Supplemental Information (SI)

Involvement of WalK$_{Spn}$ (VicK) Phosphatase Activity in Setting WalR$_{Spn}$ (VicR) Response Regulator Phosphorylation Level and Limiting Crosstalk in *Streptococcus pneumoniae* D39 Cells

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Supplemental Experimental Procedures

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Supplemental Experimental Procedure

Phos-tag SDS-PAGE and quantitative Western blotting. The Phos-tag SDS-PAGE method was based on (Barbieri and Stock, 2008) with the following additions and modifications. ***Preparation of solutions and gels.*** Phos-tag acrylamide (AAL-107) was purchased from Wako Chemicals. Solutions were prepared according to the manufacturer's instructions (http://www.phos-tag.com/english/shouh/pt_page_e_ver8.pdf). Trizma base (Sigma) was used to prepare buffers. Solution B (1.5 M Tris-HCl, 0.4% (wt/vol) SDS, pH 8.8) and solution C (0.50 M Tris-HCl, 0.4% (wt/vol) SDS, pH 6.8) were filter sterilized. Solution D (5 mM Phos-tag AAL solution containing 3% (vol/vol) methanol) was prepared using HPLC-grade methanol. Solution E (10 mM $MnCl_2$) and solution F (10% (wt/vol) diammonium peroxydisulfate) were not filter sterilized. 2× Laemmli sample buffer (Bio-Rad #161-0737) was used instead of the manufacturer's recipe. Phos-tag gels were run in Tris-glycine-SDS running buffer (10× = 10 g SDS, 29 g Trizma base, 144 g glycine per L; no pH adjustment). The transfer buffer used for Western blotting contained 3 g Trizma base, 14.4 g glycine, and 20% (vol/vol) methanol per L. Commercial 40% acrylamide solution (Sigma, A9926) was used in place of the manufacturer's recipe.

A mini-protean gel system (Bio-Rad) with 1 mm spacers was used for these experiments. The 75 μM Phos-tag acrylamide resolving gel solution was prepared by mixing together 1.75 ml of 40% acrylamide, 1.75 ml of solution B, 105 μl of solution D, 105 μl of solution E, 3.29 ml of H_2O, 75 μl of solution F, and 7.5 μl TEMED (Bio-Rad) in a 50 ml conical tube with light vortexing. 4.6 mls of

this solution was poured into the gel casting setup and overlayed with 1 ml of H_2O. For each experiment, two gels were poured and used to run the samples or the heated controls of the samples (see below). After the resolving gel polymerized, a stacking gel was prepared by mixing 0.23 ml of 40% acrylamide, 0.5 ml of solution C, 1.15 ml of H_2O, 35 µl of solution F, and 3.5 µl of TEMED. After removing the water overlay from the resolving gel, the stacking gel was poured on top of the resolving gel and the comb was added. Gels and running buffer were chilled to 4° in the electrophoresis apparatus 1 h prior to loading samples.

Growth of strains and sample preparation. 5 ml overnight cultures were grown in BHI. After 12 to 16 h of overnight incubation, strains were diluted into 30 ml of pre-warmed BHI broth in 50 ml conical tubes to OD_{620} ≈0.005. Because samples were collected at the same culture density, the starting OD_{620} was varied in experiments that included slower growing strains (OD_{620} ≈ 0.005 and OD_{620} ≈ 0.0005 for slower- and faster-growing strains, respectively). Cultures were grown to OD_{620} ≈ 0.2-0.3 and back-diluted in warm BHI, if they had grown slightly above this range. Bacteria were centrifuged at 14,500 × *g* for 5 min at 4°C in a chilled rotor, and all subsequent steps were performed at 4 °C. Supernates were decanted by pouring, and residual supernate was removed by pipetting to prevent carry-over of salts to Phos-tag SDS PAGE. Pellets were placed on ice and suspended in 1.0 ml of cold 20 mM Tris pH 7.0 (diluted from 1 M stock (Ambion)) and 8 µl of Protease Inhibitor Cocktail Set III, EDTA-free (Calbiochem) and transferred to chilled Lysing Matrix B tubes (MP Biomedicals), which were

secured in a 24 × 2 ml-tube adapter in a FastPrep-24 instrument (MP Biochemicals) stored at 4°C. Cells were disrupted by 3 consecutive runs of 40 sec each at a speed setting = 6.0 m/s. Lysed cell mixtures were placed on ice and microcentrifuged at 10,000 × *g* for 1 min at 4°C. 100 µl of supernate was transferred to a tube containing 100 µl of cold 2× Laemmli sample buffer (containing 5% (vol/vol) of freshly added β-mercaptoethanol) and vortexed lightly.

Phos-tag SDS-PAGE, Western blotting, and detection. 30 µl of each sample was loaded per well of a chilled gel and electrophoresis was performed at 150 V for 2 h. After starting the sample run, remaining sample was heated at 95°C for 10 min. Heated samples were chilled on ice for 5 min and loaded onto the duplicate chilled gel and electrophoresed at 150 V for 2 h. Gels were soaked in 150 ml of chilled transfer buffer containing 1 mM EDTA for 15 min to remove Mn^{2+} and then in 150 ml of chilled transfer buffer for 15 min to remove EDTA. Proteins were transferred to 0.45 µm nitrocellulose membranes (Bio-Rad) at 350 mA for 1 h in a wet-tank transfer apparatus (Bio-Rad).

Blocking, washing, and antibody incubation were done in 1X PBS, 0.01% (vol/vol) Tween. Blots were blocked in 5% (wt/vol) membrane blocking reagent (GE Healthcare) for 20 min at 25°C. Blots were washed twice briefly. Binding of a 1:1000 dilution of primary anti-$WalR_{Spn}$ antibody (Cocalico Biologicals; Ng *et al.*, 2003) was carried out for 1 h at 25°C, followed by 2 brief washes and an extended wash of 15 min. Binding of a 1:10,000 dilution of secondary HRP-conjugated anti-rabbit antibody (Amersham) was carried out for 1 h at 25°C, followed by 2 brief washes, an extended wash of 15 min, and 3 more washes of

5 min each. Signal detection was carried out using Amersham ECL detection reagents. After incubation with the detection solutions, luminescent signal was detected and quantitated using an IVIS imaging system (Xenogen) as described before (Wayne, *et al.,* 2010).

Table S1. Bacterial strains and plasmids used in this study[a]

***S. pneumoniae* strains**			
Strain number	Genotype (description)	Antibiotic resistance	Reference or source
EL59	R6 (unencapsulated laboratory strain R6 derived from intermediates of strain D39	None	A. Tomasz
EL1454	R6 *kan*-T1T2-P_c-*pcsB* (EL59 transformed with *kan*-T1T2-P_c-*pcsB* amplicon	Kan^R	Ng *et al.*, 2003
EL1472	R6 *walR* <>*ermAM kan*-T1T2-P_c-*pcsB* (EL1454 transformed with *walR* <>*ermAM* amplicon)	Erm^R Kan^R	Ng *et al.*, 2003
IU1690	D39 (Single colony isolate of serotype 2 strain encapsulated D39 NCTC 7466)	None	Lanie *et al.*, 2007
IU1781	D39 *rpsL1* (IU1690 transformed with *pulA*-*rpsL1*-*rpsG*-*fusA* amplicon)	Str^R	Ramos-Montanez *et al.*, 2008
IU1885	D39 *rpsL1 walK*::[kan^R-$rpsL^+$] (IU1781 transformed with *walK*::[kan^R-$rpsL^+$] amplicon)	Str^S Kan^R	Gutu *et al.,* 2010
IU1896	D39 *rpsL1* Δ*walK* (IU1885 transformed with Δ*walK* amplicon)	Str^R	Gutu *et al.*, 2010
IU2306	D39 *rpsL1* $WalK^{\Delta PAS}$ [Δ amino acids 104-198] (IU1885 transformed with WalK ΔPAS [104-198] amplicon)	Str^R	Gutu *et al.*, 2010
IU3102	D39 *rpsL1* $walK^{H218A}$ (IU1885 transformed with $walK^{H218A}$ amplicon)	Str^R	Gutu *et al.*, 2010
IU3299	D39 *rpsL1* $walK^+$-FLAG (IU1885 transformed with $walK^+$-FLAG amplicon)	Str^R	This study
IU3301	D39 *rpsL1* $walK^{H218A}$-FLAG (IU1885 transformed with $walK^{H218A}$-FLAG amplicon)	Str^R	This study
IU3307	D39 *rpsL1* $WalK^{\Delta PAS}$ [Δ amino acids 104-198]-FLAG (IU1885 transformed with $WalK^{\Delta PAS}$ [104-198]-FLAG amplicon)	Str^R	This study
IU3483	D39 *rpsL1* Δ*pnpR*::P-*ermB* (IU1781 transformed with Δ*pnpR*::P-*ermB* amplicon)	Str^R Erm^R	This study

IU4086	D39 *rpsL1* Δ *pnpRS*::P_c-*erm* (IU1781 transformed with Δ*pnpRS*::P_c-*erm* amplicon)	Str^R Erm^R	This study
IU5352	D39 *rpsL1* Δ*walK bgaA*::P_c-*cat*-T1T2-P_{fcsK}-$walK^+$ (IU1896 transformed with *bgaA*::P_c-*cat*-T1T2-P_{fcsK}-$walK^+$ amplicon)	Str^R Cm^R	This study
IU5401	D39 *rpsL1* $walK^{T222A}$ (IU1885 transformed with $walK^{T222A}$ amplicon)	Str^R	This study
IU5720	D39 *rpsL1* Δ*walK* Δ*pnpR*::P-*ermB* (IU1896 transformed with Δ*pnpR*::P-*ermB* amplicon)	Str^R Erm^R	This study
IU5728	D39 *rpsL1* Δ*walK* Δ*pnpRS*::P_c-*erm* (IU1896 transformed with Δ*pnpRS*::P_c-*erm* amplicon)	Str^R Erm^R	This study
***E. coli* strains**			
Strain Number	Genotype (plasmid)[b]	Antibiotic resistance[a]	Reference or source
EL27	BL21(DE3) pLysS (pSP001)	$Amp^R$$Cm^R$	Ng *et al.*, 2005
IU2046	DH5α (pIU136)	Kan^R	Gutu *et al.*, 2010
IU2092	BL21(DE3) pLysS (pIU140)	$Kan^R$$Cm^R$	Gutu *et al.*, 2010
IU3419	Rosetta BL21(DE3) pLysS (pIU224)	$Kan^R$$Cm^R$	This study
IU3423	Rosetta BL21(DE3) pLysS (pIU229)	$Kan^R$$Cm^R$	This study
IU3435	Rosetta BL21(DE3) pLysS (pIU241)	$Kan^R$$Cm^R$	This study
IU3456	Rosetta BL21(DE3) pLysS (pIU225)	$Kan^R$$Cm^R$	This study
IU3464	Rosetta BL21(DE3) pLysS (pIU233)	$Kan^R$$Cm^R$	This study
IU4105	Rosetta BL21(DE3) pLysS (pIU253)	$Kan^R$$Cm^R$	This study
IU4266	Tuner (pIU255)	Kan^R	This study
IU4727	Rosetta 2(DE3) (pIU262)	$Kan^R$$Cm^R$	This study
IU4730	Rosetta 2(DE3) (pIU263)	$Kan^R$$Cm^R$	This study
IU4733	Rosetta 2(DE3) (pIU265)	$Kan^R$$Cm^R$	This study
IU4821	Rosetta 2(DE3) (pIU264)	$Kan^R$$Cm^R$	This study
IU4825	Rosetta 2(DE3) (pIU268)	$Kan^R$$Cm^R$	This study
IU4848	Rosetta 2(DE3) (pIU266)	$Kan^R$$Cm^R$	This study
IU4851	Rosetta 2(DE3) (pIU267)	$Kan^R$$Cm^R$	This study
IU5019	Rosetta 2(DE3) (pIU269)	$Kan^R$$Cm^R$	This study
IU5022	Rosetta 2(DE3) (pIU270)	$Kan^R$$Cm^R$	This study

