Stk1 is required for BlaR1-mediated broad-spectrum β-lactam resistance in epidemic-causing strains of *Staphylococcus aureus*

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Results

In keeping with the binding and kinetic data, the GWX ligand in the active site binds in a highly ordered way in this structure as observed in the polder map **(Fig. S2c)**, indicating the conformational rigidity. The GWX ligand inserts itself deep into the active site cleft of the kinase allowing it to position the aromatic pyrazolopyridine moiety under Phe150. The centroids of the Phe and the pyrazolopyridine are ~3.9Å apart which is ideal for pi-stacking interactions and mimics what was seen in previous *in silico* models of this specific ligand-protein interaction. While the pyrazolopyridine group appears to be oriented similarly to the *in silico* model, there is a ~90° rotation of the rest of the molecule about the N-C bond of the aniline moiety. This rotation causes the aniline and methyl-piperazine rings to face perpendicular to what is seen in the model and drives the trifluoromethyl group to point towards the backbone of the glycine-rich loop instead of interacting with Thr. Despite this, the nearest oxygen on the γ-carboxyl of Glu97 still appears to be in close proximity (3.0 Å) to the terminal nitrogen of the methyl-piperazine group in GWX. This is an ideal range for a salt bridge between the N-O atom pair for which both would be expected to be charged in the 7.0-7.5 pH range used in this study. This positively-charged piperazine moiety was observed as critical to the strong binding of these pyrazolopyridine derivatives in the previous *in vivo* characterization.

While this structure contains many characteristics of a “DFG-out” kinase, it also shows hallmarks of an active kinase, suggesting it adopts a conformational space somewhere in-between the DFG-out and DFG-in states as described earlier for the AMP-PNP substrate analog complex[1]. In our structure, the C-helix is analogously blocked by the activation loop, preventing formation of the salt bridge between Lys and Glu. Asp of the DFG-motif, on the other hand, is positioned in the active-site in a fashion that would allow it to directly co-ordinate a Mg^{2+} ion, similar to the *M. tuberculosis* structure of PknB (PDB: 5U94). New methods to classify these kinases have been explored in detail recently due to the variety of conformational intermediates observed in structural studies. In **Fig. S2d**, the active site architecture of some Hanks-type kinases in DFG-in and DFG-

out conformations are compared, revealing the position of the DFG motif in our structure more closely matches the DFG-in motif of other kinases, while still lacking other key structural motifs that are traditionally considered necessary for activity. Our work here provides further experimental evidence that the broad spectrum of kinase structures in the literature can't be classified into two distinct states, and instead occupies a wide range of intermediates. It is worth noting the presence of additional GWX molecules outside of the active site that appear to aid in crystal packing, but likely do not play a physiological role. The latter supposition is supported by the fact that in the ITC experiments we observed an n-site of 1, and the data did not fit a two-site binding model. Additionally, in the *M. tuberculosis* PknB homolog only one binding site was observed for GWX ITC experiments and\ the reported K_d for GWX binding was similar (21nM vs 27nM). This similarity in binding constants of the *M.tuberculosis* and *S. aureus* PASTA kinases is compelling as key regions of the active site fold are preserved, suggesting the major interaction elements with GWX, i.e. the backbone interaction (at Ile90) and the highly electrostatic elements (Lys39 and Glu97/Asp), we observe in the *S. aureus* Stk1 variant will be conserved in *M. tuberculosis* PknB **(Fig. S7)**. However, there remain stark contrasts between these structures and eukaryotic kinase structures, in ligand-bound states, which could allow for the rational design of more selective inhibitors. Phe150 of STK1, which participates in the π-stacking interaction with GWX, is not conserved across Hanks-type kinases. However, in *M. tuberculosis* PknB, this position contains a methionine, for which the sulfur molecule could undergo a similar π-stacking interaction. By understanding these subtle differences in active site conservation and fold, this structure can assist in the development of more selective inhibitors that lack activity on eukaryotic kinases and could serve as a platform for adjuvant design in the treatment of *S. aureus*.

