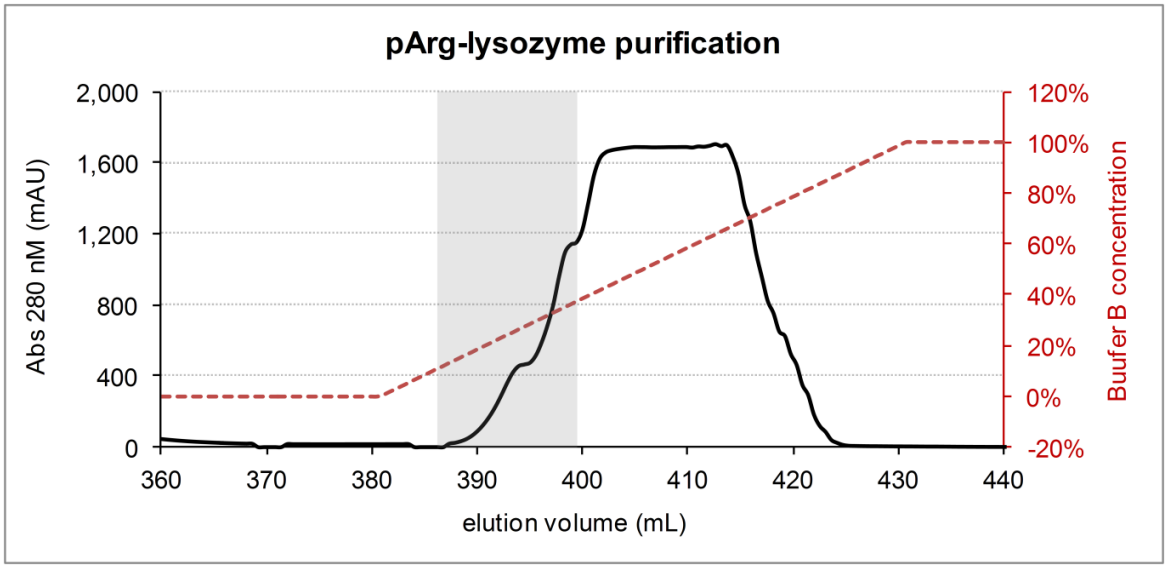
Table S1: Data collection, phasing and refinement statistics