IU5025	Rosetta 2(DE3) (pIU271)	$Kan^{R}Cm^{R}$	This study
IU5028	Rosetta 2(DE3) (pIU274)	$Kan^{R}Cm^{R}$	This study
IU5031	Rosetta 2(DE3) (pIU276)	$Kan^{R}Cm^{R}$	This study
IU5109	Rosetta 2(DE3) (pIU272)	$Kan^{R}Cm^{R}$	This study
IU5112	Rosetta 2(DE3) (pIU273)	$Kan^{R}Cm^{R}$	This study
IU5153	Rosetta 2(DE3) (pIU275)	$Kan^{R}Cm^{R}$	This study
IU5156	Rosetta 2(DE3) (pIU277)	$Kan^{R}Cm^{R}$	This study
IU5207	Rosetta 2(DE3) (pIU279)	$Kan^{R}Cm^{R}$	This study
Plasmids			
Plasmid	Description[c]	Reference or source	
pSP001	pET16b (WalR)	Ng *et al.*, 2005	
pIU136	pSumo	Gutu *et al.*, 2010	
pIU140	pSumo (ΔN35-WalK)	Gutu *et al.*, 2010	
pIU224	pSumo (ΔN210-CiaH)	This study	
pIU225	pSumo (ΔN165-HK06)	This study	
pIU229	pSumo (ΔN58-HK08)	This study	
pIU233	pSumo (ΔN164-VncS)	This study	
pIU241	pSumo (ΔN206-PnpS)	This study	
pIU253	pSumo (VncR)	This study	
pIU255	pSumo (ΔN40-PnpS)	This study	
pIU262	pSumo (ΔN35-$WalK^{V216G}$)	This study	
pIU263	pSumo (ΔN35-$WalK^{S217D}$)	This study	
pIU264	pSumo (ΔN35-$WalK^{S217A}$)	This study	
pIU265	pSumo (ΔN35-$WalK^{R221A}$)	This study	
pIU266	pSumo (ΔN35-$WalK^{P223S}$)	This study	
pIU267	pSumo (ΔN35-$WalK^{P223A}$)	This study	
pIU268	pSumo (ΔN35-$WalK^{T225A}$)	This study	
pIU269	pSumo (ΔN35-$WalK^{R221K}$)	This study	
pIU270	pSumo (ΔN35-$WalK^{R221D}$)	This study	
pIU271	pSumo (ΔN35-$WalK^{R221S}$)	This study	
pIU272	pSumo (ΔN35-$WalK^{T222A}$)	This study	
pIU273	pSumo (ΔN35-$WalK^{T222Y}$)	This study	
pIU274	pSumo (ΔN35-$WalK^{T222D}$)	This study	
pIU275	pSumo (ΔN130-PnpS)	This study	
pIU276	pSumo (ΔN150-PnpS)	This study	
pIU277	pSumo (ΔN170-PnpS)	This study	
pIU279	pSumo (RR06)	This study	

[a]Strains and plasmids were constructed as described in Experimental procedures. Antibiotic resistance markers: Kan^{R}, kanamycin; Str^{R}, streptomycin, Erm^{R}, erythromycin; Amp^{R}, ampicillin; Cm^{R}, chloramphenicol. Concentrations of antibiotics used for *S. pneumoniae* strains: 250 μg Str per mL, 250 μg Kan per mL, 0.3 μg Erm per mL, and

2.5 µg Cm per mL; for *E. coli* strains: 34 µg Cm per mL, 30 µg Kan per mL, and 100 µg Amp per mL. All antibiotics were purchased from Sigma-Aldrich.

[b]Rosetta 2(DE3), DH5α, and Tuner cells were purchased as competent cells from Novagen, Bioline, and EMD, respectively.

[c]pSumo plasmid (LifeSensors, Inc.) was used to fuse an N-terminal Sumo tag (≈12 kDa) to WalK constructs. "ΔN" indicates the deletion of the indicated number of amino acids from the N-terminus of the protein. Amino acid replacements are indicated as superscripts.

Table S2. Primers used in this study

***S. pneumoniae* strains**		
Construction of *walK*$^{+}$-FLAG amplicon (IU3299)		
Primer	Sequence (5' to 3')	Product
SR19	GTAGATGATGAGAAACCAATCTCGG	This amplicon was amplified from wild-type DNA. A single FLAG epitope was introduced into the junction site in primer KW130.
KW130	TCACTCATTCTATTTATCATCATCATCTTTATAAT CGTCTTCTACTTCAT	
SR24	CTATATCTCTGTCAATGGTGTTGCGG	This amplicon was amplified from wild-type DNA. A single FLAG epitope was introduced into the junction site in primer KW131.
KW131	ATGAAGTAGAAGACGATTATAAAGATGATGATGA TAAATAGAATGAGTGA	
Construction of *walK*^{H218A}-FLAG amplicon (IU3301)		
Primer	Sequence (5' to 3')	Product
SR19	GTAGATGATGAGAAACCAATCTCGG	This amplicon was amplified from DNA containing the His218Ala mutation. A single FLAG epitope was introduced into the junction site in primer KW130.
KW130	TCACTCATTCTATTTATCATCATCATCTTTATAAT CGTCTTCTACTTCAT	
SR24	CTATATCTCTGTCAATGGTGTTGCGG	This amplicon was amplified from DNA containing the His218Ala mutation. A single FLAG was introduced into the junction site in primer KW131.
KW131	ATGAAGTAGAAGACGATTATAAAGATGATGATGA TAAATAGAATGAGTGA	

Construction of WalK$^{\Delta PAS}$ [Δ amino acids 104-198]-FLAG amplicon (IU3307)		
Primer	Sequence (5' to 3')	Product
SR19	GTAGATGATGAGAAACCAATCTCGG	This amplicon was amplified from DNA containing the ΔPAS mutation. A single FLAG epitope was introduced into the junction site in primer KW130.
KW130	TCACTCATTCTATTTATCATCATCATCTTTATAATCGTCTTCTACTTCAT	
SR24	CTATATCTCTGTCAATGGTGTTGCGG	This amplicon was amplified from DNA containing the ΔPAS mutation. A single FLAG epitope was introduced into the junction site in primer KW131.
KW131	ATGAAGTAGAAGACGATTATAAAGATGATGATGATAAATAGAATGAGTGA	
Construction of Δ*pnpR*::P-*ermB* amplicon (IU3483, IU5720)		
Primer	Sequence (5’ to 3’)	Product
PS351	GGTAAGGGAAGGGCGCTTTATA	Upstream sequence and 5’ 171 bp of *pnpR* coding sequence
PS343	TTTCCATAACTTCTTGGTAACATGATATCAAGC	
PS345	GCTTGATATCATGTTACCAAGAAGTTATGGAAA	P-*ermB* cassette containing *ermB* coding sequence and flanking upstream and downstream sequences
PS347	GTCATGTTGGGATGTTTCTTAGCTCCTTGGAAGC	
PS349	GCTTCCAAGGAGCTAAGAAACATCCCAACATGAC	3’ 190 bp of *pnpR* coding sequence and downstream sequence
PS350	GGCCAAGACCAGTGCCACCA	
Construction of Δ*pnpRS*::P$_c$-*erm* amplicon (IU4086)		
Primer	Sequence (5’ to 3’)	Product
KW132	TACTTGAGGATATATCTTCATGAGCCCTTGATAACCACTGTCAGCCAAG	Region upstream of *pnpR*, 60 bp remaining of *pnpR*/P$_c$-*erm* junction
KW154	AGATACAAATCAAACAAATTTTGGGCCCGGGTAGTCAAGCAATTTCAGAATGTGTTCTTC	

KW155	GAAGAACACATTCTGAAATTGCTTGACTACCCGG GCCCAAAATTTGTTTGATTTGTATCT	Remaining *pnpR*, P_c-erm junction, P_c-*erm*, P_c-*erm* 60 bp remaining of 3' *pnpS* junction
KW156	TCGTGAAGCAACTGCCTCTGCCAAGTTATTTCCT CCCGTTAAATAATAGATAACT	
KW157	AGTTATCTATTATTTAACGGGAGGAAATAACTTG GCAGAGGCAGTTGCTTCACGA	P_c-*erm* 60 bp remaining of 3' *pnpS* junction, region downstream of *pnpS*
KW137	GCTTCAATAAATTTATTATCTGTGACCACTTGTTG ACCTTGTTTGGAGTG	
Construction of *bgaA*::P_{fcsK}-*walK*$^+$ amplicon (IU5352)		
20F32	ATGGTTGTCTCTTGGTTTGACTGCCG	*'bgaA* sequence
KK609	GCCCCGTTAGTTATTTTGCTTTTGCTGCGTACTC	
KK610	AGCAAAATAACTAACGGGGCAGGTTAGTGACA	P_c-*cat*-terminator cassette
KK503	GTTTTATTTGATGCCGATAAGCTTGATGAAAATTT GTTTG	
KK504	CAAGCTTATCGGCATCAAATAAAACGAAAGGCTC	T1T2-P_{fcsK}
KW119	TGGTTTGTTTCAGTAAATCAAGCATTTTTCTTCTC TCTTCGTCCTTGATT	
KW120	AATCAAGGACGAAGAGAGAAGAAAAATGCTTGA TTTACTGAAACAAACCA	*walK*
KK608	TCGCCCCAATTGGAGCGATATTTTTAGTCTTCTA CTTCATCCTCCCATAC	
KK607	CTCCAATTGGGGCGATATTTTGGGATACCTTCTT TATCATTAAAGACCAAAG	downstream sequence
20R45	CCTCCCCTTCTGATTAAATATGCC	
Construction of *walK*^{T222A} amplicon (IU5401)		
Primer	Sequence (5' to 3')[a]	Product
39R19	TGATAACTTTTCGCAAGAAGAGCAG	Upstream sequence and 5' 675 bp of *walK* coding sequence containing Thr222Ala (ACT to GCT) substitution.
KK612	AGTCAGAGG**AGC**CCGTAACTCATGGCTAAC	
KK613	TGAGTTACGG**GCT**CCTCTGACTAGCGTAAAATC	3' 697 bp of *walK* coding sequence containing Thr222Ala (ACT to GCT) substitution and downstream sequence.
39F14	GTCATCAGCAACCTCTCTTATATTGTAAGC	

Construction of Δ*pnpRS*::P_c-*erm* amplicon (IU5728)		
Primer	Sequence (5' to 3')	Product
KW132	TACTTGAGGATATATCTTCATGAGCCCTTGATAACCACTGTCAGCCAAG	Region upstream of *pnpR*, 60 bp remaining of *pnpR/erm* junction
KW133	GAGAATATTTTATATTTTTGTTCATAAAGCCTTCCTTACTTAAATGGTAG	
KW134	CTACCATTTAAGTAAGGAAGGCTTTATGAACAAAAATATAAAATATTCTC	Remaining *pnpR*/erm junction, *ermAM*, *erm*/60 bp remaining of 3' *pnpS* junction
KW135	CAAGCTGACTTGTCACCGTGACTTGAGCATTATTTCTCATTTTCCTCCCG	
KW136	CGGGAGGAAAATGAGAAATAATGCTCAAGTCACGGTGACAAGTCAGCTTG	*erm* 60 bp remaining of 3' *pnpS* junction, region downstream of *pnpS*
KW137	GCTTCAATAAATTTATTATCTGTGACCACTTGTTGACCTTGTTTGGAGTG	
Primers used in construction of plasmid inserts		
Primer	Sequence (5' to 3')	Amplicon Product
AG110	AACTGGGACAAGGAGATATAGGTCTTGAGAGTATGCA	ΔN210-CiaH for pIU224
AG111	AGTAGGATCCTTATTTTTTCTTTTTAGATGGTGTC	
AG122	AACTGGGACAAGGAGATATAGGTCGCTTGCTCAATCC	ΔN165-HK06 for pIU225
AG123	AGTAGGATCCTTACAAGCTAATCTTAAATTCCATACCG	
AG126	AACTGGTCTCAAGGTTTGCCAGTCTTTACGT	ΔN58-HK08 for pIU229
AG127	AGTAGGATCCTTAGGCTTTATTTTCACTACC	
AG116	AACTGGGACAAGGAGATATAGGTACTATTGTTGCACCGA	ΔN164-VncS for pIU233
AG117	AGTAGGATCCTTAGTCTTGGACGACTTTTGGA	
AG134	AACTGGTCTCAAGGTGATTTAACAACGATTAG	ΔN206-PnpS for pIU241
AG115	AGTAGGATCCTTAGTCCTGTGCGAAAGATTGG	
AG102	AACTGGGACAAGGAGATATAGGTATGAAAATTTTAATT	VncR for pIU253
AG103	AGTAGGATCCTTATTTTCGCTCCAATTTATAA	
AG114	AACTGCAGCATAAGTCAAGGTGGACAAGGTGGCTA	ΔN40-PnpS for pIU255
AG115	AGTAGGATCCTTAGTCCTGTGCGAAAGATTGG	
SLi05	GAAGACTCTTTGTTTCCAAT**GGT**AGCCATGAGTTACGGACTCC	ΔN35-WalK^{V216G} for pIU262
SLi06	GGAGTCCGTAACTCATGGCT**ACC**ATTGGAAACA	

