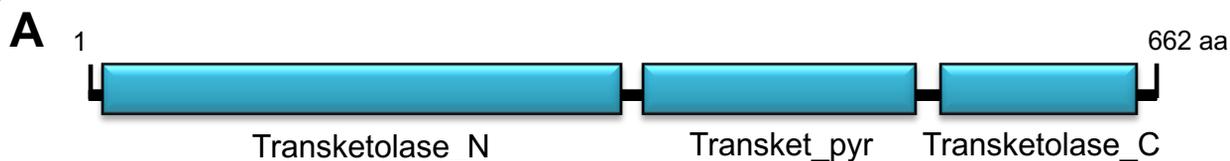
Figure S1. Characterization of the Δtkt mutant strain.

A. Schematic depiction of the Pentose Phosphate Pathway. Light blue: TKT enzymatic reaction.

B. Transketolase activity. Transketolase activity was monitored in USA300 WT, Δtkt and Cp-*tkt*. Inactivation of *tkt* gene led to a significant decrease in TKT enzyme activity (ANOVA: * $p < 0.001$).

C. Growth of USA300 wild type (WT), Δtkt mutant (Δtkt) and its complemented derivative (Cp-*tkt*). USA300 WT, Δtkt and Cp-*tkt* strains were grown in CDM supplemented with ribose as sole carbon source for 21 hours at 37°C. Optic densities were measured every-hour from 0 to 8 hours and then at 21 hours. Each panel is a representative experiment.

Figure S2



C

Protein ID	% coverage	% identity	Species
TKT_HUMAN	100.0%	100.0%	<i>Homo sapiens</i>
TKT_MOUSE	100.0%	94.7%	<i>Mus musculus</i>
TKT2_ECOLI	94.5%	24.1%	<i>Escherichia coli</i>
TKT_HAEIN	94.2%	22.9%	<i>Haemophilus influenzae</i>
TKT1_VIBCH	94.4%	24.0%	<i>Vibrio cholerae</i>
TKT_DICDI	94.2%	22.6%	<i>Dictyostelium discoideum</i>
TKT1_YEAST	94.5%	23.8%	<i>Saccharomyces cerevisiae</i>
TKT_TREPA	93.9%	21.9%	<i>Treponema pallidum</i>
TKT_MYCBO	96.6%	20.6%	<i>Mycobacterium bovis</i>
TKT_STRPN	94.2%	23.0%	<i>Streptococcus pneumoniae</i>
TKT_STAAS	94.4%	22.4%	<i>Staphylococcus aureus</i>
TKT_BACSU	94.9%	22.7%	<i>Bacillus subtilis</i>

Figure S2. Transketolase proteins domain organization and structure.

A. Conserved domains found in *S. aureus* transketolase. Transketolase_N (PF00456 domain): Transketolase, thiamine diphosphate binding domain; Transket_pyr (PF02779 domain): Transketolase, pyrimidine binding domain; Transketolase_C (PF02780): Transketolase, C-terminal domain (putative regulatory molecule binding site).

B. Protein model of *S. aureus* transketolase. Swiss-Model was used to predict the structure of *S. aureus* TKT (SAUSA300_1239) as a homodimer based on the template 3M49.1.A (Crystal Structure of Transketolase from *Bacillus anthracis*). The QMQE score and QMEAN were 0.83 and -0.59 respectively.

C. Table showing coverage percentage between transketolase amino acid sequences and percentage of identical amino acids between aligned transketolase amino acid sequences. Protein sequences were retrieved from UniProt database and aligned using Clustal Omega.

Figure S3

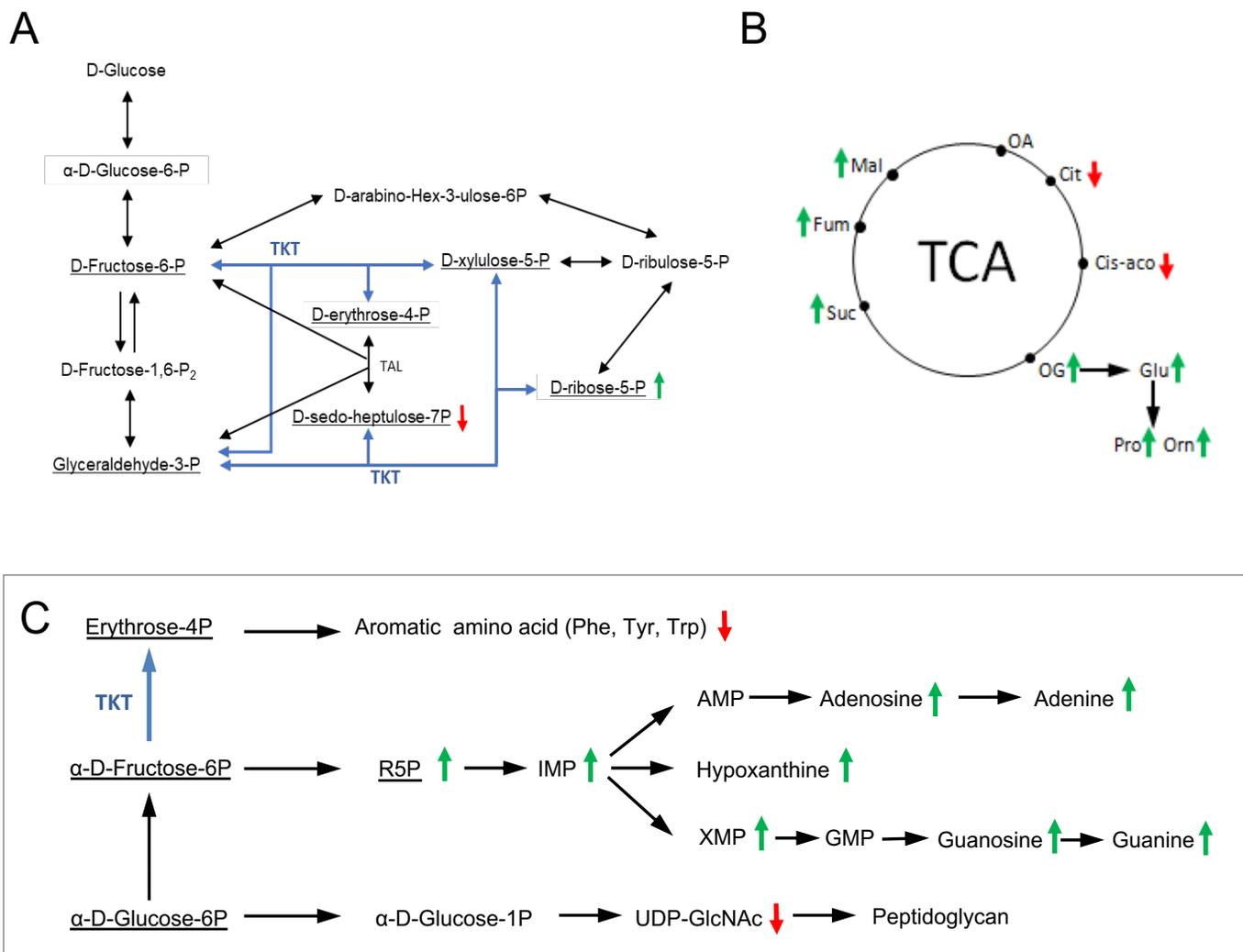
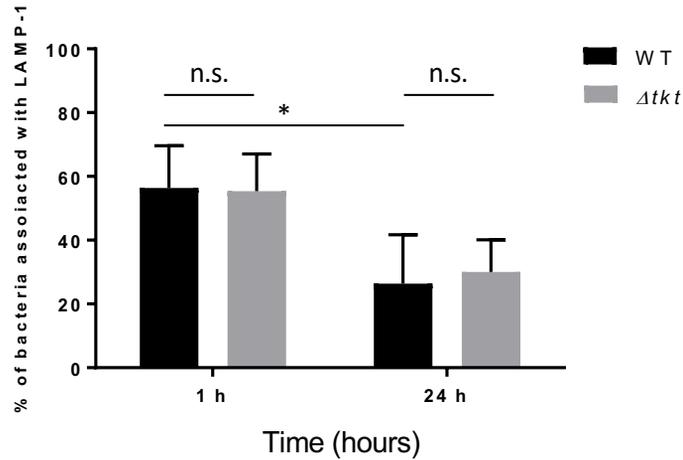