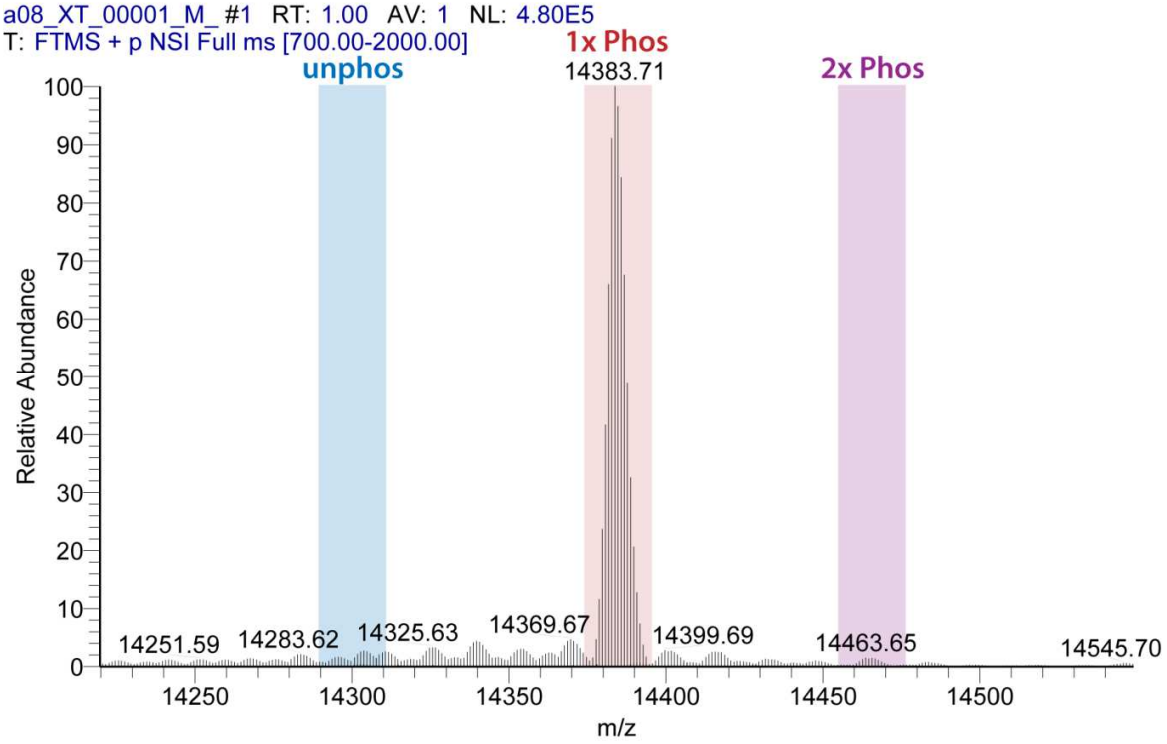
	YwlE$_{G.ste}$
Data collection	
Space group	$P2_1$
Cell dimensions	
a, *b*, *c* (Å)	44.4, 75.8, 47.7
α, β, γ (°)	90, 92.4, 90
Resolution (Å)	18.02 - 1.40 (1.48 - 1.40) [1]
R_{sym} (%) [2]	5.8 (33.6)
I / σI	14.6 (4.3)
Completeness (%)	95.3 (93.7)
Redundancy	6.1 (6.2)
Phasing	
Phasing power [3]	1.22
Figure of merit	0.87
Refinement	
Resolution (Å)	10 - 1.4
No. reflections	59,027
R_{work} / R_{free} [4]	17.8 / 20.9
No. atoms	
Protein	2,370
Ligand/ion	15
Water	376
B-factors	
Protein	16.1
Ligand/ion	35.9
Water	30.6
R.m.s. deviations	
Bond lengths (Å)	0.012
Bond angles (°)	1.72
Ramachandran statistics (%)	
Favored, allowed, disallowed region [5]	97, 2.7, 0.3

[1]Values in parentheses are for highest-resolution shell.
[2]R_{sym} is the unweighted R-value on I between symmetry mates.
[3]Phasing power is the root mean squared value of F_H divided by the root mean squared lack-of-closure.
[4]$R_{work} = \Sigma_{hkl} \mid |F_{obs}(hkl)| - k|F_{calc}(hkl)| \mid / \Sigma_{hkl}|F_{obs}(hkl)|$ for the working set of reflections; R_{free} is the R-value for 5% of the reflections excluded from refinement.
[5]The stereochemistry of the model was validated with MolProbity (Chen et al., 2010).

A


pArg-lysozyme purification
Abs 280 nM (mAU)
2,000
1,600
1,200
800
400
0
120%
100%
80%
60%
40%
20%
0%
-20%
Buufer B concentration
360
370
380
390
400
410
420
430
440
elution volume (mL)

B


a08_XT_00001_M_ #1 RT: 1.00 AV: 1 NL: 4.80E5
T: FTMS + p NSI Full ms [700.00-2000.00]
unphos
1x Phos
14383.71
2x Phos
Relative Abundance
100
90
80
70
60
50
40
30
20
10
0
14251.59
14283.62
14325.63
14369.67
14399.69
14463.65
14545.70
14250
14300
14350
14400
14450
14500
m/z

C

KVFGRCELAA AMK**R**HGLDNY **R**GYSLGNWVC AAKFESNFNT QATNRNTDGS
TDYGILQINS RWWCNDG**R**TP GS**R**NLCNIPC SALLSSDITA SVNCAKKIVS
DGNGMNAWVA W**R**NRCKGTDV QAWI**R**GC**R**L