Supplementary tables:

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Table S1: List of strains used in this study

Strain	Description	Reference
S. aureus RN4220	Laboratory *S. aureus* strain	BEI resources
SF8300	USA300 MRSA Background Strain	2
SF8300 Δ*stk1*	SF8300 with *stk1* gene deletion	This Study
SF8300 Δ*stp1*	SF8300 with *stp1* gene deletion	This Study
SF8300 Δ*stk1* pTX_{Δ}[E]	SF8300 Δ*stk1* with an empty pTX_{Δ} vector	This Study
SF8300 Δ*stk1* pTX_{Δ} [*stk1*(WT)]	SF8300 Δ*stk1* with pTX_{Δ} constitutively expressing wild-type Stk1	This Study
SF8300 Δ*stk1* pTX_{Δ} [*stk1*(K39G)]	SF8300Δ*stk1* with pTX_{Δ} constitutively expressing Stk1 with a K39G loss-of-function mutation	This Study
SF8300 p*TX15*	SF8300 with an empty p*TX15* vector	This Study
SF8300 Δ*stk1* pTX_{Δ}[E]	SF8300 Δ*stk1* with an empty p*TX15* vector	This Study
SF8300 Δ*stk1* P*TX15* [*stk1*(WT)]	SF8300 Δ*stk1* with a inducible wild-type Stk1 in a p*TX15* vector	This Study
SF8300 Δ*stk1* p*TX15* [*stk1*(K39G)]	SF8300 Δ*stk1* with a inducible Stk1 K39G loss-of-function mutation in a p*TX15* vector	This Study
SF8300 Δ*stp1* pTX_{Δ}[E]	SF8300 Δ*stp1* with an empty pTX_{Δ} vector	This Study
SF8300 Δ*stp1* pTX_{Δ} [*stp1*(WT)]	SF8300 Δ*stp1* with pTX_{Δ} constitutively expressing wild-type Stp1	This Study
SF8300 Δ*stp1* pTX_{Δ} [*stp1*(G40A)]	SF8300 Δ*stp1* with pTX_{Δ} constitutively expressing Stp1 with a G40A loss-of-function mutation	This Study
SF8300 pTX_{Δ} [E]	SF8300 with an empty pTX_{Δ} vector	
SF8300 pTX_{Δ}[*blaI*(WT)]	SF8300 with pTX_{Δ} constitutively expressing wild-type BlaI	This Study
SF8300 Dstk1 pTX_{Δ} [*blaI*(WT)]	SF8300 Dstk1 with pTX_{Δ} constitutively expressing wild-type BlaI	This Study
SF8300 pTX_{Δ} [*blaI*(N101A,F102A)]	SF8300 with pTX_{Δ} constitutively expressing BlaI with a N101A, F102A cleavage-defective mutation	This Study
SF8300 Δ*stk1* pTX_{Δ} [*blaI*(N101A,F102A)]	SF8300 Δ*stk1* with pTX_{Δ} constitutively expressing BlaI with a N101A,F102A cleavage defective mutation	This Study
Mu50	ATCC 700699	
SF8300ex	SF8300 devoid of *mecA* gene and *blaZ* plasmid	3
SF8300ex pTX_{Δ}[E]	SF8300ex with an empty pTX_{Δ} vector	4
SF8300ex pTX_{Δ} [*blaI*(WT)]	SF8300ex with pTX_{Δ} constitutively expressing wild-type BlaI	This Study
SF8300ex pTX_{Δ}[*blaR1* (C-His)]	SF8300ex with pTX_{Δ} constitutively expressing wild-type BlaR1 with a C-terminal 6x His-tag	This Study
SF8300ex Δ*spa* Δ*sbi*	SF8300ex with *spa* and *sbi* gene deletion	This Study
SF8300ex Δ*spa* Δ*sbi* pTX_{Δ}[E]	SF8300ex Δ*spa* Δ*sbi* with an empty pTX_{Δ} vector	This Study

	SF8300ex Δ*spa* Δ*sbi* pTX_{Δ} [*blaR1*]	SF8300ex Δ*spa* Δ*sbi* with pTX_{Δ} constitutively expressing wild-type BlaR1	This Study
E. coli	DH5a	Laboratory *E. coli* strain	Invitrogen
	BL21	*E. coli* strain used for protein expression	Invitrogen
	BL21 pET28b (+) [*stk1* (KD)]	Used for expressing Stk1 KD, residues 1-291	This Study