	AAGAGTCTTC	
AG26	GATGGATCCTTACTAGTCTTCTACTTCATCCTCC CATAC	
AG28	ACTGGGACATTCTGTCAGAGGTCGTGATAATATT CAGTTG	
SLi07	GACTCTTTGTTTCCAATGTT**GAC**CATGAGTTACG GACTCCTCT	ΔN35-WalK^{S217D} for pIU263
SLi08	AGAGGAGTCCGTAACTCATG**GTC**AACATTGGAA ACAAAGAGTC	
AG26	GATGGATCCTTACTAGTCTTCTACTTCATCCTCC CATAC	
AG28	ACTGGGACATTCTGTCAGAGGTCGTGATAATATT CAGTTG	
SLi09	GACTCTTTGTTTCCAATGTT**GCG**CATGAGTTACG GACTCCTCT	ΔN35-WalK^{S217A} for pIU264
SLi10	AGAGGAGTCCGTAACTCATG**CGC**AACATTGGAA ACAAAGAGTC	
AG26	GATGGATCCTTACTAGTCTTCTACTTCATCCTCC CATAC	
AG28	ACTGGGACATTCTGTCAGAGGTCGTGATAATATT CAGTTG	
SLi11	CCAATGTTAGCCATGAGTTA**GCG**ACTCCTCTGA CTAGCGTAAA	ΔN35-WalK^{R221A} for pIU265
SLi12	TTTACGCTAGTCAGAGGAGT**CGC**TAACTCATGG CTAACATTGG	
AG26	GATGGATCCTTACTAGTCTTCTACTTCATCCTCC CATAC	
AG28	ACTGGGACATTCTGTCAGAGGTCGTGATAATATT CAGTTG	
SLi13	TTAGCCATGAGTTACGGACT**TCT**CTGACTAGCGT AAAATCCTA	ΔN35-WalK^{P223S} for pIU266
SLi14	TAGGATTTTACGCTAGTCAG**AGA**AGTCCGTAACT CATGGCTAA	
AG26	GATGGATCCTTACTAGTCTTCTACTTCATCCTCC CATAC	
AG28	ACTGGGACATTCTGTCAGAGGTCGTGATAATATT CAGTTG	
SLi15	TTAGCCATGAGTTACGGACT**GCT**CTGACTAGCG TAAAATCCTA	ΔN35-WalK^{P223A} for pIU267
SLi16	TAGGATTTTACGCTAGTCAG**AGC**AGTCCGTAACT CATGGCTAA	
AG26	GATGGATCCTTACTAGTCTTCTACTTCATCCTCC CATAC	
AG28	ACTGGGACATTCTGTCAGAGGTCGTGATAATATT CAGTTG	

SLi17	ATGAGTTACGGACTCCTCTG**GCT**AGCGTAAAATCCTATCTTGA	ΔN35-WalK^{T225A} for pIU268
SLi18	TCAAGATAGGATTTTACGCT**AGC**CAGAGGAGTCCGTAACTCAT	
AG26	GATGGATCCTTACTAGTCTTCTACTTCATCCTCCCATAC	
AG28	ACTGGGACATTCTGTCAGAGGTCGTGATAATATTCAGTTG	
SLi25	CCAATGTTAGCCATGAGTTA**AAA**ACTCCTCTGACTAGCGTAAA	ΔN35-WalK^{R221K} for pIU269
SLi26	TTTACGCTAGTCAGAGGAGT**TTT**TAACTCATGGCTAACATTGG	
AG26	GATGGATCCTTACTAGTCTTCTACTTCATCCTCCCATAC	
AG28	ACTGGGACATTCTGTCAGAGGTCGTGATAATATTCAGTTG	
SLi27	CCAATGTTAGCCATGAGTTA**GAC**ACTCCTCTGACTAGCGTAAA	ΔN35-WalK^{R221D} for pIU270
SLi28	TTTACGCTAGTCAGAGGAGT**GTC**TAACTCATGGCTAACATTGG	
AG26	GATGGATCCTTACTAGTCTTCTACTTCATCCTCCCATAC	
AG28	ACTGGGACATTCTGTCAGAGGTCGTGATAATATTCAGTTG	
SLi29	CCAATGTTAGCCATGAGTTA**TCT**ACTCCTCTGACTAGCGTAAA	ΔN35-WalK^{R221S} for pIU271
SLi30	TTTACGCTAGTCAGAGGAGT**AGA**TAACTCATGGCTAACATTGG	
AG26	GATGGATCCTTACTAGTCTTCTACTTCATCCTCCCATAC	
AG28	ACTGGGACATTCTGTCAGAGGTCGTGATAATATTCAGTTG	
SLi31	ATGTTAGCCATGAGTTACGG**GCG**CCTCTGACTAGCGTAAAATC	ΔN35-WalK^{T222A} for pIU272
SLi32	GATTTTACGCTAGTCAGAGG**CGC**CCGTAACTCATGGCTAACAT	
AG26	GATGGATCCTTACTAGTCTTCTACTTCATCCTCCCATAC	
AG28	ACTGGGACATTCTGTCAGAGGTCGTGATAATATTCAGTTG	
SLi33	ATGTTAGCCATGAGTTACGG**TAC**CCTCTGACTAGCGTAAAATC	ΔN35-WalK^{T222Y} for pIU273
SLi34	GATTTTACGCTAGTCAGAGG**GTA**CCGTAACTCATGGCTAACAT	
AG26	GATGGATCCTTACTAGTCTTCTACTTCATCCTCCCATAC	

AG28	ACTGGGACATTCTGTCAGAGGTCGTGATAATATTCAGTTG	
SLi35 SLi36 AG26 AG28	ATGTTAGCCATGAGTTACGG**GAC**CCTCTGACTAGCGTAAAATC GATTTTACGCTAGTCAGAGG**GTC**CCGTAACTCATGGCTAACAT GATGGATCCTTACTAGTCTTCTACTTCATCCTCCCATAC ACTGGGACATTCTGTCAGAGGTCGTGATAATATTCAGTTG	ΔN35-WalK^{T222D} for pIU274
SLi41 SLi44	AACTGCAGCATAAGTCAAGGTAGTCGCTCTCTGCCTCATTA AGTAGGATCCTTAGTCCTGTGCGAAAGATTGG	ΔN130-PnpS for pIU275
SLi42 SLi44	AACTGCAGCATAAGTCAAGGTCTCAAACGGATGGATATTCG AGTAGGATCCTTAGTCCTGTGCGAAAGATTGG	ΔN150-PnpS for pIU276
SLi43 SLi44	AACTGCAGCATAAGTCAAGGTAAACAAGAAGTAAGTGGGTT AGTAGGATCCTTAGTCCTGTGCGAAAGATTGG	ΔN170-PnpS for pIU277
AG86 AG87	AACTGGGACAAGGAGATATAGGTATGAACATTTTAGT AGTAGGATCCTTATAAGCTAATCTTATACCCAACATT	RR06 for pIU279
Primers used for QPCR analyses		
Primer	Sequence (5’ to 3’)	Target transcript
AL16	AGTATCTGAATCTGCAGCAGCACC	*pcsB*
AL17	TGCTCCAACTTGAGGTGTTGAACC	*pcsB*
AL33	AGTGGTTATCGTCCAGGAGACAGT	*spd_1874*
AL34	ATTGGGTTCCAAGTGTTAGCTGGC	*spd_1874*
KK489	AAAGGTCGTGGTGGTAAGGGAATG	*gyrA*
KK490	GCATCTTGATCCAGGCGCATTACT	*gyrA*

[a]Sequences in bold letters represent introduced codon changes.

Table S3. Proteins purified for this study[a]

Protein	Source	Storage buffer[b]
(N)-Sumo-ΔN35-WalK	Gutu *et al*, 2010	A
(N)-Sumo-ΔN210-CiaH	This study	A
(N)-Sumo-ΔN165-HK06	This study	A
(N)-Sumo-ΔN58-HK08	This study	C2
(N)-Sumo-ΔN164-VncS	This study	A
(N)-Sumo-ΔN206-PnpS	This study	A
(N)-Sumo-ΔN40-PnpS	This study	A
(N)-Sumo-ΔN130-PnpS	This study	C2
(N)-Sumo-ΔN150-PnpS	This study	C2
(N)-Sumo-ΔN170-PnpS	This study	A
(N)-Sumo-ΔN35-WalK^{V216G}	This study	A
(N)-Sumo-ΔN35-WalK^{S217D}	This study	C2
(N)-Sumo-ΔN35-WalK^{S217A}	This study	A
(N)-Sumo-ΔN35-WalK^{R221A}	This study	C2
(N)-Sumo-ΔN35-WalK^{P223S}	This study	C2
(N)-Sumo-ΔN35-WalK^{P223A}	This study	C2
(N)-Sumo-ΔN35-WalK^{T225A}	This study	A
(N)-Sumo-ΔN35-WalK^{R221K}	This study	C2
(N)-Sumo-ΔN35-WalK^{R221D}	This study	A
(N)-Sumo-ΔN35-WalK^{R221S}	This study	A
(N)-Sumo-ΔN35-WalK^{T222A}	This study	A

(N)-Sumo-ΔN35-WalK^{T222Y}	This study	C2
(N)-Sumo-ΔN35-WalK^{T222D}	This study	C2
(N)-His-WalR	Gutu *et al*, 2010	D
(N)-Sumo-VncR	This study	D
(N)-Sumo-RR06	This study	C2

[a]Protein purification is described in Experimental procedures. *E. coli* strains used to express proteins from recombinant plasmids are listed in Table S1.

[b]Purified proteins were stored in the following buffers from Gutu *et al*., 2010:

Buffer A: 10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 50 mM KCl, 12% glycerol;

Buffer C2: 50 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 200 mM KCl, 30% glycerol;

Buffer D: 10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 300 mM KCl, 0.1 mM DTT, 50% glycerol.

Fig. S1. Changes introduced into the DHp domain of pneumococcal WalK to identify amino acids important for phosphatase activity. Amino acids in black type on light gray (first row) are predicted to contact WalR (see (Howell *et al.*, 2006)). Amino acids in white type on charcoal were changed in this study to examine their effects on WalK autophosphorylation, phosphoryltransfer, and phosphatase activities (see Fig. 2, S2, S3, S4, S5, S6, and S7). His218 is autophosphorylated in WalK, and WalK M11 corresponds to the WalK^{T222A} protein with strongly reduced phosphatase activity characterized in this study.

Fig. S2. Phosphorimages showing autophosphorylation of WalK mutant derivatives with amino acid changes in the DHp recognition helix (see Fig. S1). Autophosphorylation reactions containing 2.0 μM of WalK mutant proteins were performed at 25°C as described in Experimental procedures. Samples were removed after 10, 30, and 60 min (shown left to right) and analyzed by 10% SDS-PAGE.

Fig. S3. Phosphorimages of combined assays showing phosphoryltransfer from WalK~P constructs to WalR and dephosphorylation of WalR~P. Reactions were performed at 25°C in reaction mixtures containing Mg^{2+} or Ca^{2+}, 2.0 μM of purified truncated sensor kinase, and 6.6 μM of (N)-His-WalR as described in Experimental procedures and Fig. 2. (A) and (E) (N)-Sumo-ΔN35-WalK^{+}; (B) (N)-Sumo-ΔN35-WalK^{R221A}; (C) (N)-Sumo-ΔN35- WalK^{T225A}; (D) (N)-Sumo-ΔN35-WalK^{S217A}; (F) (N)-Sumo-ΔN35-WalK^{R221S}; (G) (N)-Sumo-ΔN35-WalK^{T222Y}; and (H) (N)-Sumo-ΔN35-WalK^{R221K}. Panels (A) through (D) also show samples removed after 1 min and 10 min of WalK autophosphorylation.

Fig. S4. Autophosphorylation reaction progress curves used to calculate kinetic parameters of truncated WalK^{T222A}. Representative curves are shown. Upper right: typical reaction time courses used to calculate initial rates of WalK^{T222A} autophosphorylation. Reactions containing 1.0 μM of (N)-Sumo-ΔN35-WalK^{T222A} protein and the indicated concentrations of ATP were carried out at 25°C as described in Experimental procedures. Lower graph: velocity versus ATP concentration curve used to calculate K_m (ATP) and k_{cat} for truncated WalK^{T222A} listed in the table. Similar assays (not shown) were performed to determine the kinetic parameters listed for (N)-Sumo-ΔN35-WalK^{+}. Means with standard errors based on the number (n) of independent determinations are indicated.

Fig. S5. Half-lives of WalK^{+}~P and WalK^{T222A}~P in phosphoryltransferase reactions to WalR. Reactions were performed at 25°C in mixtures containing Mg^{2+} or Ca^{2+} buffer as described in Experimental procedures. Autophosphorylation reactions containing 3.0 μM of purified (N)-Sumo-ΔN35-WalK^{+} or (N)-Sumo-ΔN35-WalK^{T222A} protein proceeded for 20 min. Free ATP was removed from reactions using a spin-desalting column, after which (N)-His-WalR (final concn = 0.5 or 1.0 μM) was added to the reaction to initiate phosphoryltransfer. Half-lives of WalK~P and WalK^{T222A}~P were determined from exponential decay plots after the addition of WalR. Means and standard errors of two independent experiments are shown. Under the reaction conditions used, the half-lives of WalK~P reflect the kinetic preference (k_{cat}/K_m) of the phosphoryltransferase reaction (see (Gutu *et. al.*, 2010)).