Figure S3. Metabolomic analysis of the Δtkt mutant strain.

Quantitative metabolomic analysis was performed by ion chromatography and tandem mass spectrometry (IC-MS/MS) after overnight cultures, in BHI Broth, from WT USA300 and Δtkt mutant strains (see **Table S1**). A, B and C. Highlight on metabolic pathways. Light blue: TKT enzymatic reaction. Green arrow: increased concentration in Δtkt mutant compared to WT strain. Red arrow: decreased concentration in Δtkt mutant compared to WT strain.

A

LAMP-1 colocalization assay

**Figure S4. LAMP-1 colocalization assay**

LAMP-1 colocalization assay. Endothelial EA.hy926 cells were infected with GFP-expressing WT and *Dtkl* mutant strains. One hour after infection, cells were washed several times with gentamicin containing medium to eliminate extracellular bacteria. A gentamicin concentration of 50 μ g/ml was maintained throughout the experiment. At 1 hour and 24 hours after infection cells were fixed using 4% PFA and processed for immunohistochemistry. Lysosomes were stained using anti-LAMP1 antibodies and images were acquired by confocal microscopy. Quantification of colocalization between GFP expressing USA300 WT or Δtkl bacteria and LAMP-1 staining was obtained using ImageJ software. Data were obtained from more than 200 infected cells for each condition and expressed as % of bacteria associated with LAMP-1 \pm SD. (ANOVA; n.s.: not statistically different; * $p < 0.05$).

Figure S5

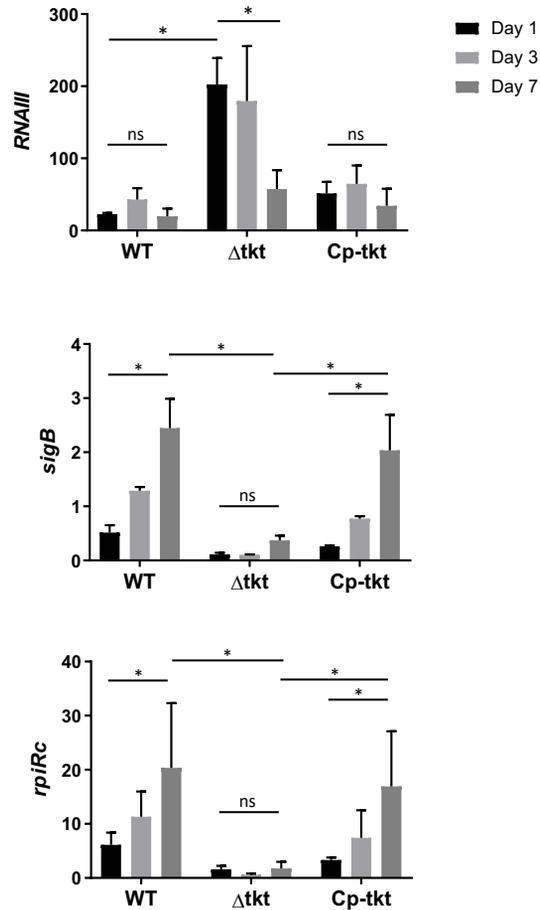


Figure S5. Transcription profile of intracellular *S. aureus*.

Endothelial EA.hy926 cells were infected with USA300 WT, Δ tkk and Cp-tkk strains as described in figure 2. One, three or seven days after infection cells were washed and collected. RNA from intracellular *S. aureus* were prepared and gene expression of *RNAIII*, *rpiRc* and *sigB* was analysed by quantitative RT-PCR. Gene expression level was normalized by that of *gyrA* at days 1, 3 and 7 after infection of EA-hy296 cells. Data are shown as mean normalized expression \pm SD of triplicate measurements from two independent experiments. (ANOVA, ns : not statistically different; * $p < 0.01$, ** $p < 0.001$).

Experiments presented in Figure S5 were independent of those presented in Figure 3.

Supplementary Methods

Strains, Cells, Culture conditions and Infection.