Table S2: List of plasmids used in this study

Plasmid	Description	Notes	Reference
pJB38 [Δ*stk1*]	Used to delete *stk1* in SF8300	*E. coli* ampR; *S. aureus* cmR	This Study
pJB38[Δ*stp1*]	Used to delete *stp1* in SF8300	*E. coli* ampR; *S. aureus* cmR	4
p*TX*$_{Δ}$ *[E]*	Empty vector for p*TX*$_{Δ}$	*S. aureus* tetR	4
p*TX*$_{Δ}$ [*stk1*(WT)]	Constitutively expressing wild-type Stk1	*S. aureus* tetR	This Study
p*TX*$_{Δ}$ [*stk1*(K39G)]	Constitutively expressing Stk1 with a K39G loss-of-function mutation	*S. aureus* tetR	This Study
p*TX15* [E]	Empty vector for p*TX15*	*S. aureus* tetR; Xylose inducible	This Study
p*TX15* [*stk1*(WT)]	Xylose-inducible expression wild-type Stk1	*S. aureus* tetR; Xylose inducible	This Study
p*TX15* [*stk1*(K39G)]	Xylose-inducible expression Stk1 with a K39G loss-of-function mutation	S. aureus tetR; Xylose inducible	This Study
p*TX*$_{Δ}$ [*stp1*(WT)]	Constitutively expressing wild-type Stp1	*S. aureus* tetR	4
p*TX*$_{Δ}$ [*stp1*(G40A)]	Constitutively expressing Stp1 with a G40A loss-of-function mutation	*S. aureus* tetR	This Study
p*TX*$_{Δ}$ [*blaI*(WT)]	Constitutively expressing wild-type BlaI	*S. aureus* tetR	This Study
p*TX*$_{Δ}$ [*blaI*(N101A,F102A)]	Constitutively expressing BlaI with a N101A, F102A cleavage-defective mutation	*S. aureus* tetR	This Study
p*TX*$_{Δ}$ [*blaR1* (C-His)]	Constitutively expressing wild-type BlaR1 with a C-terminal 6x His-tag	*S. aureus* tetR	This Study
pJB38 [Δ*spa*]	Used to delete *spa* in SF8300	*E. coli* ampR; *S. aureus* cmR	This Study
pJB38 [Δ*sbi*]	Used to delete *sbi* in SF8300	*E. coli* ampR; *S. aureus* cmR	This Study
p*TX*$_{Δ}$ [*blaR1*]	Constitutively expressing wild-type BlaR1	*S. aureus* tetR	This Study
pET28b (+) [*stk1* (KD)]	E. coli IPTG inducible protein expression vector expressing Wild-type Stk1 Kinase domain, residues 1-291	*E. coli* kanR; IPTG inducible	This Study