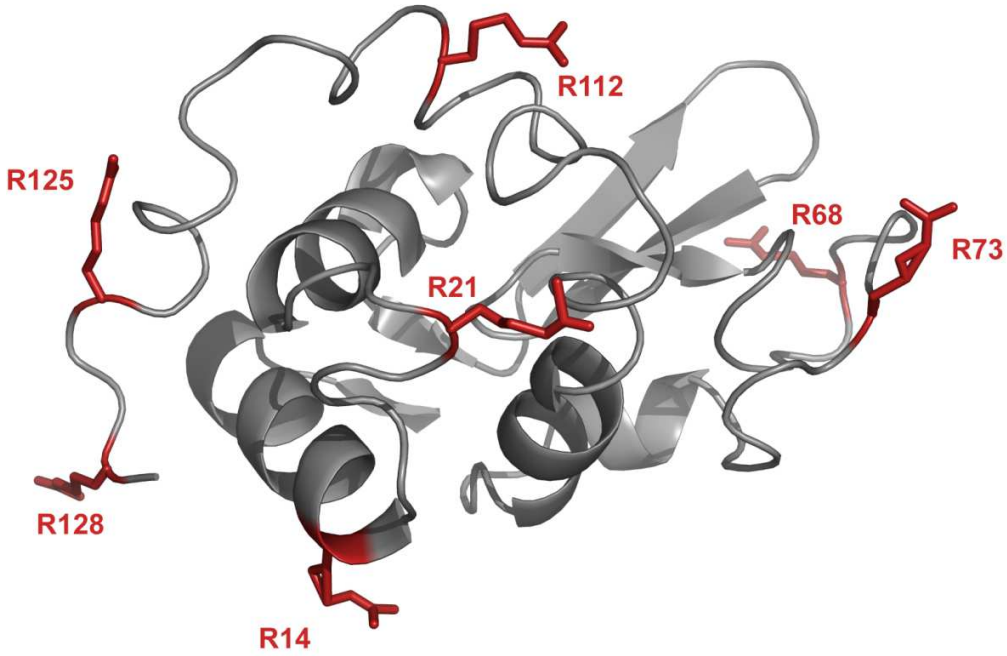


Figure S1: Production of an arginine-phosphorylated model protein. A) Cation exchange chromatography was used to purify the arginine-phosphorylated lysozyme. The recombinant McsB kinase used to phosphorylate chicken egg lysozyme was washed away with low salt (50 mM KCl) concentrations, and the purely phosphorylated form of lysozyme was recovered in the initial fractions (marked in grey background) of a salt gradient ranging from 50 mM to 1M KCl. **B)** Deconvoluted spectra of the offline mass measurement of the marked elution fraction shows that the obtained phosphoarginine-lysozyme protein was 100% monophosphorylated. **C)** Tryptic digestion and LC-MS/MS analysis showed that the phosphorylations occurred in at least 7 different arginine residues, indicated on the sequence (Uniprot P00698) and on the crystal structure (PDB 1LYZ) of lysozyme.

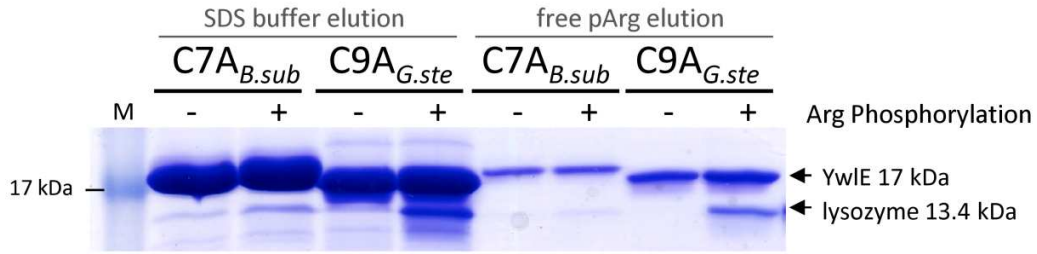


Figure S2: Phosphoarginine binding activity of *B. subtilis* and *G. stearothermophilus* YwlE C/A mutants. Pull-down experiments were used to assess the binding of the YwlE C/A mutants towards an arginine-phosphorylated model protein, lysozyme. Unphosphorylated lysozyme was used as control for the assessment of unspecific binding. The results obtained with two elution methods were applied, either the magnetic beads were boiled in SDS-PAGE sample buffer (left) or incubated with 10 mM phospho-L-arginine trisodium salt (right), resulting in competitive elution of the proteins bound to the catalytic pocket of YwlE.