S. aureus. The epidemic clone *S. aureus* USA300-LAC (designated USA300-WT) was provided by the Biodefense and Emerging Infections Research Resources (BEI). The GFP-expressing strain was generated by curing the p03 plasmid from USA300-WT and introducing the pCN57-GFP recombinant plasmid (obtained from the BEI) by electroporation as described before [1]. The Δtkt mutant strain and the complemented strain were constructed as described below. Strains and plasmids used are summarized at the end of these supplementary methods.

Before experiments bacteria were grown 24 hours at 37°C on BHI agar plates supplemented with antibiotics when required and then overnight in BHI Broth from an optical density 600 nm (OD₆₀₀) of 0.05. The day of experiment, bacteria were resuspended in BHI broth to an OD₆₀₀ of 0.05 and grown at 37°C until the culture reached the initial-middle log phase (OD₆₀₀ of 0.6).

Growth curve were performed in brain heart infusion, DMEM and chemically defined medium (CDM) supplemented with ribose or fructose or glycerol when it was required [2].

Internalization rate and survival inside the EA.hy926 endothelial cell line were similar between the GFP strain and the parental strain.

Cell line. EA.hy926 cell line (ATCC CRL-2922), originally derived from human umbilical vein, were grown in Dulbecco's modified Eagle high glucose medium (DMEM, Dominique Dutscher) supplemented with 10 % fetal bovine serum (FBS, Thermofisher scientific) in a humidified 5 % CO₂ atmosphere at 37 °C. When indicated cells were incubated with no glucose DMEM supplemented with glucose, ribose or fructose at a concentration of 25mM. To facilitate cell identification and counting during time lapse experiments, EA.hy926 cells were transduced with the IncuCyte[®] NuLight Red Lentivirus, following the manufacturer's

recommendations, to obtain red nuclear labelling of living cells due to the expression of the red fluorescent protein mKate2 containing a nuclear localization signal.

Infection.

When fresh cultures reached OD₆₀₀ of 0.6, bacteria were added to endothelial cells at a multiplicity of infection (MOI) of 1 and placed in a humidified 5 % CO₂ atmosphere at 37 °C for 1 h. One hour after infection, each well was washed three times with 100 µL of phosphate buffer saline (PBS) containing 300 µg/mL gentamicin, to remove extracellular bacteria. Cells were then incubated with 100 µL of cell culture medium containing 50 µg.mL⁻¹ gentamicin. Infected cells were kept in a humidified 5 % CO₂ atmosphere at 37 °C. Importantly, gentamicin is an antibiotic with a high bactericidal effect on *S. aureus* USA300 (CMI=2 µg.mL⁻¹) and a very poor penetration inside eukaryotic cells. In our model the use of gentamicin in the extracellular medium was necessary to abolish extracellular proliferation.

Construction of the *S. aureus* Δ *tkt* chromosomal deletion mutant

We inactivated the *tkt* gene in wild-type *S. aureus* USA300 strain and substituted it by the kanamycin resistance gene *nptII* fused with p*Gro* promoter. For this, we used the pMAD-temperature-sensitive shuttle vector system [3]. Briefly, the recombinant plasmid pMAD- Δ *tkt* was constructed by overlap PCR. First, the two regions (upstream 694bp, downstream 567bp) flanking *tkt* and the *nptII* gene (fused with p*Gro* promoter 1,091bp) were amplified by PCR. The region (2,352 bp) was then amplified by two-step overlap PCR. The resulting PCR product was cloned in pMiniT 2.0 (to yield recombinant plasmid pMiniT/*tktUp-npt-tktDown*). This recombinant plasmid was digested with *Bam*HI and *Eco*RI (New England BioLabs) and the *Bam*HI-*Eco*RI *tktUp-npt-tktDown* DNA fragment was finally subcloned into *Bam*HI-*Eco*RI-digested pMAD. All PCR reactions were realized using Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific) and PCR products were purified using QIAquick PCR purification kit (Qiagen). The pMAD- Δ *tkt* plasmid was first introduced into *S. aureus* RN4220 prior to electroporation into *S. aureus* USA300. We used a standard two-step allelic

exchange procedure [3] to create the chromosomal *S. aureus* USA300 Δtkt strain. The Δtkt strain was finally checked for loss of the corresponding wild-type genes by PCR sequencing (Eurofins Genomics) using specific primers (described at the end of these supplementary methods).

Construction of the complementation of *S. aureus* Cp-*tkt* strain

The plasmid pCN57-CP*tkt* was constructed by homologous recombination [4]. The pCN57 linear vector and *tkt* gene fragments were amplified by PCR. To allow recombination, overlapping region of 20pb was added at each ends. PCN57 was linearized using SphI (New England BioLabs). *E. coli* DH5 α were chemically transfected with a mix of both PCR product (insert: vector stoichiometry 2:1, 100ng of vector) in 10 μ l MilliQ water. The colonies containing plasmid pCN57-CP-*tkt* were checked by PCR and the construct was sequenced. The pCN57-CP-*tkt* vector was then transfected into *S. aureus* RN4220 prior to electroporation into *S. aureus* USA300 Δtkt . The Δtkt -Cp-*tkt* (Cp-*tkt*) strain was finally checked for complementation of wild-type genes by PCR sequencing (Eurofins Genomics) using specific primers.

Time lapse microscopy

Four days prior to infection, NuLight Red labelled EA.hy926 cells were seeded in ImageLock 96-well plates (Essen BioScience Inc) in culture medium without antibiotic. Cells were infected with wild-type and mutant of the GFP-expressing USA300 strain as described above. Plates were incubated and monitored at 5% CO₂ and 37°C for 24 hours in the fully automated microscope Incucyte[®] S3 (Essen BioScience). Images were taken every 30 minutes with the 20X objective. The medium was changed once during the observation period. Analysis and time-lapse videos (from which images were extracted) were generated by using Incucyte[®] S3 software.

Detailed analysis: Green items were analysed with a threshold of 1 GCU and using Top-hat segmentation mode. Red item were analysed with a threshold of 3 RCU and using Top-hat

segmentation mode and edge sensitivity of -25. A spectral unmixing of 10% was applied to remove red signal from green signal. Green item counts were considered as an approximation of the number of intracellular bacteria, and red item counts considered as the number of EA-hy296 cells. (Green items/Red items) represents the number of intracellular bacteria per cell during the time of infection.