Table S3: List of primers used in this study

Primer	Sequence	Description
stp1-*stk1*- P1	ACAAGAGCTCCAAAAATTTCGAGCGAAACAG ATTTTTGAATGG	*stp1* & *stk1* deletion
stk1-P2	TACATTTACTTCAATTATATTCATACTTTATCA CCTTCAATAGCCGCGAGTATGAAAGTAACG	*stk1* deletion
stk1-P3	ATATAATTGAAGTAAATGTACCGAGGTTTCTA TTTGGAAGTC	*stk1* deletion
stk1-P4	TATTCCCGGGTATCTCTAATTGATGCTTAAC ATTACAATTAGG	*stk1* deletion
stp1-P2	TTTATTATTTTACCTATCATTTGTCTTTACCTC GTTTCTACTTGTCGTTCCTTTGC	*stp1* deletion
stp1-P3	ATGATAGGTAAAATAATAAATGAACGATATAA AATTGTAGATAAGC	*stp1* deletion
stp1-P4	TACACCCCCGGGTCTGGCTTTTGGAATTGCT GATGATGAGCAGGCCC	*stp1* deletion
Stk1-for	ACTTGGATCCTCGCGGCTATTGAAGGTGATA AAGTATGATAGG	*stk1* cloning in p*TX*$_{\Delta}$ and p*TX15*
Stk1-rev	TCAAACGCGTTTATACATCATCATAGCTGAC TTCTTTTTCAGCTAC	*stk1* cloning in p*TX*$_{\Delta}$ and p*TX15*
Stk1-K39G-for	CTTAACATTAAAGTTGCAATTGGGGCGATT	*stk1* (K39G) loss-of-function mutant cloning in p*TX*$_{\Delta}$
Stk1-K39G-rev	TCTAGGTGGTATAAAAATCGCCCCAATTGC	*stk1*(K39G) loss-of-function mutant cloning in p*TX*$_{\Delta}$
Stp1-for	AAAGGATCCACAAGTAGAAACGAGGTAAAG ACAAATGC	*stp1* cloning in p*TX*$_{\Delta}$
Stp1-rev	ATTACGCGTATCATACTTTATCACCTTCAATA GCCGC	*stp1* cloning in p*TX*$_{\Delta}$
Stp1-G40A-for	TTTTAGTTCTGTGTGATGGTATGGCTGGCCA TAAAGC	*stp1*(G40A) loss-of-function mutant cloning in p*TX*$_{\Delta}$
Stp1-G40A-rev	TTGCAACTTCTCCTGCTTTATGGCCAGCCAT ACCATC	*stp1*(G40A) loss-of-function mutant cloning in p*TX*$_{\Delta}$
BlaI-for	CCGGATCCATATTAAAAGGAATGGGTGTTTT AAATGACC	*blaI* cloning in p*TX*$_{\Delta}$
BlaI-rev	CCACGCGTTTACTTTTTACTAATATCATTTAA AATGTC	*blaI* cloning in p*TX*$_{\Delta}$
BlaI-N101A,F102A-for	ATATGAAAAGTTTAGTGCTGGCTGCTGCGAA AAATG	*blaI* (N101A, F102A) non-cleavable mutant cloning in p*TX*$_{\Delta}$
BlaI-N101A,F102A-rev	GTTATTTAATTCTTCATTTTTCGCAGCAGCCA GCAC	*blaI* (N101A, F102A) non-cleavable mutant cloning in p*TX*$_{\Delta}$
BlaR1-for	TTAGGATCCATATTTGAAAGAAGGTGTCAAA ATGGC	*blaR1* cloning in p*TX*$_{\Delta}$
BlaR1-rev	TTCACGCGTAGATATTTCAACTTGCTTATTG GTC	*blaR1* cloning in p*TX*$_{\Delta}$

BlaR1-C-His-rev	TTCACGCGTTTAATGATGATGATGATGATGTGATGAACCTTGGTCATTTAAAACACCCATTTGAAGGTACCATGTTTCGTTTTAAGAAAAAA	*blaR1*(C-His) cloning in p*TX*$_{\Delta}$
spa-P1	GTCAAGCCTGAAGTCG	*spa* deletion
spa-P2	AATATAACGAATTATGTATTGCAATACTAAAATC	*spa* deletion
spa-P3	TGCAATACATAATTCGTTATATTAAACAAACAATACACAACGATAGATATCATTTTATCC	*spa* deletion
spa-P4	AAGGTCGACTGCTTTATTGGTCGAAGCAATTAAAGAATTATGG	*spa* deletion
sbi-P1	TTGCGTTAGGTACAACGTCATCTTCTTTGAACAAACGAGGTGCATTTTGAATAACTACTTGG	*sbi* deletion
sbi-P2	GTGTATTCCCTTTCTTTTTACATATTAAATTTATTGTATGCTTTTAGAATTTATC	*sbi* deletion
sbi-P3	TCAATCAAAAATATCTTCTCTAGTTTTACATCATTTTTTAAATAATTTTCGTAACAAACCG	*sbi* deletion
sbi-P4	TGATGGCGCTGATTGGAAGTTTCCGCCGATATTATAGCCTAATTTCTGAC	*sbi* deletion
pET-Stk1-KD-for	CCGTCGACATAGGTAAAATAATAAATGAACGATATAAAATTG	Cloning Stk1 Kinase domain into pET28 (+) IPTG inducible expression vector
pET-Stk1-KD-rev	CCGCGGCCGCTTAATCTTCTTTTTTCAAAGGTACCG	Cloning Stk1 Kinase domain into pET28 (+) IPTG inducible expression vector