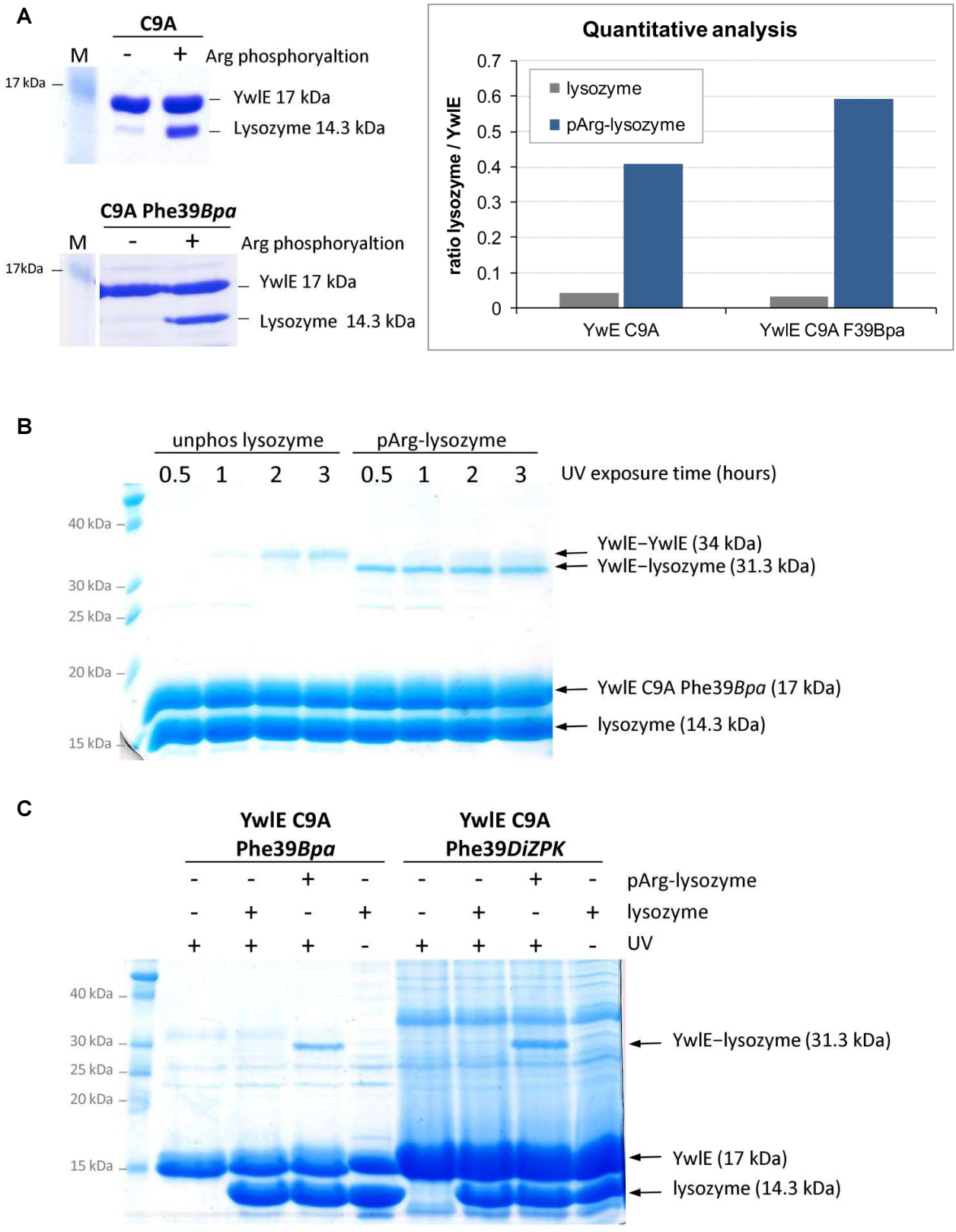

A
C9A
M
-
+
Arg phosphoryaltion
17 kDa
YwlE 17 kDa
Lysozyme 14.3 kDa
C9A Phe39Bpa
M
-
+
Arg phosphoryaltion
17kDa
YwlE 17 kDa
Lysozyme 14.3 kDa
Quantitative analysis
ratio lysozyme / YwlE
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
lysozyme
pArg-lysozyme
YwE C9A
YwlE C9A F39Bpa
B
unphos lysozyme
pArg-lysozyme
0.5
1
2
3
0.5
1
2
3
UV exposure time (hours)
40 kDa
30 kDa
25 kDa
20 kDa
15 kDa
YwlE–YwlE (34 kDa)
YwlE–lysozyme (31.3 kDa)
YwlE C9A Phe39Bpa (17 kDa)
lysozyme (14.3 kDa)
C
YwlE C9A Phe39Bpa
YwlE C9A Phe39DiZPK
pArg-lysozyme
lysozyme
UV
40 kDa
30 kDa
25 kDa
20 kDa
15 kDa
YwlE–lysozyme (31.3 kDa)
YwlE (17 kDa)
lysozyme (14.3 kDa)

Figure S3: Testing the photocrosslinking approach using an arginine-phosphorylated model protein. A) Testing of the YwlE C9A F39*Bpa* binding to the arginine phosphorylated model protein. The gels show the results of pull-down experiments using either the $YwlE_{G.ste}$ C9A or the $YwlE_{G.ste}$ C9A F39*Bpa* trapping mutants, incubated with Lysozyme (unphosphorylated control or pArg) at 12-14 μM concentration. The graph on the left depicts the quantitative