Fig. S6. Representative HPLC chromatograms of dephosphorylation of WalR~P by WalK^{+} and WalK^{T222A} after a 1 h incubation. (A) No WalK (WalR~P

autodephosphorylation); (B) WalK^{+}; and (C) WalK^{T222A}. Dephosphorylation analysis is described in Experimental procedures. The identities of the protein peaks are indicated. The No WalK and WalK^{+} or WalK^{T222A} experiments were performed twice or four times, respectively, with similar results.

Fig. S7. Phosphatase reaction progress curves showing dephosphorylation of WalR~P mediated by truncated WalK^{T222A}. Representative curves are shown. Reaction mixtures containing 3.0 μM to 6.0 μM of WalR~P, 2.0 μM of WalK^{T222A}, and 13.2 μM of ADP were incubated at 25°C and amounts of remaining WalR~P were determined by HPLC as described in Experimental procedures. The experiment was performed independently 5 times, and the mean half-life of WalR~P with standard error is listed. For comparison, WalR~P autodephosphorylation (no WalK^{+}) and dephosphorylation by WalK^{+} determined previously (Gutu *et al.*, 2010) are shown in the graph and listed in the table. Reduced WalK^{T222A} phosphatase activity compared to that of WalK^{+} was confirmed directly for the 60 min incubation time (Fig. S6).

Fig. S8. Growth and survival of *walK*$^{+}$ and *walK* mutant strains. (A) Representative growth curves of *walK*$^{+}$ parent strain IU1781 (D39 *rpsL1*) and isogenic mutant strains IU3102 (D39 *rpsL1 walK*^{H218A}) and IU5401 (D39 *rpsL1 walK*^{T222A}) in static BHI broth cultures at 37°C in an atmosphere of 5% CO_2. Samples were removed for live-dead staining as described in Experimental procedures at mid-exponential (E^{M}), late-exponential (E^{L}), transition (T), and stationary (S) growth phases (arrows). (B) Percentage of live cells in cultures of the *walK*$^{+}$, *walK*^{H218A}, and *walK*^{T222A} strains at different phases of growth determined by live-dead staining. Averages and standard errors for each growth point reflect data from three independent cultures of each strain.

Total number of cells counted for each growth point: *walK*$^{+}$ E^{M} (1,022), E^{L} (2,123), T (2,226), S (2,819); *walK*^{H218A} E^{M} (1,047), E^{L} (2,326), T (1,782), S (1,679); *walK*^{T222A} E^{M} (681), E^{L} (1,235), T (2,500), S (2,710). (C) Relative cellular amounts of WalK^{+}-FLAG (IU3299), WalK^{H218A}-FLAG (IU3301), and WalK$^{\Delta PAS}$-FLAG (IU3307) in exponentially growing bacteria. Strains were grown to an OD_{620} ≈0.2. Samples were prepared, and equal volumes of cell extracts (normalized for OD_{620}) were analyzed by 10% SDS-PAGE and Western blotting with anti-FLAG antibody as described previously in (Wayne *et al.*, 2010). Chemiluminescent images were captured and quantitated in arbitrary units. Predicted sizes of proteins: WalK^{+}-FLAG and WalK^{H218A}-FLAG , 52.7 kDa; WalK$^{\Delta PAS}$-FLAG, 41.9 kDa.

Fig. S9. Viability of *walK*$^{+}$ and *walK* mutant strains during exponential growth and stationary phase. *walK*$^{+}$ (IU1781), *walK*^{H218A} (IU3102), and *walK*^{T222A} (IU5401) strains were grown in static BHI broth culture at 37°C in an atmosphere of 5% CO_2. Samples were removed for staining with live-dead fluorescent dyes as described in Experimental procedures at the growth phases indicated in Fig. S8A. OD_{620} of cultures sampled during mid-exponential (E^{M}) phase: 0.10 to 0.12; transition (T) phase: 0.51 to 0.61 (*walK*$^{+}$ and *walK*^{T222A}) or 0.28 to 0.30 (*walK*^{H218A}); stationary (S) phase: 0.54 to 0.67 (*walK*$^{+}$ and *walK*^{T222A}) or 0.30 to 0.35 (*walK*^{H218A}). Representative phase-contrast and fluorescent micrographs are shown of *walK*$^{+}$ and *walK* mutant strains at mid-exponential and stationary phases of growth. Bacteria are false-colored green (live; stained with SYTO-9) and red (dead; stained with propidium iodide). Scale bar = 1 micron. We did not detect significant differences in the number of intact cells in cultures of the *walK*$^{+}$

and *walK* mutant strains at the times sampled in the exponential, transition, or stationary growth phase (data not shown).

Fig. S10. WalRK regulon expression in *walK*$^{+}$, *walK* autokinase-deficient, and Δ*walK* //*walK*$^{+}$ complementation strains during exponential growth. Strains were grown statically in BHI broth culture to OD_{620} ≈0.2 at 37°C in an atmosphere of 5% CO_2. RNA samples for QRT-PCR analysis were prepared and analyzed as described in Experimental procedures. Amount of *pcsB* or *spd_1874* transcript was normalized to that of *gyrA* for each sample. Transcript amounts are expressed relative to that of the *walK*$^{+}$ parent and represent averages from duplicate samples from at least two independent experiments. Unpaired two-tailed t tests were performed using GraphPad Prism 5 software. Strains used: *walK*$^{+}$ parent (IU1781); *walK*^{H218A} mutant (IU3102), Δ*walK* mutant (IU1896); Δ*walK* //*bgaA*::P_{fcsK}-*walK*$^{+}$ complemented strain (IU5352) (Table S1). (A) Relative *pcsB* transcript amounts in *walK*$^{+}$ and *walK*^{H218A} strains during exponential growth. (B) Relative *spd_1874* transcript amounts in *walK*$^{+}$, Δ*walK,* and complemented Δ*walK bgaA*::P_{fcsK}-*walK*$^{+}$ strains during exponential growth. IU5352 was cultured in BHI broth supplemented with 1% fucose during overnight and final growths.

Fig. S11. Validation of Phos-tag SDS-PAGE method for detection of WalR~P in biochemical reactions and cellular extracts. (A) Separation of WalR~P and WalR by 25 μM Phos-tag SDS-PAGE. WalR was phosphorylated by acetyl phosphate in a biochemical reaction as described in Experimental procedures and (Gutu *et al.*, 2010). Proteins were detected by staining with Coomassie brilliant blue. (B) Detection of different relative amounts of WalR~P and WalR by Western blotting. WalR was phosphorylated by acetyl phosphate, and WalR~P and WalR were mixed in the

proportions shown and analyzed by 25 μM Phos-tag SDS-PAGE and Western blotting as described in Experimental procedures. Subsequent gels contained 50 or 75 μM Phos-tag acrylamide to increase resolution. (C) Demonstration that WalR and WalR~P can be detected in cellular extracts prepared as described in Experimental procedures. Proteins were resolved on 50 μM Phos-tag SDS-PAGE and detected by Western blotting with anti-WalR antibody. Left 2 lanes, extracts of a Δ*walR* P_c-*pcsB*$^+$ control strain (EL1472) (Ng *et al.*, 2003) showing that WalR is not detected. Right 2 lanes, extracts of encapsulated *walRK*$^+$ *pcsB*$^+$ strain (IU1781) grown exponentially (lane 4) or treated with a sublethal concentration of an antibiotic (lane 3) to stimulate WalR phosphorylation (K. M. Kazmierczak, in preparation). The WalR~P band in lane 3 was heat-labile (data not shown; see Fig. 5 and S12). The faint contaminant band indicated ran just above WalR~P or between WalR~P and WalR on 50 or 75 μM Phos-tag SDS-PAGE, respectively (see Fig. 5). Titration experiments indicated the distance of the shift of WalR~P from WalR was approximately linear with the concentration of Phos-tag acrylamide in the gels (data not shown). 75 μM Phos-tag SDS-PAGE gave the best and most consistent resolution of WalR~P from WalR for quantitation. See text for additional details.

Fig. S12. Heated controls for samples in Fig. 5. Samples were heated at 95°C for 10 min before 75 μM Phos-tag SDS-PAGE as described in Experimental procedures. The expected position of WalR~P is indicated. See Fig. 5 and the text for additional details.

Fig. S13. Phosphorimages showing dephosphorylation of WalR~P mediated by WalK and WalK^{T222A} in combined assays containing physiologically relevant molar ratios of WalK sensor kinase to WalR response regulator (1: 6.7). Combined assays were

performed at the indicated molar ratios of $WalK^{+}$ or $WalK^{T222A}$ to WalR as described in Experimental procedures. The accumulation of WalR~P after 30 min is graphed in Fig. 6, and similar trends were seen on graphs of the 1 min and 60 min time points (data not shown). The experiment was performed 3 times independently with similar results.

Fig. S14. Comparison of amino acid sequence of the DHp helix sequence of WalK with that of other related pneumococcal HisKA-family sensor kinases. Amino acids in black type on light gray are predicted to contact WalR (see (Howell et al., 2006)). Amino acids with similar physico-chemical properties are indicated in light gray type. Dissimilar amino acids are indicated in white type on a charcoal background. The last column summarizes whether the indicated phosphorylated sensor kinase engaged in phosphoryltransfer to WalR in biochemical reactions (see Fig. S15 and Experimental procedures). +/- indicates low levels of WalR~P were detected in phosphoryltransferase reactions after long incubation times of 10 and 30 min. +++ indicates that WalR~P was detected within 1 min in phosphoryltransferase reactions containing cognate WalK~P, despite the WalK phosphatase activity (Fig. 2 and S7).

Fig. S15. Phosphorimages showing phosphorylation of WalR to WalR~P by cognate WalK~P and non-cognate phosphorylated HisKA-family sensor kinases of *S. pneumoniae* (Fig. S14). Combined assays were carried out at 25°C in reaction mixtures containing Mg^{2+} or Ca^{2+} as described in Experimental procedures. Experiments were repeated multiple times, and representative time courses are shown. WalR was incubated with autophosphorylated: (A) (N)-Sumo-ΔN35-WalK; (B) (N)-Sumo-ΔN206-PnpS; (C) (N)-Sumo-ΔN58-HK08; (D) (N)-Sumo-ΔN210-CiaH; (E) (N)-Sumo-ΔN164-VncS; and (F) (N)-Sumo-ΔN165-HK06. Arrows indicate low levels of WalR~P formed in

these reactions. The lack of accumulation of WalR~P in reactions containing WalK reflects WalK phosphatase activity (see Fig. 2 and S7). For non-cognate sensor kinases, low levels of WalR~P formation reflect kinetically unfavorable kinetics of phosphoryltransfer. See text for additional details. Autophosphorylated (N)-Sumo-ΔN164-VncS and (N)-Sumo-ΔN165-HK06 did not transfer a phosphoryl group to WalR, but could phosphorylate their cognate response regulators, (N)-Sumo-VncR (G) and (N)-Sumo-RR06 (H), respectively.

Fig. S16. Model for regulation of WalK phosphatase activity by its PAS domain. (A) structure of the interaction between ThkA histidine kinase and its cognate response regulator TrrA from *Thermotoga martima* reported in (Yamada *et al.*, 2009). In this structural model, the PAS domain of the ThkA histidine kinase contacts the TrrA response regulator. This interaction may contribute to the requirement of the PAS domain for optimal ThkA-mediated phosphatase activity on TrrA~P (Yamada *et al.*, 2009). (B) By analogy, results presented here and in (Gutu *et al*., 2010) indicate that the predominant default activity of WalK is its phosphatase activity against WalR~P, both in biochemical reactions (Fig. 2 and 6) and in exponentially growing cells (Fig.1, 4, 5). Stress conditions like cell wall damage may cause a ligand or protein, or both, to bind to the PAS domain of WalK, thereby turning the phosphatase activity down or off to allow accumulation of WalR~P formed by phosphoryltransfer from WalK~P, whose formation might also increase in response to the stress (Fig. 1). This model will be tested in future studies.