Table S4: List of qRTPCR primers used in this study

Primer	Sequence	Description
gyrB-for	ATTGCTCTAGTAAAAGTCCTGAAGAATG	forward qRT-PCR primer for *gyrB*
gyrB-rev	TAATCGTGCTTTTTCAACATTTAATATC	reverse qRT-PCR primer for *gyrB*
pbp2a-for	ACTTAAAACAAGCAATAGAATCATCAG	forward qRT-PCR primer for *mecA*
pbp2a-rev	AATTTGAGCATTATAAAATGGATAATCAC	reverse qRT-PCR primer for *mecA*
blaZ-for	TGCTTTAAATACTAAAAGTGGTAAGG	forward qRT-PCR primer for *blaZ*
blaZ-rev	AGCAACTATATCATCTTTGTTAATATG	reverse qRT-PCR primer for *blaZ*
blaR1-qPCR-F	AGTTTGGTATCTAACTATGCTTGCTGG	forward qRT-PCR primer for *blaR1*
blaR1-qPCR-R	ATAGGTTTGGTGGTATTTATGTTATGGTTC	reverse qRT-PCR primer for *blaR1*
blaI-qPCR-F	TGATAAAACGATTAGAACATTAATCACAAG	forward qRT-PCR primer for *blaI*
blaI-qPCR-R	TCCATACAGTTTATTAAGAAAGGTTTTAGC	reverse qRT-PCR primer for *blaI*

Table S5: Data collection and refinement statistics

Data collection and refinement statistics for PDB: 9YMT as generated through Phenix' model statistics program. Anisotropic cut-off applied to merged intensity data through StarAniso. See tables S6a and S6b.

PDB: 9YMT	$STK1^{1-291}$+ GWX
Data Collection Statistics	
Resolution range	45.08 – 2.07 (2.144 – 2.07)
Space group	P 4_3 2_1 2
Cell dimensions	
a,b,c (Å)	100.792 100.792 91.698
α,β,ɣ (°)	90 90 90
Total reflections	*727220 (56898)**
Unique reflections	*29327 (340)*
Multiplicity	*24.8 (20.1)*
Completeness (%)	*74.91 (11.87)*
Mean I/ σ(I)	*23.96 (0.48)*
Wilson B-factor (Å²)	*42.62*
R-merge	*0.1134 (6.17)*
R-meas	*0.1158 (6.332)*
R-pim	*0.02309 (1.381)*
CC1/2	*1.000 (0.217)*
CC*	*1.000 (0.597)*
Refinement Statistics	
Reflections used in refinement	22003 (341)
Reflections used for R-free	2000 (31)
R-work	0.2130 (0.4184)
R-free	0.2494 (0.5569)
CC (work)	0.926 (0.553)

CC (free)	0.907 (0.295)
Number of non-H atoms	2350
Macromolecules	2166
Ligands	112
Solvent	72
Protein Residues	273
RMS (bonds) (Å)	0.002
RMS (angles) (°)	0.54
Ramachandran favored	97.77
(%)	2.23
Ramachandran allowed	0.00
(%)	1.23
Ramachandran outliers	4.06
(%)	59.2
Rotamer outliers (%)	59.7
Clashscore	53.8
Average B-factor (Å²)	53.8
Macromolecules	3
Ligands	1.45
Solvent	
Number of TLS groups	
MolProbity Score	

* Italicized values are statistics calculated with respect to unmerged data and assume spherical truncation of the data based on the selected cut-off. For a comparison with these statistics for the elliptically truncated data, please refer to Supplementary Table 6b.

Table S6a: Data processing and anisotropy information extracted from the STARANISO log file:

Diffraction limits (Å) and corresponding principal axes of the ellipsoid fitted to the diffraction cut-off surface as direction cosines in the orthogonal basis, and in terms of reciprocal unit-cell vectors:					Eigenvalues of the overall anisotropy tensor on |F|s (Å 2) and corresponding eigenvectors of the overall anisotropy tensor as direction cosines in the orthogonal basis, and in terms of reciprocal unit-cell vectors:				
2.393	1.0000	0.0000	0.0000	a*	66.26	1.0000	0.0000	0.0000	a*
2.393	0.0000	1.0000	0.0000	b*	66.26	0.0000	1.0000	0.0000	b*
1.981	0.0000	0.0000	1.0000	c*	39.24	0.0000	0.0000	1.0000	c*

Table S6b: Data processing statistics with comparison of spherical and ellipsoidal truncation of data for PDB: 9YMT following anisotropic cut-off.