Transcriptional analyses

Intracellular bacteria RNA extraction: EA.hy926 cells were grown to confluence in 75 cm² flasks and infected with *S. aureus* as described above. At selected time points, the culture medium was removed and the flasks were washed twice with PBS without antibiotics. Cells were lysed with 5 mL of distilled water for 10 min and the surface of the flask was scraped to detach the cells. The contents were recovered and centrifuged for 5 min at 3000 *g*. The supernatant was carefully removed and the pellet (which contained cell debris and bacteria) was resuspended in 100 µL of TE buffer containing 20 µg of lysostaphin and incubated for 5 minutes at 37 °C [5] to allow cell wall peptidoglycan degradation.

Planktonic bacteria RNA extraction: *S. aureus* was resuspended in CDM supplemented with glucose, ribose or fructose to an OD₆₀₀ of 0.05 and grown overnight at 37°C. Bacteria were centrifuged at 12 000 *g* for 2 min and incubated in 50 µL of TE buffer containing 10 µg of lysostaphin for 5 minutes at 37 °C. The samples were resuspended in Trizol (ThermoFisher Scientific) and treated with chloroform. RNAs were recovered using RNeasy Clean-up column (Qiagen) and DNA was digested using Turbo Dnase (ThermoFisher Scientific). RNA concentration was measured using NanoDrop 1000 (ThermoFisher Scientific). Reverse transcription was carried out with 500 ng of RNA and 0.5 µg of random primers (Promega). After denaturation at 70 °C for 5 min, 15 µL of the mix containing 4 µL M-MLV 5 X reaction buffer, 1 µL of 0.5 mM PCR Nucleotide Mix, 0.5 µL of RNasin Ribonuclease Inhibitor, and 1 µL M-MLV Reverse Transcriptase (Promega) was added. Samples were incubated 5 min at 25 °C, 1 h at 42 °C, then 15 min at 70 °C and chilled on ice. Quantitative RT-PCR was performed using FastStart SYBR Green qPCR Master Mix (ThermoFischer Scientific) or

Luna Universal qPCR Master Mix (New England Biolabs) according to the manufacturer's recommendations. Transcript levels were analyzed using a 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the standard settings of the system software. The thermal cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Gene specific primer sequences are presented at the end of these supplementary methods. Expression of the gene *gyrA* was chosen as a reference. We used the "The Relative Standard Curve Method" for analyzing the qRT-PCR data.

Metabolomic analyses

Bacteria: Metabolite profiling of *S. aureus* isolates was performed by liquid chromatography–mass spectrometry (LC-MS) as described [6, 7]. Two independent experiments with three biological replicates were performed for each isolate. Briefly, *S. aureus* strains were grown in BHI Broth overnight to stationary phase and pelleted by centrifugation. Metabolic activity was blocked by immersion in liquid nitrogen for 10 sec. Metabolites were extracted using a solvent mixture of Methanol/ACN/H₂O (50:30:20) at –20 °C (Extraction solution). Samples were vortexed for 5 min at 4°C, and then centrifuged at 16,000 g for 15 minutes at 4°C.

Endothelial cells: EA.hy926 endothelial cells were grown to confluence on a 12-well plate in no glucose DMEM supplemented with glucose or fructose or ribose (25 mM). One independent experiment with three biological replicates was performed for each condition. At each time points the cells were washed with cold PBS. Metabolism was blocked by plunging the plate into liquid nitrogen for 10 seconds. The cell metabolites were extracted from the samples by placing the cell culture plate on a rocking shaker at 4°C for 10 minutes. The extraction solution from each well was then collected and centrifuged at 16,000 g for 15 minutes at 4°C.

The supernatants were collected and analysed by LC-MS using SeQuant ZIC-pHilic column (Millipore) for the liquid chromatography separation [6]. The aqueous mobile-phase solvent was 20 mM ammonium carbonate plus 0.1% ammonium hydroxide solution and the organic mobile phase was acetonitrile. The metabolites were separated over a linear gradient from

80% organic to 80% aqueous phase for 15 min. The column temperature was 48 °C and the flow rate was 200 µl/min. The metabolites were detected across a mass range of 75–1,000 m/z using the Q-Exactive Plus mass spectrometer (Thermo) at a resolution of 35,000 (at 200 m/z) with electrospray ionization and polarity switching mode. Lock masses were used to insure mass accuracy below 5 ppm. The peak areas of different metabolites were determined using TraceFinder software (Thermo) using the exact mass of the singly charged ion and known retention time on the HPLC column. By this approach we were able to identify and measure: Glucose, Glucose-6P (G6P), UDP-glucose (UDP-Glc), Sedoheptulose-7-P (SH-7P), Ribose 5-phosphate, Glyceraldehyde 3-phosphate, Lactate, Pyruvate, Phospho-Enol-Pyruvate (PEP), Citrate (Cit), cis-aconitate (cis-aco), Succinate (Suc), Fumarate (Fum), Malate (Mal), alpha ketoglutarate (aKG), dihydroxyacetone phosphate (DHAP), N-carbamoyl-L-aspartate, Nicotinamide adenine dinucleotide (NAD), NADH, Nicotinamide adenine dinucleotide phosphate (NADP), NADPH, Adenine, Adenosine, Adenosine Mono, Di and Tri-phosphate (AMP, ADP, ATP), Cytidine, Cytidine Mono, Di and Tri-phosphate (CMP, CDP, CTP), Uridine Mono, Di and Tri-phosphate (UMP, UDP, UTP), Hypoxanthine, Guanine, Guanosine, Guanidine Mono and Di-phosphate (GMP, GDP), Inosine monophosphate (IMP), Pantothenate, Dephospho-Coenzyme A, Coenzyme A (CoA), Flavin adenine dinucleotide (FAD), Niacin, Nicotinamide, 3-hydroxybutyrate, Urate, 5-oxo-L-Proline, Acetyl-aspartate, Acetyl-glutamine, Alanine, Arginine, Argininosuccinate, Asparagine, Aspartate, Betaine, Citrulline, Cystathionine, Cystine, Glutamine, Glutamate, Glycine, Histidine, IsoLeucine, Leucine, Sarcosine, Lysine, Methionine, Ornithine, Phenylalanine, Proline, Serine, Taurine, S-Adenosyl-L-methionine, Threonine, Tryptophan, Tyrosine, Valine, Butyric acid, Carnitine, Decanoic acid, Lauric acid, Hexanoic acid, Linoleic acid, Myristic acid, Octanoic acid, Oleic acid, Palmitic acid.