analysis of the presented gel bands, plotted as the lysozyne/YwlE ratios in the control and phosphoarginine pull-downs. **B)** Testing of different UV-exposure times in the crosslinking efficiency of the YwlE C9A F39*Bpa* trapping mutant to unphosphorylated (control) or phosphoarginine lysozyme. SDS-PAGE analysis reveals equal formation of YwlE-(pArg)lysozyme crosslinked products in all exposure times, indicating that after 30 minutes the reaction reached its saturation limit. **C)** Comparison of crosslinking efficiency of the Bpa and DiZPK photoactivatable amino acids. SDS-PAGE shows formation of similar amounts of YwlE-(pArg)lysozyme crosslinked products for both $YwlE_{G.ste}$ C9A F39*Bpa* and F39*DiZPK* mutants.

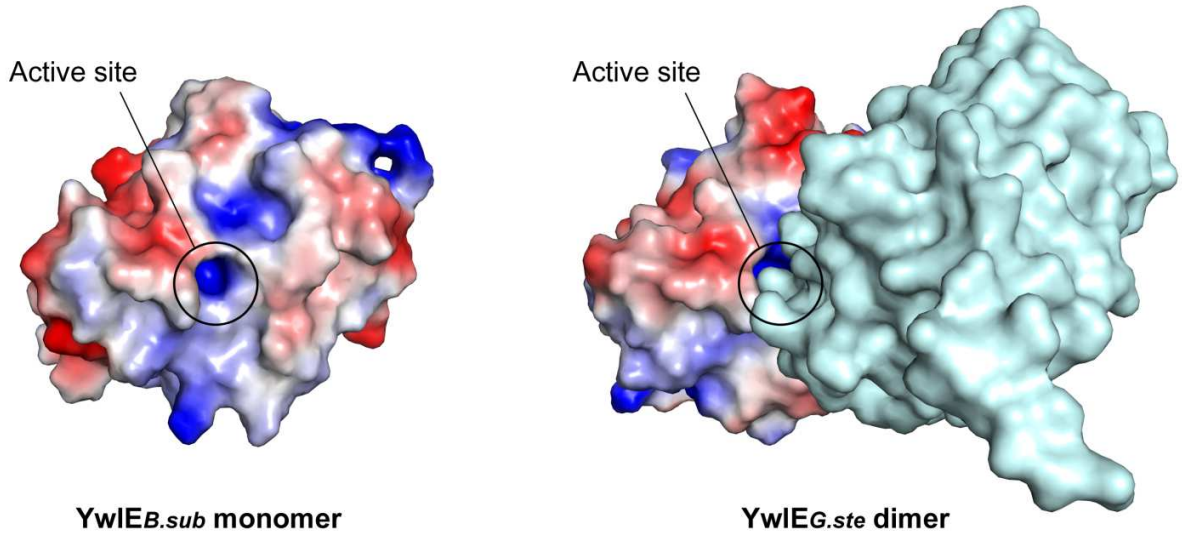


Figure S4: YwlE dimerization blocks access to the active site. The electrostatic surface potential of monomeric *B. subtilis* YwlE (Pdb code: 4KK3), left site. The dimeric *G. stearothermophilus* YwlE is depicted as electrostatic surface potential (protomer B) and surface representation (protomer A, pale cyan), right side. The active site is highlighted by a black circle.