	Overall	**Inner Shell**	**Outer Shell**
Low resolution limit (Å)	45.076	45.076	2.235
High resolution limit (Å)	2.066	6.437	2.066
Rmerge (all I+ & I-)	0.091	0.023	2.010
Rmerge (within I+/I-)	0.095	0.022	1.983
Rmeas (all I+ & I-)	0.093	0.023	2.059
Rmeas (within I+/I-)	0.098	0.023	2.075
Rpim (all I+ & I-)	0.018	0.005	0.436
Rpim (within I+/I-)	0.027	0.006	0.603
Total number of observations	557554	24378	23811
Total number of unique	22007	1100	1100
Mean(I)/sd(I)	28.7	114.0	1.8
Completeness (spherical)	74.5	100.0	18.1
Completeness (ellipsoidal)	95.8	100.0	80.2
Multiplicity	25.3	22.2	21.6
CC (1/2)	1.000	1.000	0.643
Anomalous completeness (spherical)	73.4	100.0	17.2
Anomalous completeness (ellipsoidal)	95.7	100.0	80.3
Anomalous multiplicity	13.7	13.6	11.7

References

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Figure S1:

a) Coomassie-stained gel as loading control for **Fig. 1c**

b) Coomassie-stained gel as loading control for **Fig. 1d**

c) Growth curve assay of *S. aureus* MW2 Wt and its isogenic Δ*stk1* and Δ*stp1* mutants without antibiotics (untreated), or with nafcillin ([Naf] 2 µg/mL) or oxacillin ([Oxa] 4 µg/mL).

d) Growth curve demonstrating conditions of sample collection for EM studies. 8 µg/mL Nafcillin was added 1 hour of growth, following which cells were collected at the final time point of 6 hours.

e) Coomassie-stained gel as loading control for **Fig. 2a**

f) Coomassie-stained gel as loading control for **Fig. 2b**

Figure S2:

a) Coomassie-stained gel as loading control for **Figs. 3c - e**

b) ITC thermograms to assess GWX binding to $Stk1^{1-291}$. Normalized binding isotherm (top) and normalized binding heats (bottom) with a curve depicting a least squares regression fit for the non-linear data.

c) mFo-dFc Polder omit map of the GWX ligand in the active site contoured at 3.0 σ

d) Ligplot analysis results depicting a 2D representation of the protein-ligand interactions between GWX and $Stk1^{1-291}$

e) Coomassie-stained gel as loading control for **Fig. 3k**

f) qRT-PCR for relative induction of *blaZ* in Wt and Δ*stk1* at the mid-exponential stage (2 hours of growth) when treated with 2 µg/mL nafcillin (Naf), 2.5 µg/mL GWX inhibitor, or co-treated with nafcillin and GWX inhibitor for 30 min in comparison to samples untreated for 30 min. qRT-PCR was performed in triplicate, in two independent experiments. Data from representative independent experiments are presented as mean ± SD. Statistical analysis using an unpaired Student's t-test. ns = not significant with P> 0.05, *** P ≤ 0.001.

Figure S3:

a) Immunoblotting validation with custom-generated BlaI antibody. Validation of anti-BlaI antibody (α-BlaI) by detecting intact BlaI in SF8300 Wt at ~15 kDa in whole cell lysate. Samples were grown until the mid-exponential stage (2 hours of growth), then untreated or treated nafcillin (2 µg/mL and 8 µg/mL) for 1 hour. Nafcillin exposure led to BlaI cleavage, resulting in loss of the BlaI band. SF8300ex (Wtex), which is devoid of BlaI, was included as a negative control. Wtex constitutively expressing BlaI through pTX_{Δ}, Wtex [*blaI*], was included as a positive control. Sortase A (SrtA) was used as a loading control

b) Coomassie-stained gel as loading control for **Fig. S3a**

c) Coomassie-stained gels as loading control for **Fig. 4a - c**

d) Coomassie-stained gels as loading control for **Fig. 4d - e**

Figure S4:

a) Immunoblotting validation with custom-generated BlaR1 antibody. Validation of anti-BlaR1 antibody (α-BlaR1) by detecting full-length BlaR1 in SF8300 Wt at ~55 kDa in membrane fractions. Samples were grown until the mid-exponential stage (2 hours of growth), then untreated or treated nafcillin (2 µg/mL and 8 µg/mL) for 1 hour. Nafcillin exposure led to BlaR1 degradation, resulting in loss of the BlaR1 band. SF8300ex (Wtex), which is devoid of BlaR1, was included as a negative control. Wtex constitutively expressing C-terminal His-tagged BlaR1 through p*TX*$_{\Delta}$, Wtex [*blaR1* (C-His)], was included as a positive control. Sortase A (SrtA) was used as a loading control.