Statistical analyses were performed using MetaboAnalyst 4.0 software [8, 9]. The algorithm for heatmap clustering was based on the Pearson distance measure for similarity and the Ward linkage method for biotype clustering. Metabolites with similar abundance patterns were positioned closer together.

Transketolase activity assay

Bacteria were grown to stationary phase in BHI Broth and 1 ml of culture was harvested. Bacteria were washed in PBS, pelleted by centrifugation, and resuspended in 300 μ l of 50 mM Tris with 60 μ g lysostaphin, pH 7.5. Samples were incubated at 37 °C for 5 min and then sonicated at 4 °C (30s on, 30s off, repeated 30 times) (Diagenode). Samples were centrifuged at 12,000 g and 4 °C for 10 min to remove cell debris and protein concentration was determined using BCA assay with BSA as a standard (Thermo Fisher Scientific). TKT activity was analysed as described previously [10]. Briefly, each enzymatic reactions were performed at a final volume of 100 μ l containing 20 μ l of supernatant, 50 mM Tris (pH 7.5), 0.24 mM $MgCl_2$, 10 μ M thiamine pyrophosphate, 0.5 mM NADH, 3 units of glycerol-3-phosphate dehydrogenase, 10 units of triosephosphate isomerase, 0.5mM D-xylulose-5-phosphate, and 0.5mM D-erythrose-4-phosphate (all reagents obtained from Sigma-Aldrich). The reaction proceeded at 37 °C for 5 min, and the decrease in $A_{340\text{ nm}}$ was monitored with a microplate reader (Molecular Devices). Transketolase enzyme activity in bacteria lysates was expressed as units per microgram of total protein. One unit of enzyme was defined as the amount of enzyme that oxidized 1 μ mol of NADH per minute.

LAMP-1 colocalization assay

EA.hy926 endothelial cells were grown to confluence on 8-well Slides (Lab Tek II) and infected with *S. aureus* as described above. For each point, cells were washed 3 times with PBS and fixed 20 min in PBS-4% paraformaldehyde at 4 °C. Fixation was stopped in PBS- NH_4Cl 50 mM. Cells were permeabilized with PBS containing 0.1% saponin and 5% goat serum for 10 min on ice. Cells were incubated for 30 min with anti-LAMP-1 (Lysosomal-associated membrane protein 1) rabbit polyclonal antibody (1/200e, AbCam) diluted in PBS containing 5% goat serum (staining buffer). After washing, cells were incubated for 30 min with Alexa-568 conjugated donkey anti-rabbit secondary antibodies (1/400e, AbCam) diluted in staining buffer. After washing, 4,6-diamidino-2-phenylindole (DAPI) was added (1/10,000e)

for 20 min and glass coverslips were mounted in Mowiol (Cityfluor Ltd.). Cells were examined using an X63 oil-immersion objective on an Apotome microscope (Zeiss™). Colocalization of GFP expressing bacteria with LAMP-1 staining was quantified by using Image J software (plug in: Coloc based Sur-vol _ Beta v6). Data were obtained from more than 200 infected cells for each condition and expressed as % of bacteria associated with LAMP-1 ± SD.

***In vivo* infection**

For all experiments we used 6 to 10 weeks-old female BALB/c (Janvier Labs) with access to food and water ad libidum.

Infection. Overnight cultures of *S. aureus* in BHI Broth were diluted in BHI Broth to an OD₆₀₀ of 0.05. Bacteria were grown at 37°C until the culture reached the initial-middle log phase (OD₆₀₀=0.6) and resuspended in a sterile isotonic saline solution to an OD₆₀₀ of 0.4.

Mice were infected with 10⁷ bacteria intravenously (iv) in the tail vein. Inoculum titers were checked by spreading dilutions of the preparations on BHI agar plates. For bacterial burden determination, mice were euthanized after 4, 24, 48 and 72 hours. Sequential dilutions of homogenized preparations from spleen and kidney were spread onto BHI agar plates.

Mice infected with the complemented strain were treated with erythromycin to avoid loss of the complementing plasmid. For this, mice received one dose of erythromycin 16 hours before infection and 2 doses of erythromycin per day until they were sacrificed (500 µg of erythromycin per dose).

Statistics

Data were analyzed using GraphPad Prism software. Results are presented either with one representative experiment for clarity or as means ± standard deviation (SD). The number of biological and technical replicates is indicated in the legend of each figure. For two-sample comparisons statistical significance was measured using unpaired two-tail Student's t-test. For comparisons between more than two groups, statistical significance was measured using

one-way analysis of variance (ANOVA) with Dunnett's correction. P values of $p < 0.05$ were considered to indicate statistical significance.

Ethics statement

All experimental procedures involving animals were conducted in accordance with guidelines established by the French and European regulations for the care and use of laboratory animals (Decree 87–848, 2001–464, 2001–486 and 2001–131 and European Directive 2010/63/UE) and approved by the INSERM Ethics Committee (Authorization Number: 75-906).

Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)	Source of reference
Strains		
<i>E.coli</i>		
DH5 α	$\Phi 80'$ <i>lacZ</i> Δ M15 <i>gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	Invitrogen
<i>S.aureus</i>		
USA300 LAC	MRSA containing phage Φ SA2usa (PVL), Ery ^R	BEI Resources
USA300-Ery ^S	Erythromycin-sensitive derivative of USA300	Laboratory collection
RN4220	Restriction-deficient transformation recipient <i>S. aureus</i>	Laboratory collection
USA300 Δ <i>tkl</i>	<i>tkl</i> defective mutant, Kan ^R	This study
USA300 Δ <i>tkl</i> -CP <i>tkl</i>	Complemented strain, USA300 Δ <i>tkl</i> containing pCN57-CP <i>tkl</i> , Ery ^R Kan ^R	This study
USA300 LAC- <i>gfp</i>	Strain containing pCN57-GFP, Ery ^R	Laboratory collection
USA300 Δ <i>tkl</i> - <i>gfp</i>	USA300 Δ <i>tkl</i> containing pCN57, Ery ^R Kan ^R	This study
Plasmids		
pMAD	Plasmid with a thermosensitive origin, used for gene deletion; Amp ^R Ery ^R	Laboratory collection
pMAD- Δ <i>tkl</i>	Derivative of pMAD with upstream and downstream sequences from <i>tkl</i> gene and <i>nptII^a</i> , Amp ^R Ery ^R Kan ^R	This study
pCN57	Plasmid containing <i>gfp</i> expression cassette (constitutive expression), Ery ^R	Laboratory collection
pXT02	Derivative of pCN57. The <i>gfp</i> expression cassette was replaced by the sequence of the <i>tkl</i> gene with its own promoter.	This study

pMiniT 2.0

Plasmid used to clone upstream and downstream regions from *tkt* gene and *nptII*, Amp^R New England Biolabs