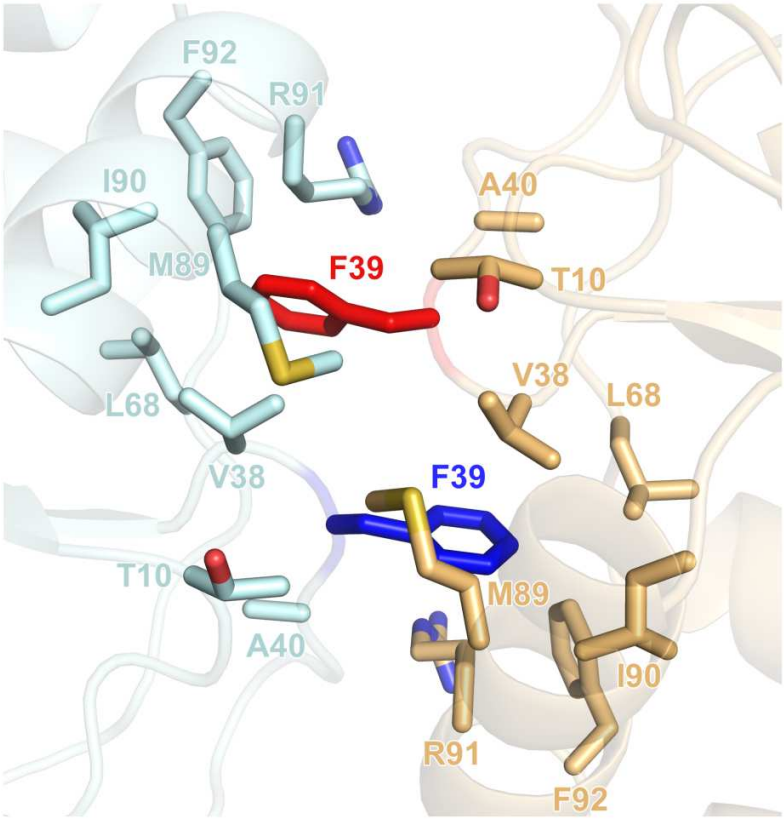


Figure S5: Close up view of the YwlE$_{G.ste}$ dimer interface. Residue F39 is buried in a hydrophobic pocket of the neighboring protomer. Residues constituting the dimer interface are highlighted in stick representation; protomer A (blue and pale cyan), protomer B (red and light orange).