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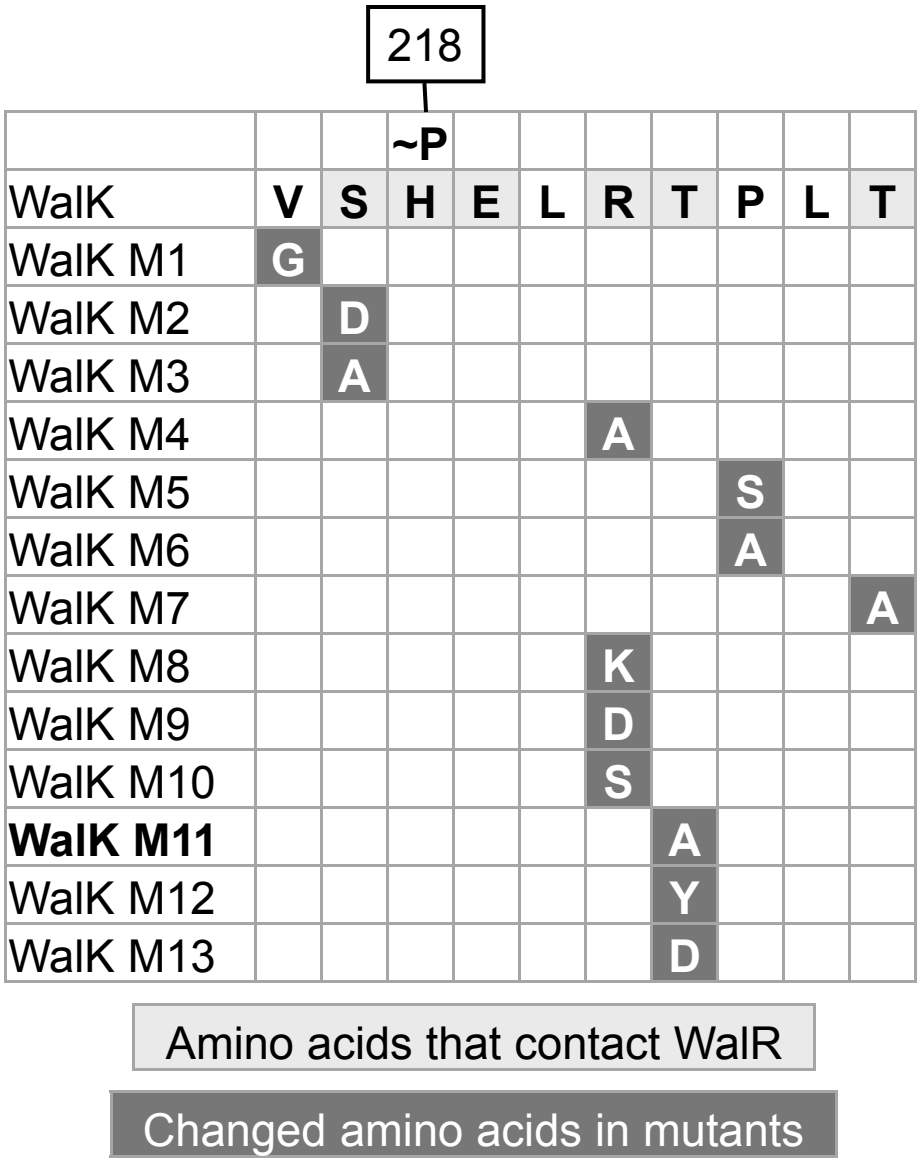
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WalK M8 K
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Amino acids that contact WalR
Changed amino acids in mutants

Fig. S1

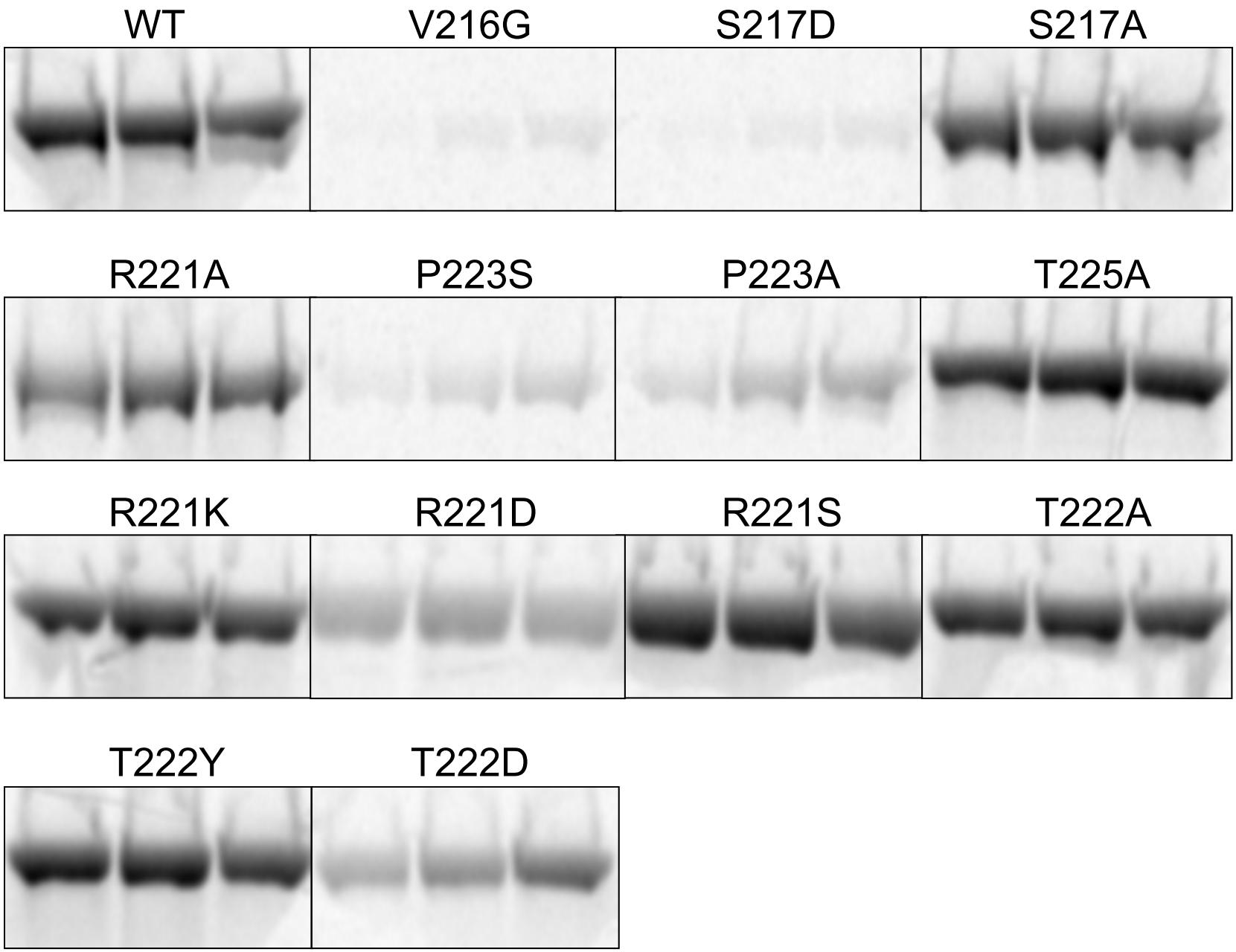
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Fig. S2

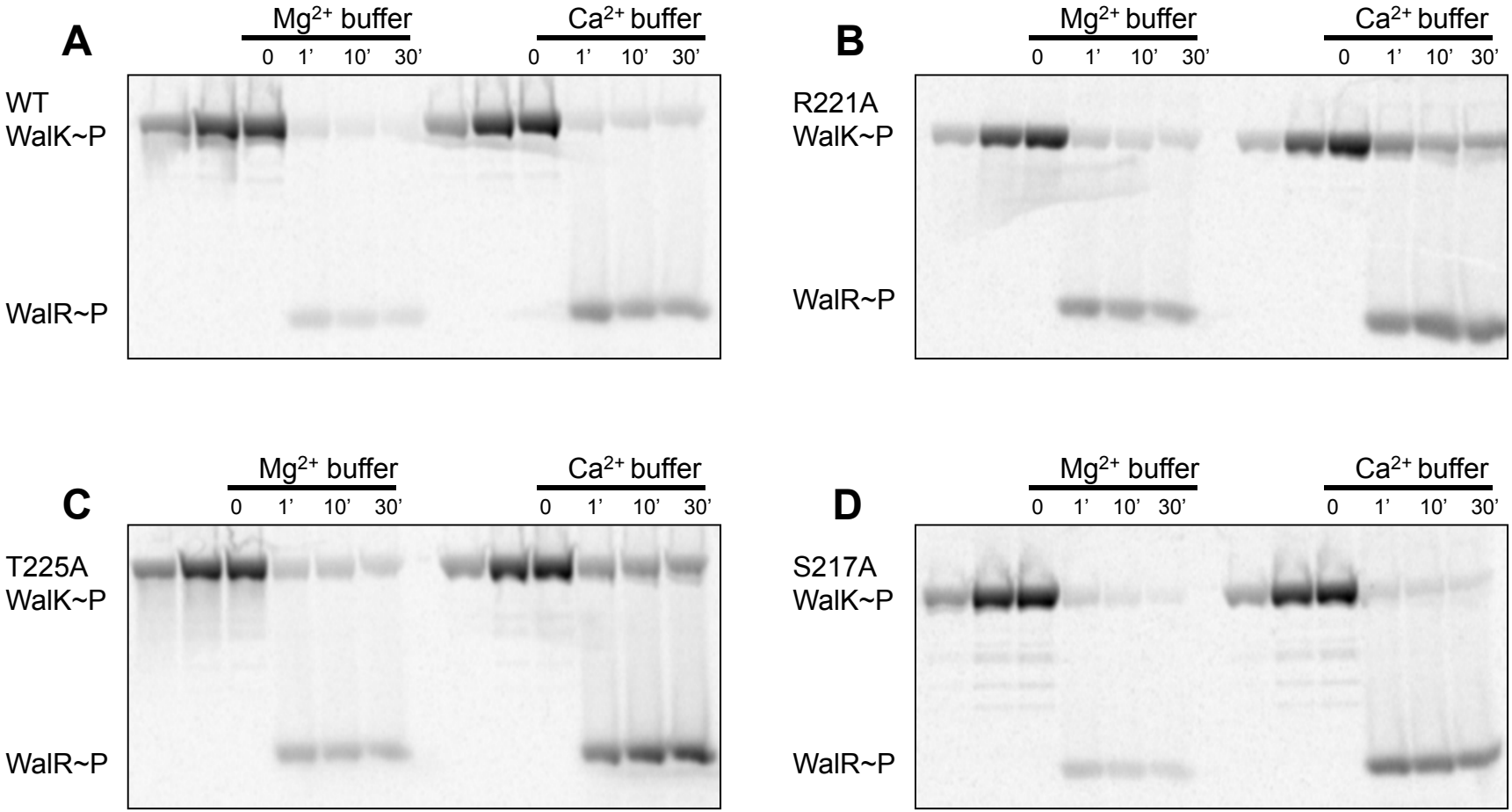
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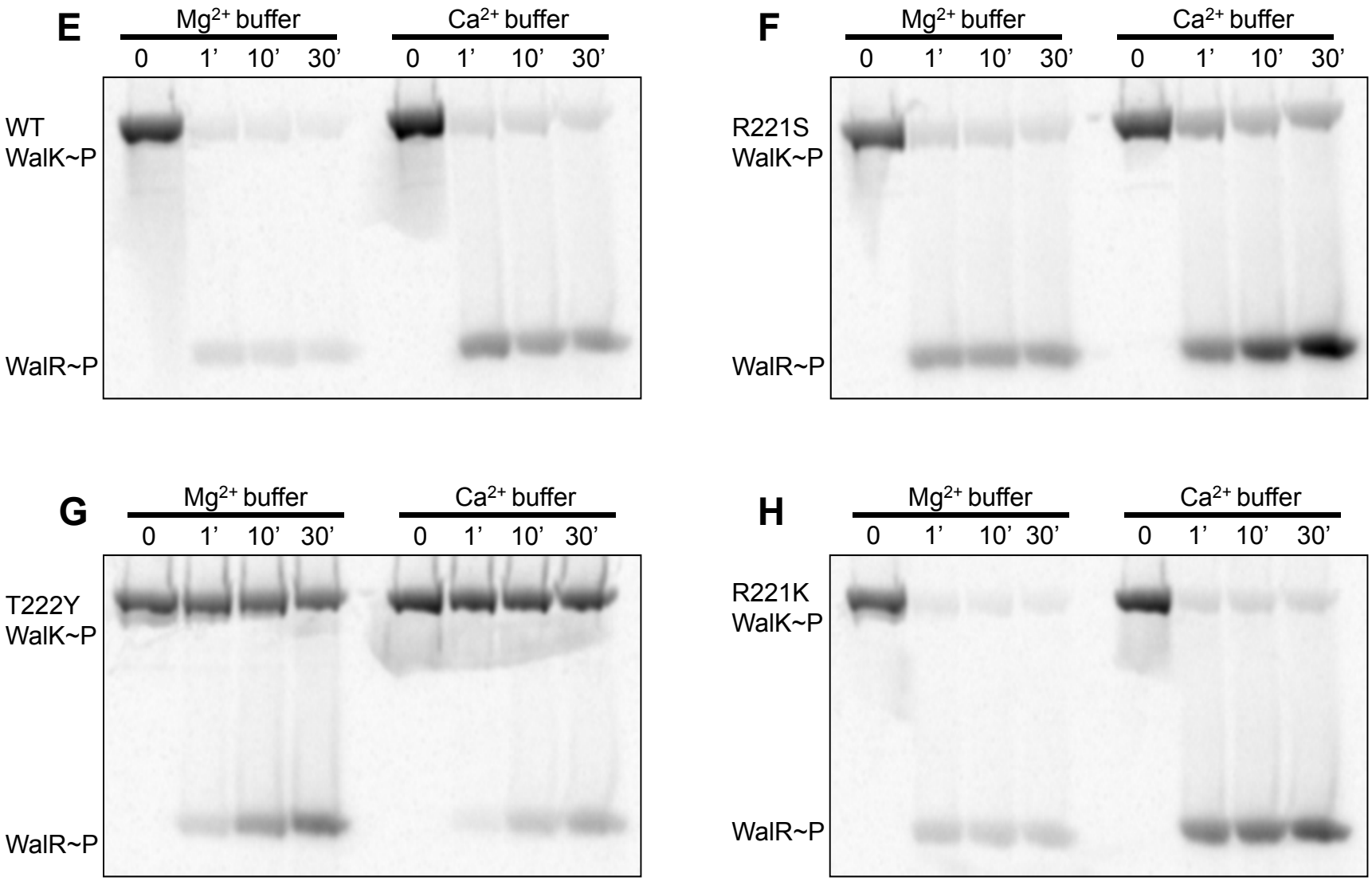
Fig. S3