^a. *nptII*: neomycin phosphotransferase gene, determining kanamycin resistance.

Primers used for Δ tkt mutant construction and complementation

Primer name	Primer sequence (From 5' to 3')
tktup	F: CGGAATTCGAAGTAGGATTAAGTCAAGAAGAAGC R: TTTAGCTCGACTAATCCATACAAGTCTAAAGCTAATCCTACTGAC
tktdn	F: CCTTCTTGACGAGTTCTTCTGAGGATGGCATAAATATGTAGGTACTG R: CGGGATCCATTTGCATCATCATACGAAGC
nptII	F: TTGTATGGATTAGTCGAGCTAAA R: TCAGAAGAAGTCTCAAGAAGG
s_tkt	F: GAATATCGAAAGAATTAACGAGTTAGC R: CGCACTCTTCATATTTTGATCCAT
s_pMAD	F: TCTGGCCATTGCTCTGGGTTA R: GCTGTCCCTGATGGTCGTCAT
HRtkt	F: CTTTGAGTGAGCTGGCGGCCGGATCCGGGTTTAAACAACAAATTGAAAATAC R: CATAGTAAGCTGCATGCAGCCCCGGGTTATAAGCTCATAACTTGGTTTAAAGATATTTTC
HRpCN57	F: ACCAAGTTATGAGCTTATAACCCGGGGCTGCATGCAGCTTACTATG R: TCAATTTGTTGTTTAAACCCGGATCCGGCCGCCAGCTCACTCAAAG

Primers used for RT-qPCR.

Gene name	Primer sequence (From 5' to 3')
<i>RNAIII</i>	F: GCCATCCCAACTTAATAACCATGTAAAATTAGC R: GCGATGTTGTTTACGATAGCTTACATGCTAG
<i>rpirC</i>	F: CATAACGACTACTACACGCGCACTTAATC R: GTCGGTAGCAACTACAAAAGATGCG
<i>tkt</i>	F: GGTCATAATGGTGCATCTCGTGC R: CATTGGATTTGTTTGAACCAGCAAGG
<i>gyrA</i>	F: GGTGGATTTGAAAACCTTAGAGGACGAAGAC R: CATACTTGAACACCACGACCACC
<i>sigB</i>	F: GGTGCCATAAATAGATTCGATATGTCCTT R: CTTTTGATTTCAACCGATTACAGTAGGTACT
<i>rsbU</i>	F: CGCGTGAAGATGTGTTCAAGAC R: CTATCTCTTTATCGTGAACCTTGAAG
<i>rsbW</i>	F: GAAGATTTTATCGAAATGCGC R: CATCATATGTAGCACCAGCTCTC