b) Immunoblotting validation with custom-generated BlaR1 antibody against non-specific binding. Validation of anti-BlaR1 antibody (α-BlaR1) by detecting full-length BlaR1 in SF8300 Wt at ~55 kDa in membrane fractions. Samples were grown until the mid-exponential stage (2 hours of growth), then untreated or treated nafcillin (2 µg/mL) for 1 hour. Nafcillin exposure (2 µg/mL) led to BlaR1degradation, resulting in loss of the BlaR1 band. Deletion of IgG-binding proteins Spa and Sbi in SF8300ex (Wtex Δ*spa* Δ*sbi),* which is devoid of BlaR1, was included as a negative control. Wtex Δ*spa* Δ*sbi* constitutively expressing BlaR1 through p*TX*$_{\Delta}$, Wtex Δ*spa* Δ*sbi* [*blaR1* (C-His), was included as a positive control. Sortase A (SrtA) was used as a loading control.

c) Coomassie-stained gel as loading control for **Fig. S4a**

d) Coomassie-stained gel as loading control for **Fig. S4b**

e) Coomassie-stained gel as loading control for **Fig. 5a**

f) Coomassie-stained gel as loading control for **Fig. 5b**

Figure S5:

a) Coomassie-stained gel as loading control for **Fig. 5c**

b) Coomassie-stained gel as loading control for **Fig. 5d**

c) Coomassie-stained gel as loading control for **Fig. 5f**

Figure S6:

a) qRT-PCR for basal *blaI* expression in Wt, Δ*stk1*, and Δ*stp1* at the mid-exponential stage (2 hours of growth) and late-exponential stage (3 hours of growth). qRT-PCR was performed in triplicate, in two independent experiments. Data from representative independent experiments are presented as mean ± SD. Statistical analysis using an unpaired Student's t-test. ns = not significant with P> 0.05, ** P≤0.01, *** P ≤ 0.001.

b) Immunoblotting and corresponding densitometry analysis for basal protein levels of BlaI in Wt, Δ*stk1*, and Δ*stp1* in whole cell lysates at the late-exponential stage (3 hours of growth). Sortase A (SrtA) was used as a loading control. Immunoblotting was performed in three independent experiments, the blots of which were used for densitometry, represented as mean ± SD. Statistical analysis using an unpaired Student's t-test. ns = not significant with P> 0.05, * P ≤0.05, ** P≤0.01.

c) Coomassie-stained gel as loading control for Fig. S6b

Figure S7:

Comparison of key active-site features.

a) The active site of *S. aureus* $Stk1^{1-291}$ in complex with GW779439X aligned with the active site of Hanks-type kinases.

b) *M. tuberculosis* PknB in complex with GSK690693 (PDB: 5U94).

c) *H. sapiens* ABL1 in complex with Dastinib.

d) *H. sapiens* ABL1 in complex with Imatinib (PDB: 2GQG and 2HYY).

To capture the relative location of the DFG-Phe residue, the outermost atom of the Phe ring was measured with respect to conserved residues. The Cα atom of the conserved β3 Lys and the Cα atom of the residue 4 AA downstream of the conserved Glu in the C-helix were used for distance measurements[4]. The measured distances for ABL1 kinase with imatinib match the measurements seen in the DFG-out conformation, while the other structures contain distances which correspond to DFG-in conformations

e) Multiple sequence alignment: Sequence alignment of the S. aureus Stk1 (Strain Mu50, identical to USA300) kinase domain against the kinase domains of various Hanks-type kinases. The aligned sequences include *B. subtilis* PrkC (strain 168), *M. tuberculosis* PknB (strain H37Rv*), H. sapiens* ABL1 (Uniprot: P00519), and *E. faecalis* IreK (GenBank: WP_002387383). Residues are numbered based on the primary sequence of Stk1 and key conserved elements are labelled below.