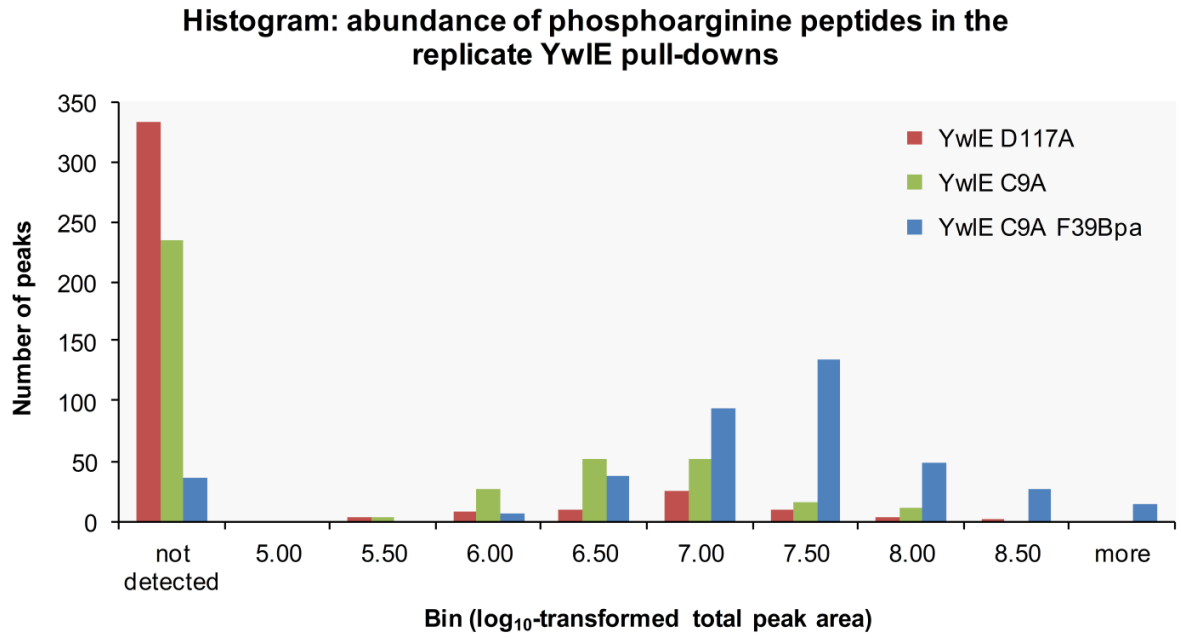


Figure S6: Histogram showing the abundance distribution of 132 phosphoarginine peptides identified in the YwlE pull-downs. An extracted ion chromatogram was computed for each peptide in each replicate of the YwlE D117A, YwlE C9A and YwlE C9A F39*Bpa* pull-downs using the Skyline software. In sum, 1188 extracted ion chromatograms were examined (396 for each YwlE mutant, representing the sum of 132 peak examinations for each of the 3 pull-down replicates). The bin "not detected" shows the number of times a peak could not be observed, indicating that the corresponding phosphoarginine peptide was not present in the respective sample.

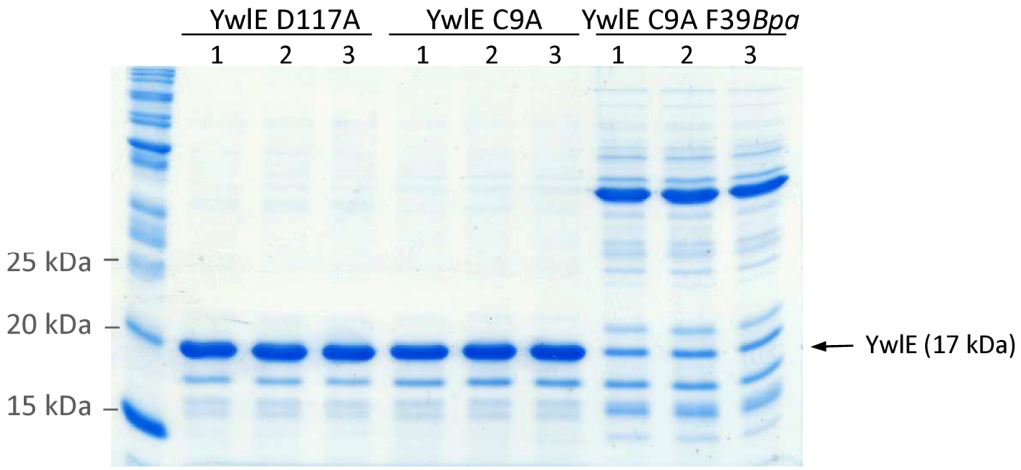


Figure S7: SDS-PAGE analysis of the YwlE *in vitro* pull-downs using *B. subtilis* Δ*ywlE* cell extracts. Gels show the result of each triplicate pull-down experiment (2% aliquot) using either the YwlE D117A, C9A or C9A F39*Bpa* mutants, demonstrating excellent reproducibility between replicates. While the recovery of YwlE was the same for the C9A and D117A mutants, a pronounced decrease in the amount of YwlE C9A F39*Bpa* bound to the His-tag beads is observed.