<i>asp23</i>	F: TCGCTGCACGTGAAGTAAA
	R: CACCAACTTCAACAGATACACC

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Figure S1

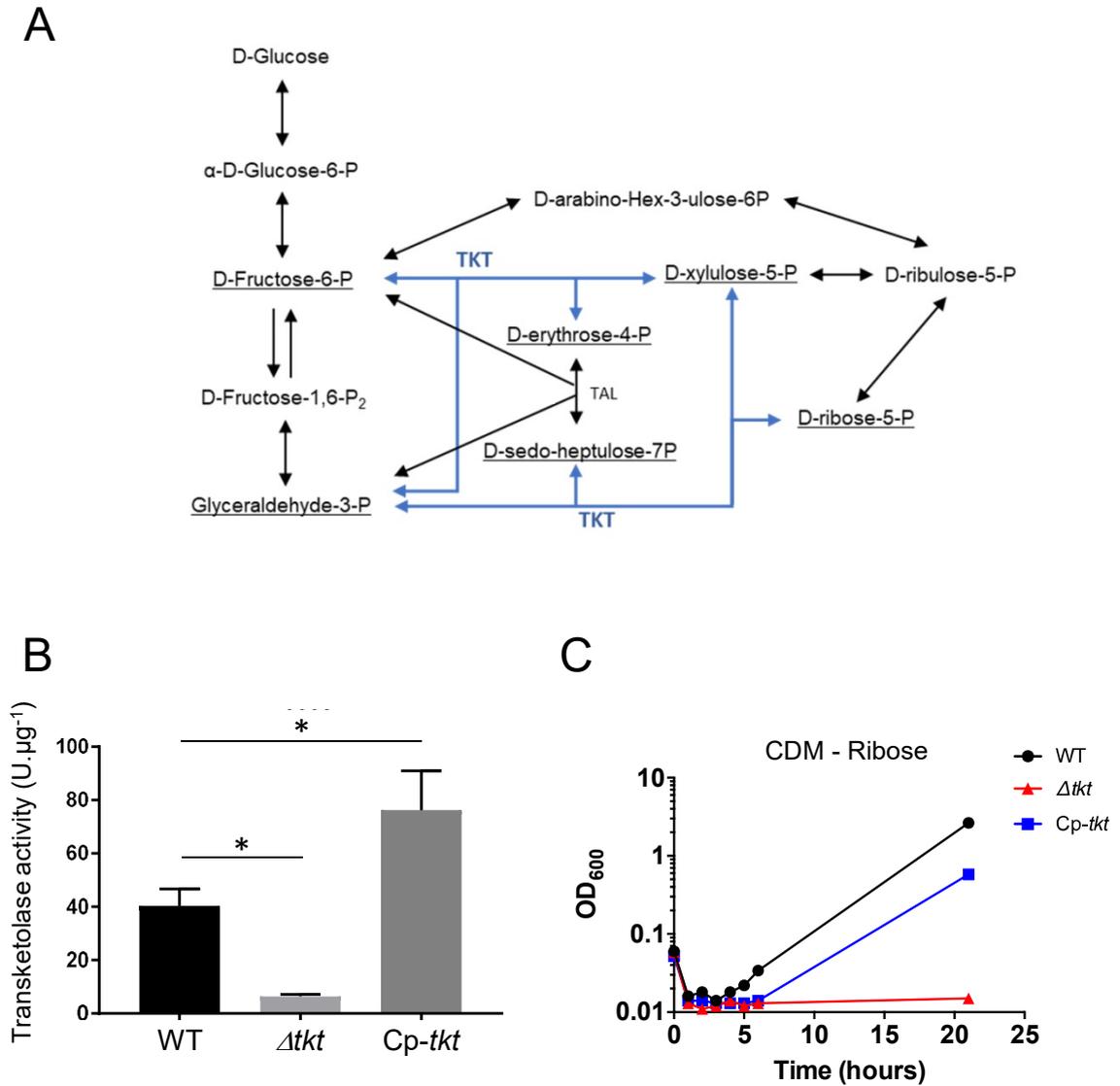


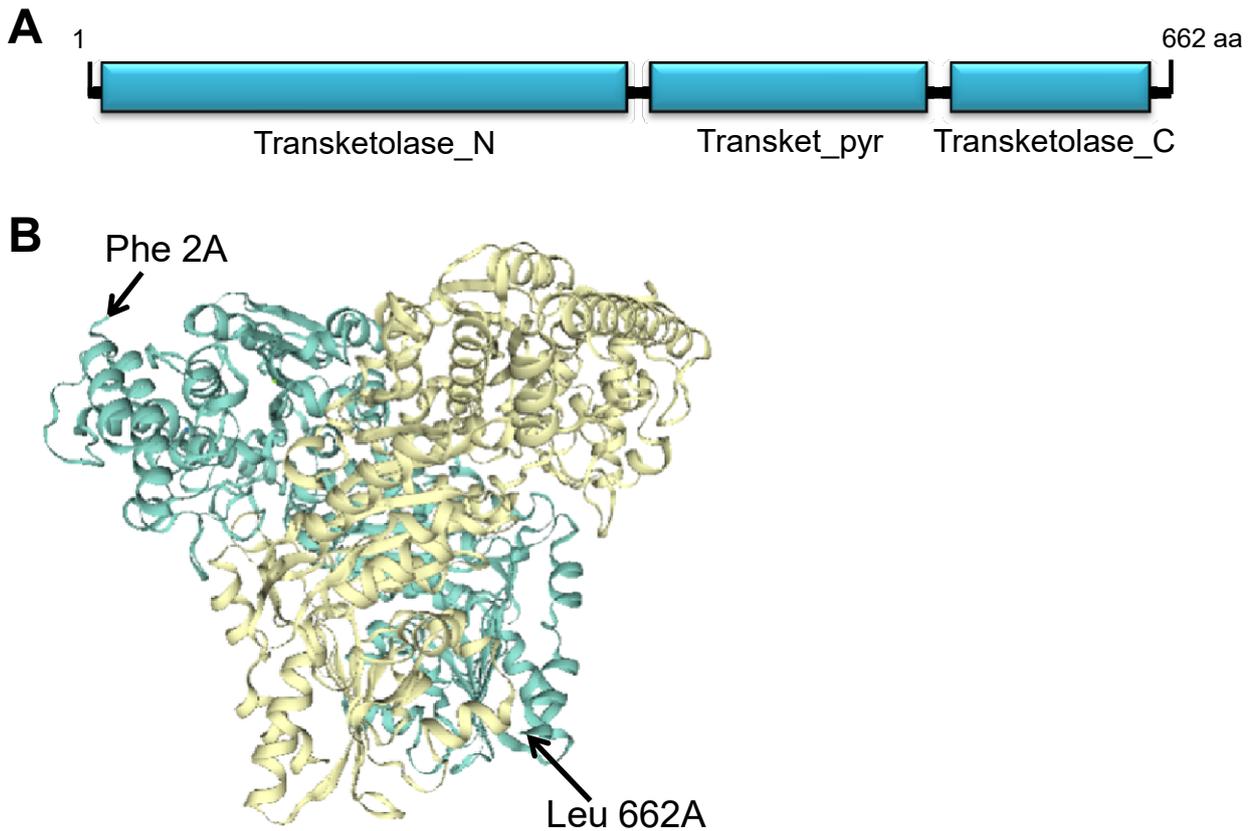
Figure S1. Characterization of the Δtkt mutant strain.

A. Schematic depiction of the Pentose Phosphate Pathway. Light blue: TKT enzymatic reaction.

B. Transketolase activity. Transketolase activity was monitored in USA300 WT, Δtkt and Cp-*tkt*. Inactivation of *tkt* gene led to a significant decrease in TKT enzyme activity (ANOVA: * $p < 0.001$).

C. Growth of USA300 wild type (WT), Δtkt mutant (Δtkt) and its complemented derivative (Cp-*tkt*). USA300 WT, Δtkt and Cp-*tkt* strains were grown in CDM supplemented with ribose as sole carbon source for 21 hours at 37°C. Optic densities were measured every-hour from 0 to 8 hours and then at 21 hours. Each panel is a representative experiment.

Figure S2



C

Protein ID	% coverage	% identity	Species
TKT_HUMAN	100.0%	100.0%	<i>Homo sapiens</i>
TKT_MOUSE	100.0%	94.7%	<i>Mus musculus</i>
TKT2_ECOLI	94.5%	24.1%	<i>Escherichia coli</i>
TKT_HAEIN	94.2%	22.9%	<i>Haemophilus influenzae</i>
TKT1_VIBCH	94.4%	24.0%	<i>Vibrio cholerae</i>
TKT_DICDI	94.2%	22.6%	<i>Dictyostelium discoideum</i>
TKT1_YEAST	94.5%	23.8%	<i>Saccharomyces cerevisiae</i>
TKT_TREPA	93.9%	21.9%	<i>Treponema pallidum</i>
TKT_MYCBO	96.6%	20.6%	<i>Mycobacterium bovis</i>
TKT_STRPN	94.2%	23.0%	<i>Streptococcus pneumoniae</i>
TKT_STAAS	94.4%	22.4%	<i>Staphylococcus aureus</i>
TKT_BACSU	94.9%	22.7%	<i>Bacillus subtilis</i>

Figure S2. Transketolase proteins domain organization and structure.