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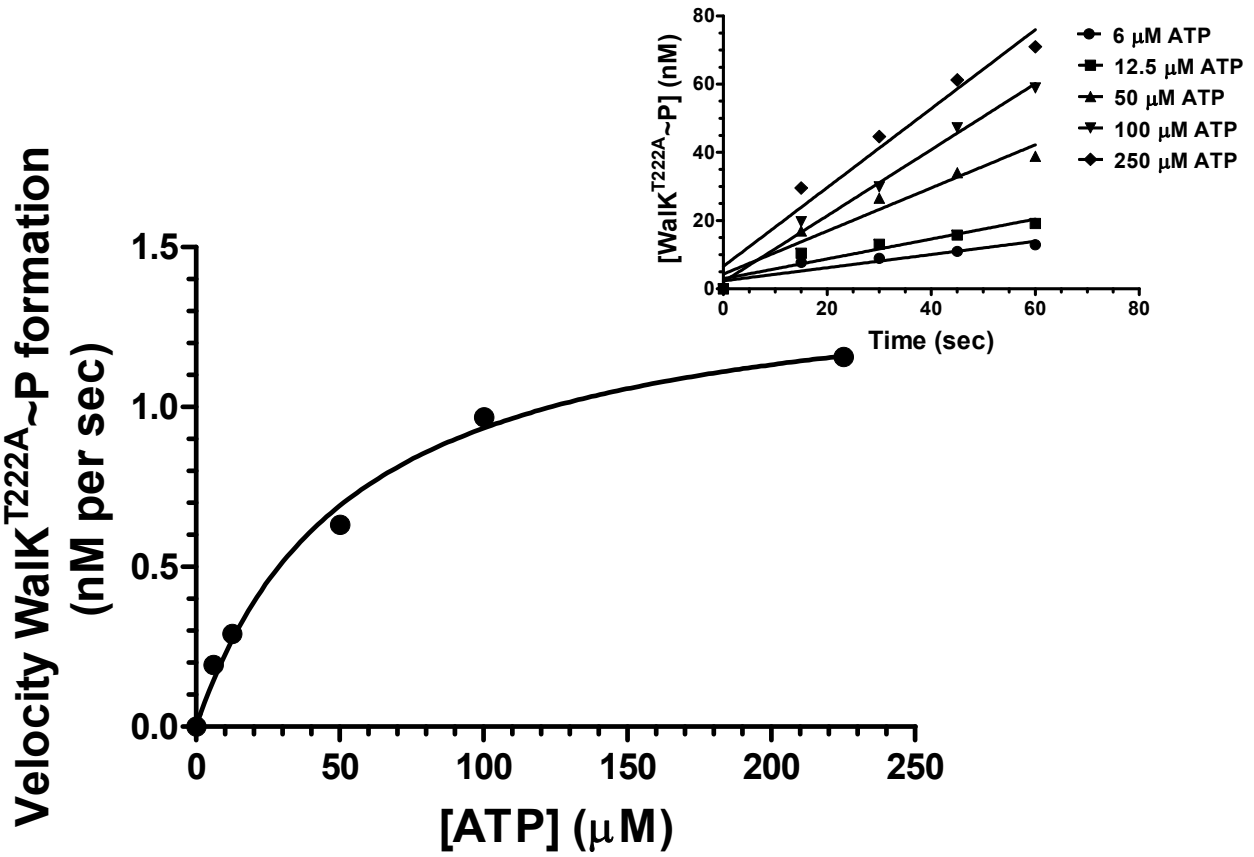
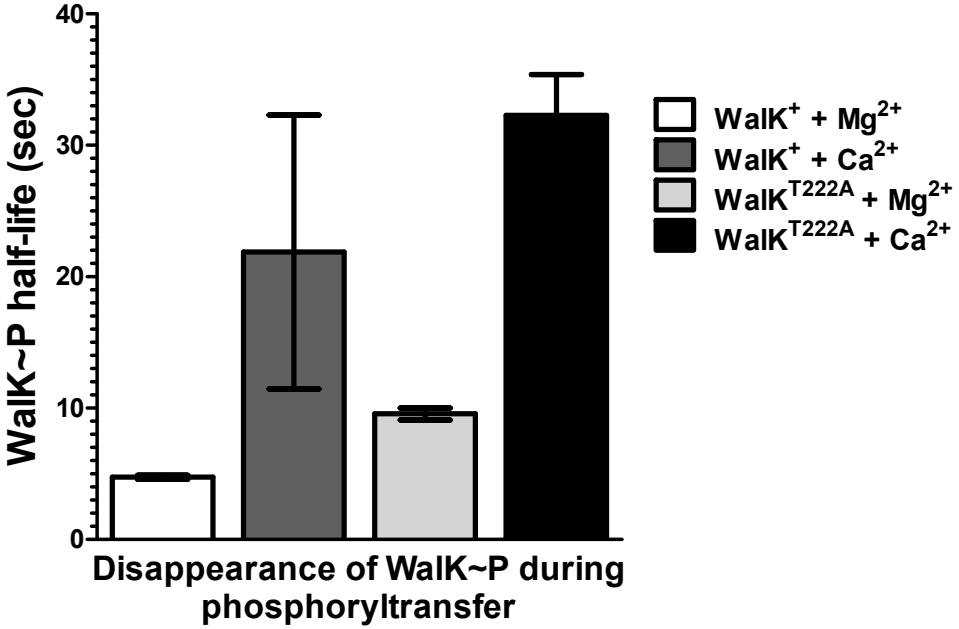


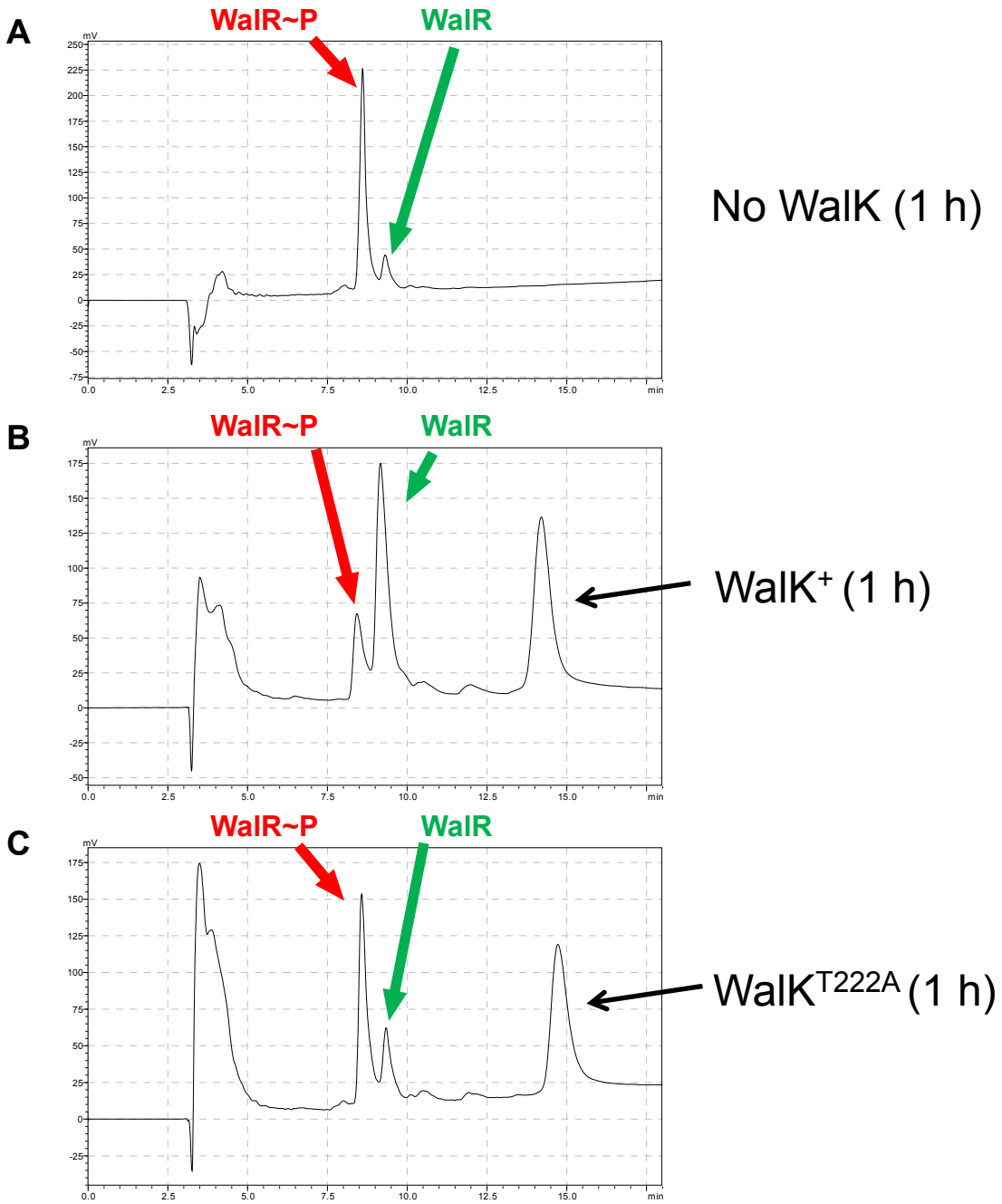
WalK construct	K_m(ATP)(μM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})
(N)-Sumo-ΔN35-$WalK_{Spn}^{+}$	64.1 ± 8.3 (n=4)	0.175 ± 0.012 (n=4)	2,800 ± 172
(N)-Sumo-ΔN35-$WalK_{Spn}^{T222A}$	71.9 ± 5.7 (n=8)	0.079 ± 0.004 (n=8)	1130 ± 74

Fig. S4



WalK construct	WalK~P half life in Mg^{2+} buffer (sec)	WalK~P half life in Ca^{2+} buffer (sec)
(N)-Sumo-ΔN35- $WalK_{Spn}^{+}$	4.7 ± 0.2	22 ± 10
(N)-Sumo-ΔN35- $WalK_{Spn}^{T222A}$	9.6 ± 0.5	32 ± 3

Fig. S5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mV
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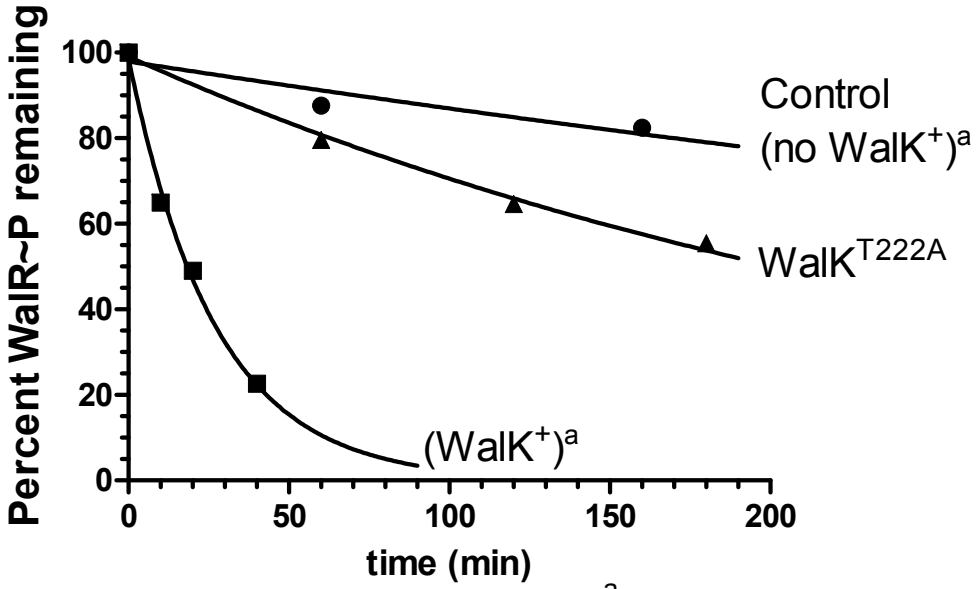
WalKT222A (1 h)
min