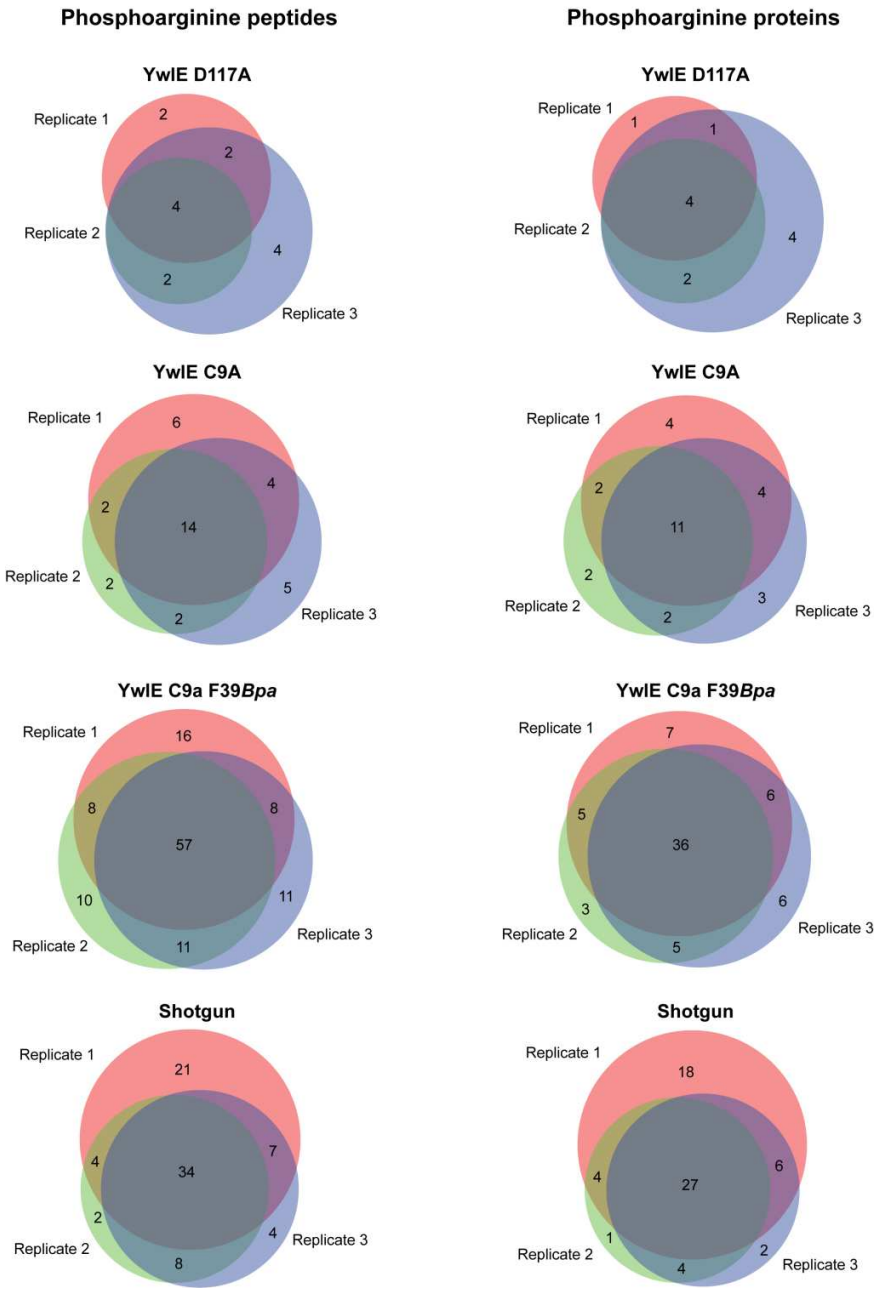


Figure S8: Reproducibility analysis of the YwlE *in vitro* pull-downs using *B. subtilis* Δ*ywlE* cell extracts. Venn diagrams show the overlap between technical replicates. The numbers of identified arginine-phosphorylated peptides (left diagrams) or proteins (right diagrams) are shown. For comparison, the results obtained for triplicate shotgun analysis of *B. subtilis* Δ*ywlE* cell extract are included. As reproducibility of pull-down and shotgun experiments are comparable, the MS analysis rather than the pull-down procedure is the main source of variation.