A. Conserved domains found in *S. aureus* transketolase. Transketolase_N (PF00456 domain): Transketolase, thiamine diphosphate binding domain; Transket_pyr (PF02779 domain): Transketolase, pyrimidine binding domain; Transketolase_C (PF02780): Transketolase, C-terminal domain (putative regulatory molecule binding site).

B. Protein model of *S. aureus* transketolase. Swiss-Model was used to predict the structure of *S. aureus* TKT (SAUSA300_1239) as a homodimer based on the template 3M49.1.A (Crystal Structure of Transketolase from *Bacillus anthracis*). The QMQE score and QMEAN were 0.83 and -0.59 respectively.

C. Table showing coverage percentage between transketolase amino acid sequences and percentage of identical amino acids between aligned transketolase amino acid sequences. Protein sequences were retrieved from UniProt database and aligned using Clustal Omega.

Figure S4

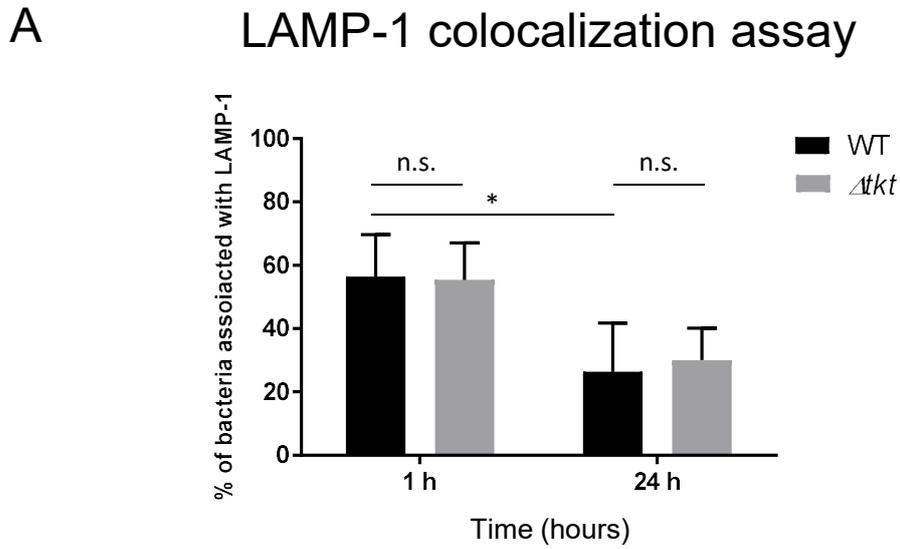


Figure S4. LAMP-1 colocalization assay

LAMP-1 colocalization assay. Endothelial EA.hy926 cells were infected with GFP-expressing WT and *Dtkk* mutant strains. One hour after infection, cells were washed several times with gentamicin containing medium to eliminate extracellular bacteria. A gentamicin concentration of 50 μ g/ml was maintained throughout the experiment. At 1 hour and 24 hours after infection cells were fixed using 4% PFA and processed for immunohistochemistry. Lysosomes were stained using anti-LAMP1 antibodies and images were acquired by confocal microscopy. Quantification of colocalization between GFP expressing USA300 WT or Δtkk bacteria and LAMP-1 staining was obtained using ImageJ software. Data were obtained from more than 200 infected cells for each condition and expressed as % of bacteria associated with LAMP-1 \pm SD. (ANOVA; n.s.: not statistically different; * $p < 0.05$).

Figure S5

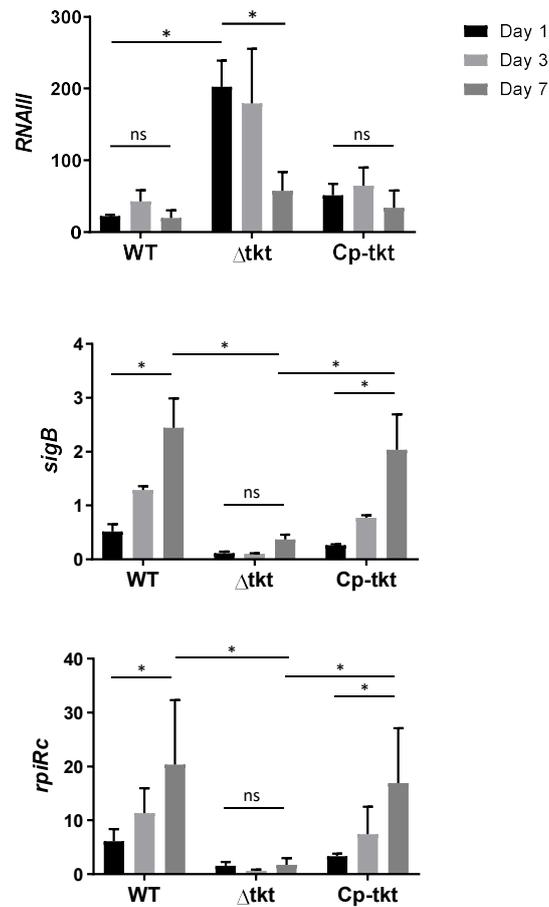


Figure S5. Transcription profile of intracellular *S. aureus*.

Endothelial EA.hy926 cells were infected with USA300 WT, Δtkt and Cp-*tkt* strains as described in figure 2. One, three or seven days after infection cells were washed and collected. RNA from intracellular *S. aureus* were prepared and gene expression of *RNAIII*, *rpiRc* and *sigB* was analysed by quantitative RT-PCR. Gene expression level was normalized by that of *gyrA* at days 1, 3 and 7 after infection of EA-hy296 cells. Data are shown as mean normalized expression \pm SD of triplicate measurements from two independent experiments. (ANOVA, ns : not statistically different; * $p < 0.01$, ** $p < 0.001$).

Experiments presented in Figure S5 were independent of those presented in Figure 3.