Fig. S6



[a]Data from Gutu *et al.,* 2010

	k (min^{-1})	WalR~P half life (min)
[a](N)-His-$WalR_{Spn}^{+}$	0.0006 ± 0.0001	1,370 ± 320
[a](N)-Sumo-ΔN35-$WalK_{Spn}^{+}$	0.036 ± 0.001	18.9 ± 0.4
(N)-Sumo-ΔN35-$WalK_{Spn}^{T222A}$	0.0029 ± 0.0002 (n=5)	240 ± 15 (n=5)

Fig. S7

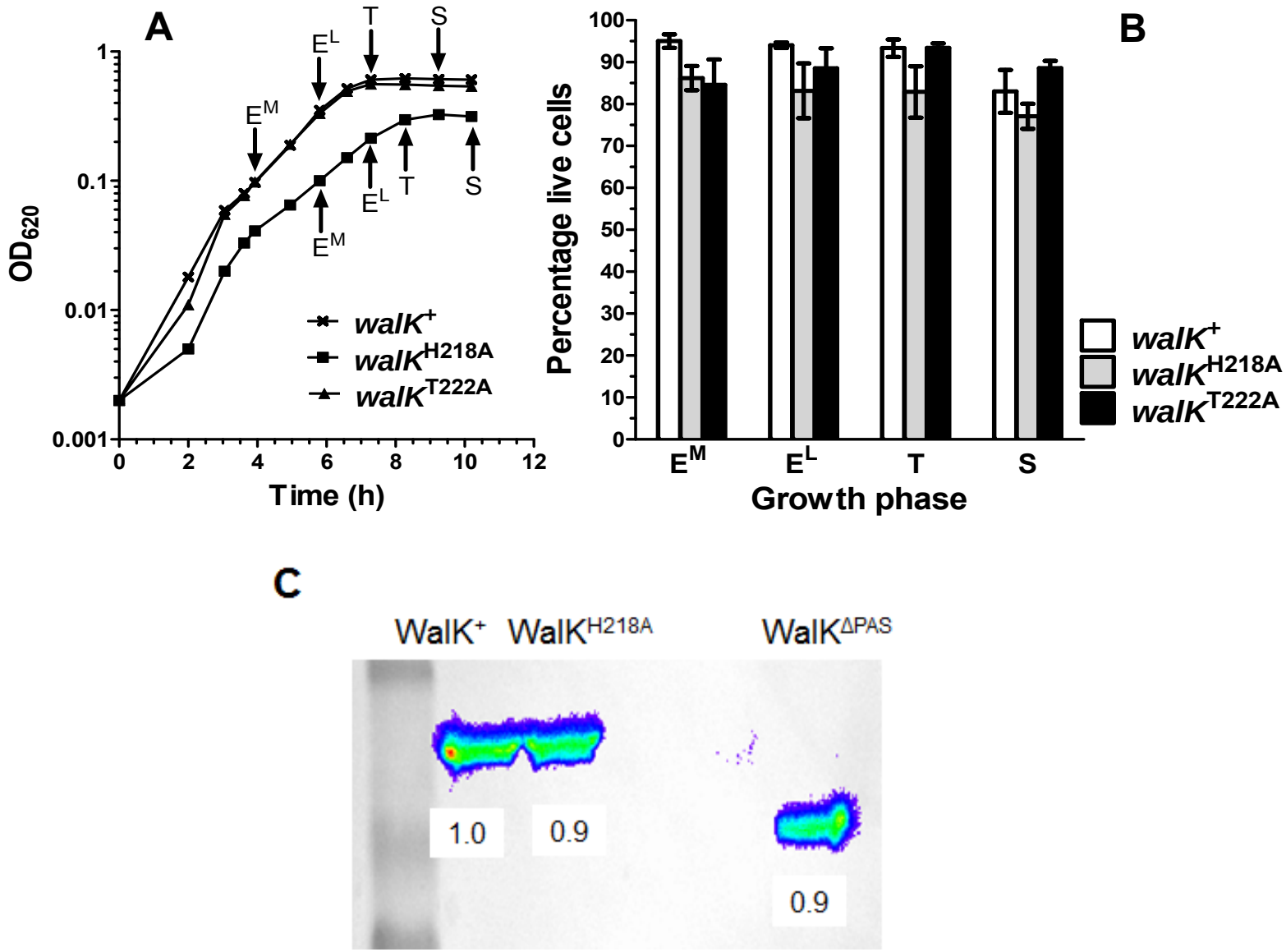

A
B
C
OD620
Time (h)
Percentage live cells
Growth phase
walK+
walKH218A
walKT222A
EM
EL
T
S
WalK+
WalKH218A
WalKΔPAS
1.0
0.9
0.9

Fig. S8

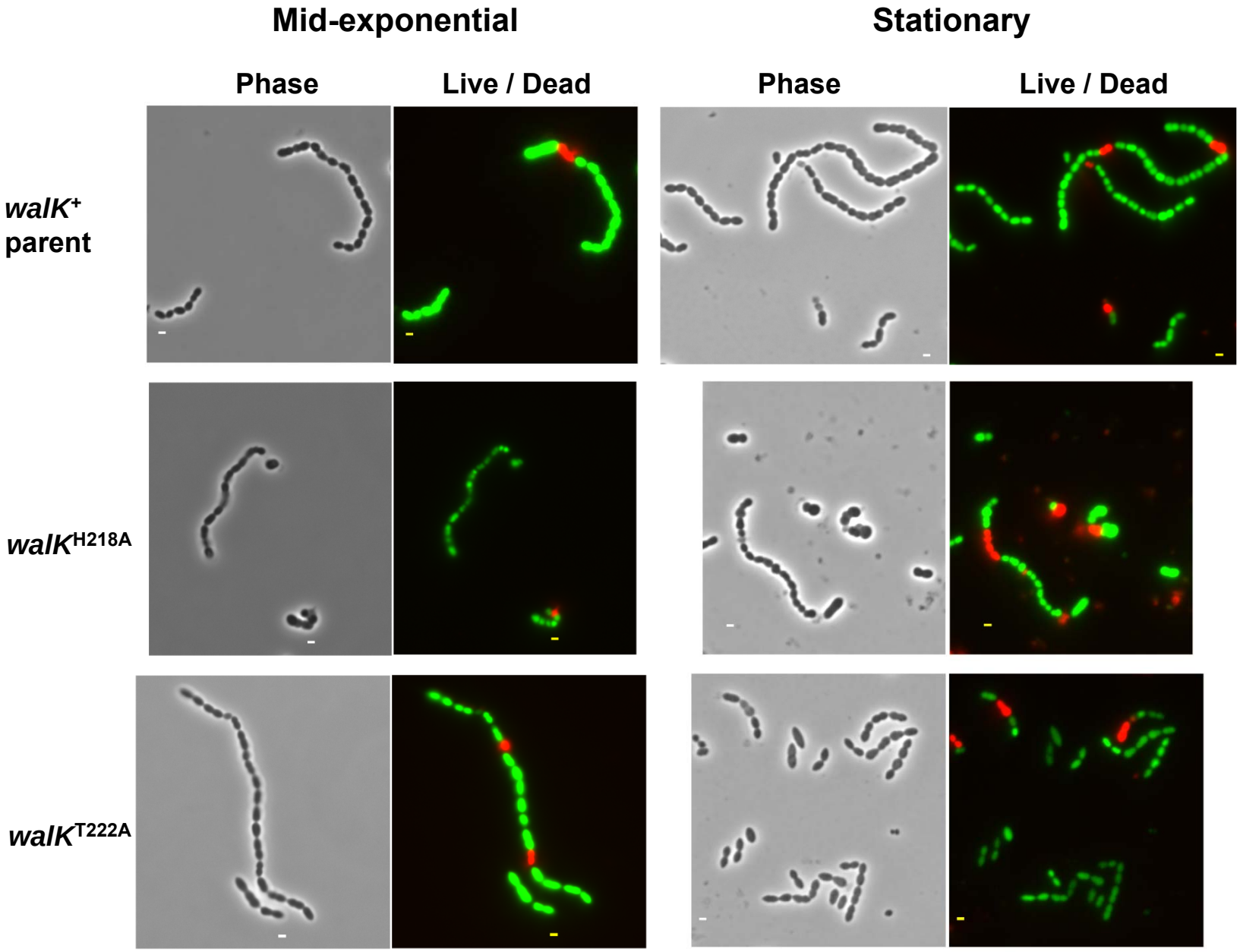

Mid-exponential
Stationary
Phase
Live / Dead
Phase
Live / Dead
walK+ parent
walKH218A
walKT222A

Fig. S9

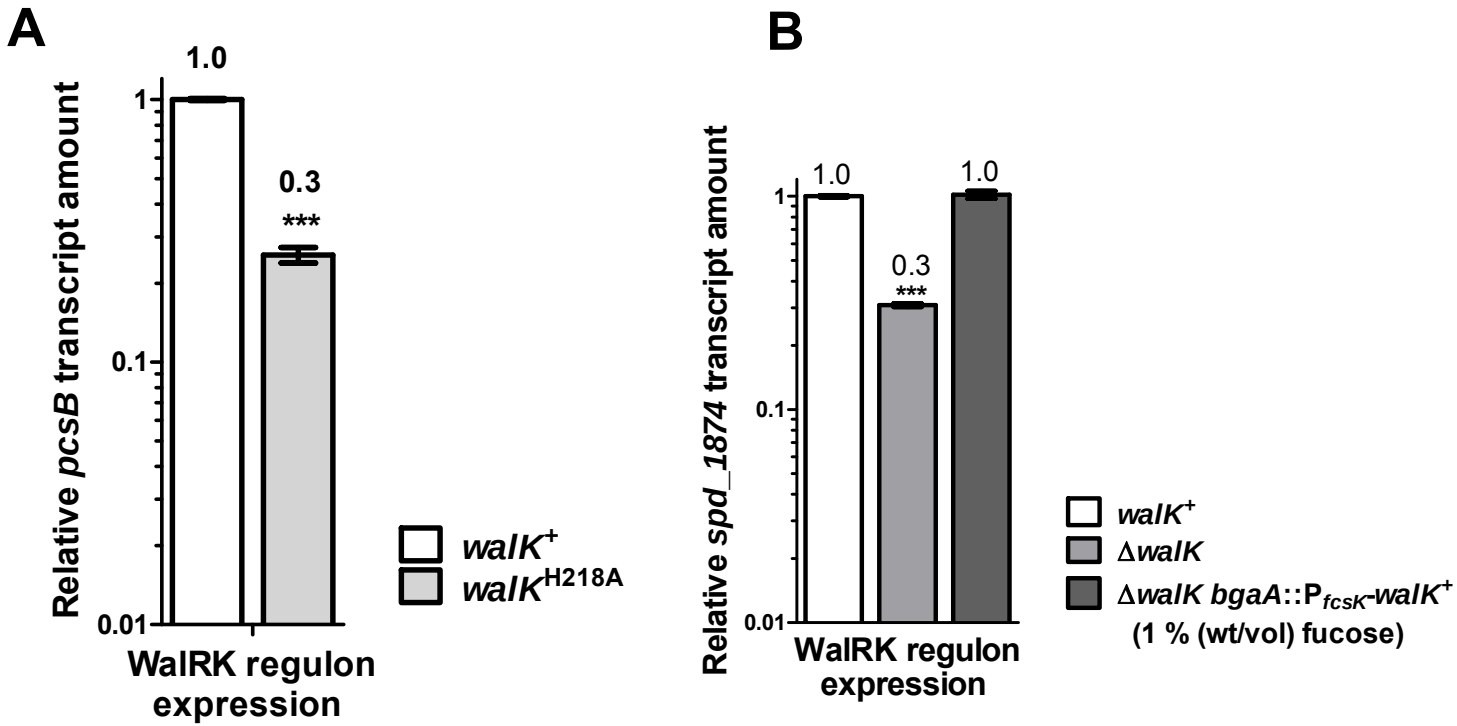

A
1.0
0.3
***
1
0.1
0.01
Relative pcsB transcript amount
WalRK regulon expression
walK+
walKH218A
B
1.0
0.3
***
1.0
1
0.1
0.01
Relative spd_1874 transcript amount
WalRK regulon expression
walK+
ΔwalK
ΔwalK bgaA::PfcsK-walK+
(1 % (wt/vol) fucose)

Fig. S10

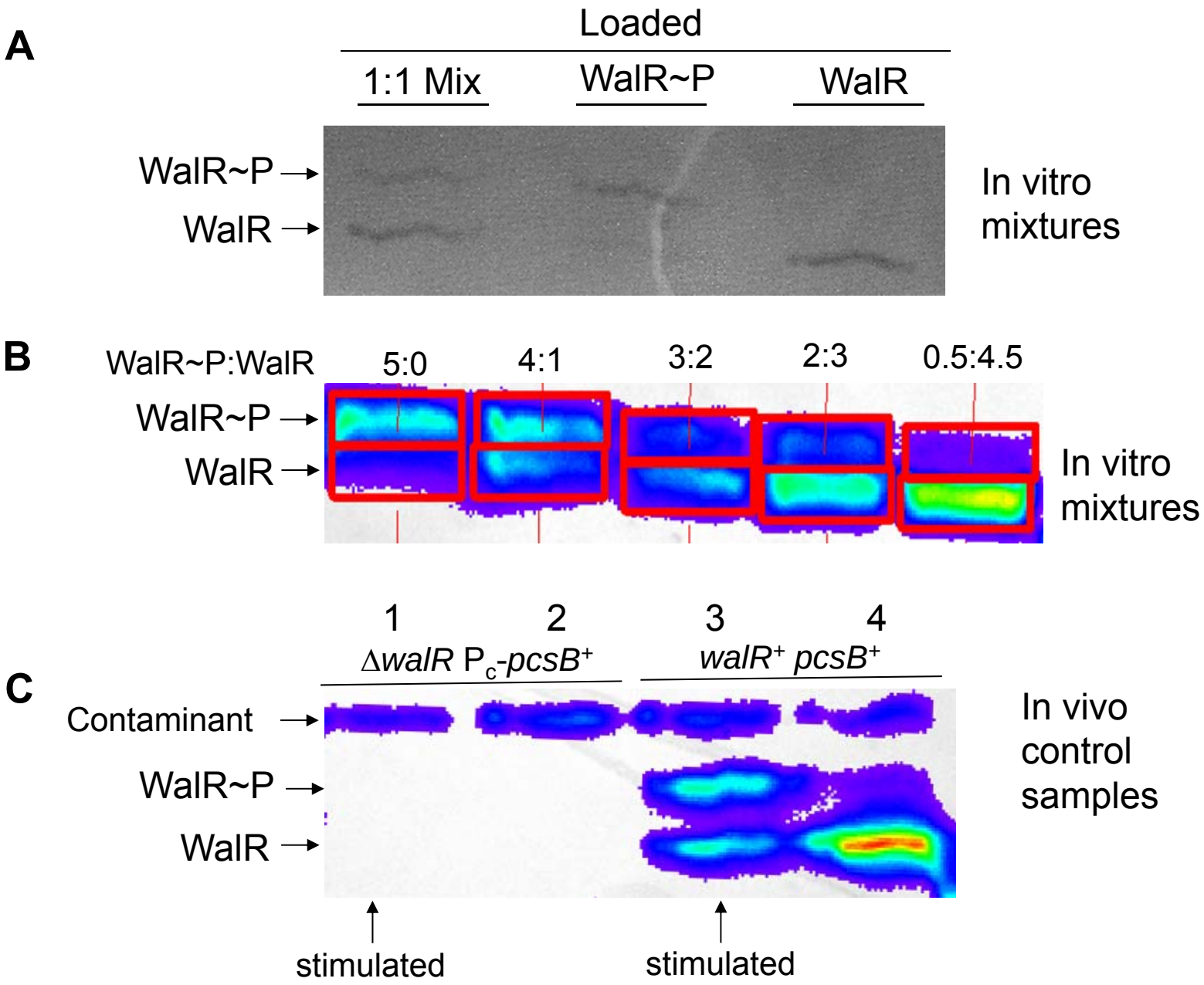
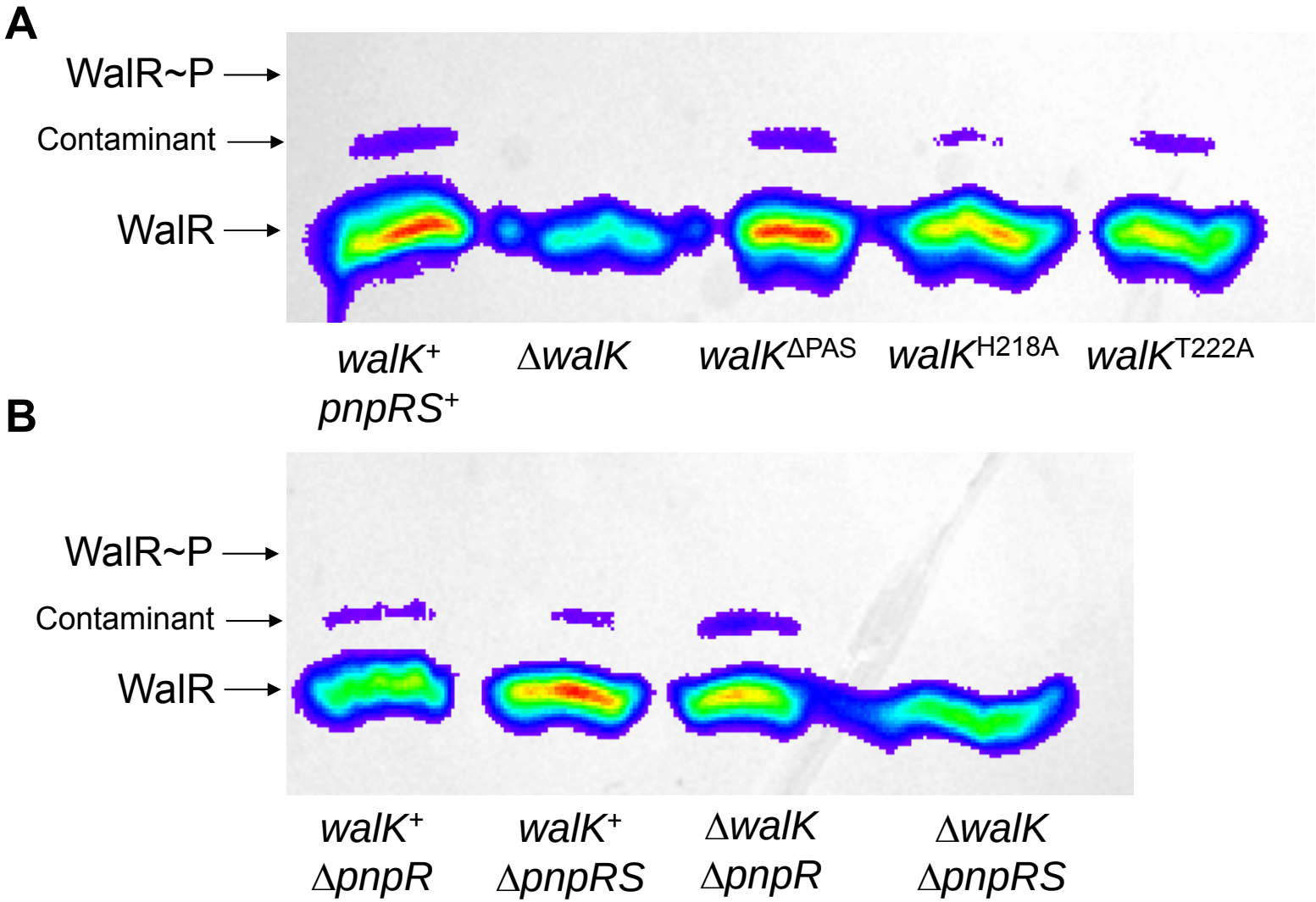

A
Loaded
1:1 Mix
WalR~P
WalR
WalR~P
WalR
In vitro mixtures
B
WalR~P:WalR
5:0
4:1
3:2
2:3
0.5:4.5
WalR~P
WalR
In vitro mixtures
C
1
2
3
4
ΔwalR Pc-pcsB+
walR+ pcsB+
Contaminant
WalR~P
WalR
In vivo control samples
stimulated
stimulated

Fig. S11



Heated controls corresponding to samples in Fig. 5; 75 μM Phos-tag SDS-PAGE

Fig. S12

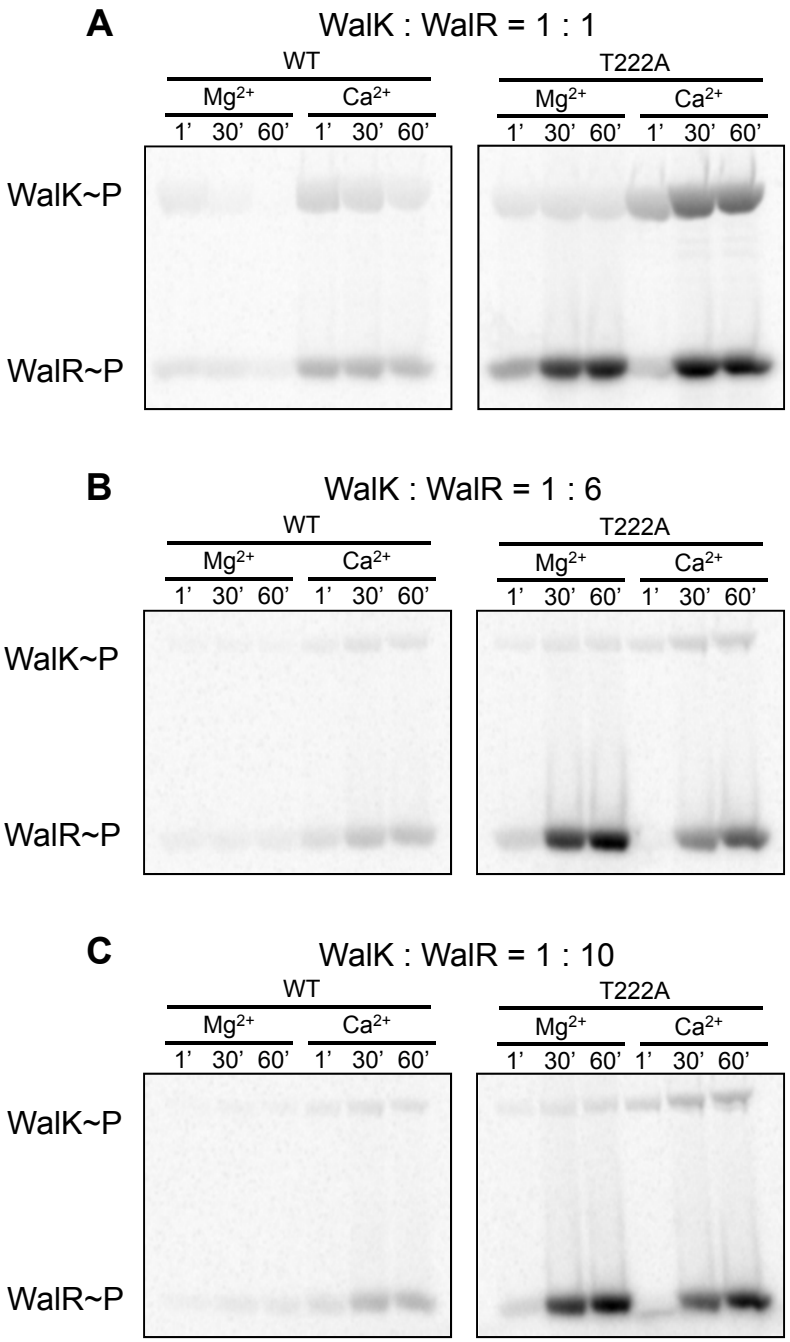

A
WalK : WalR = 1 : 1
WT
T222A
Mg2+
Ca2+
1’ 30’ 60’ 1’ 30’ 60’
WalK~P
WalR~P
B
WalK : WalR = 1 : 6
C
WalK : WalR = 1 : 10

Fig. S13

	-4				~P			3											14	~P transfer to WalR
HK02 WalK	S	N	V	S	H	E	L	R	T	P	L	T	S	V	K	S	Y	L	E	+++
HK04 PnpS	S	N	A	S	H	E	L	R	T	P	V	T	S	I	K	G	F	A	E	+/-
HK06	R	G	A	S	H	E	L	K	T	P	L	A	S	L	R	I	I	L	E	-
HK10 VncS	R	G	A	S	H	E	L	K	T	P	L	A	S	L	K	I	L	I	E	-
HK05 CiaH	E	N	A	S	H	E	L	R	T	P	L	A	V	L	Q	N	R	L	E	+/-
HK08	A	Q	L	S	H	D	I	K	T	P	I	T	S	I	Q	A	T	V	E	+/-

Amino acids that contact WalR	Same amino acid	Similar amino acid	Different amino acid

Fig. S14

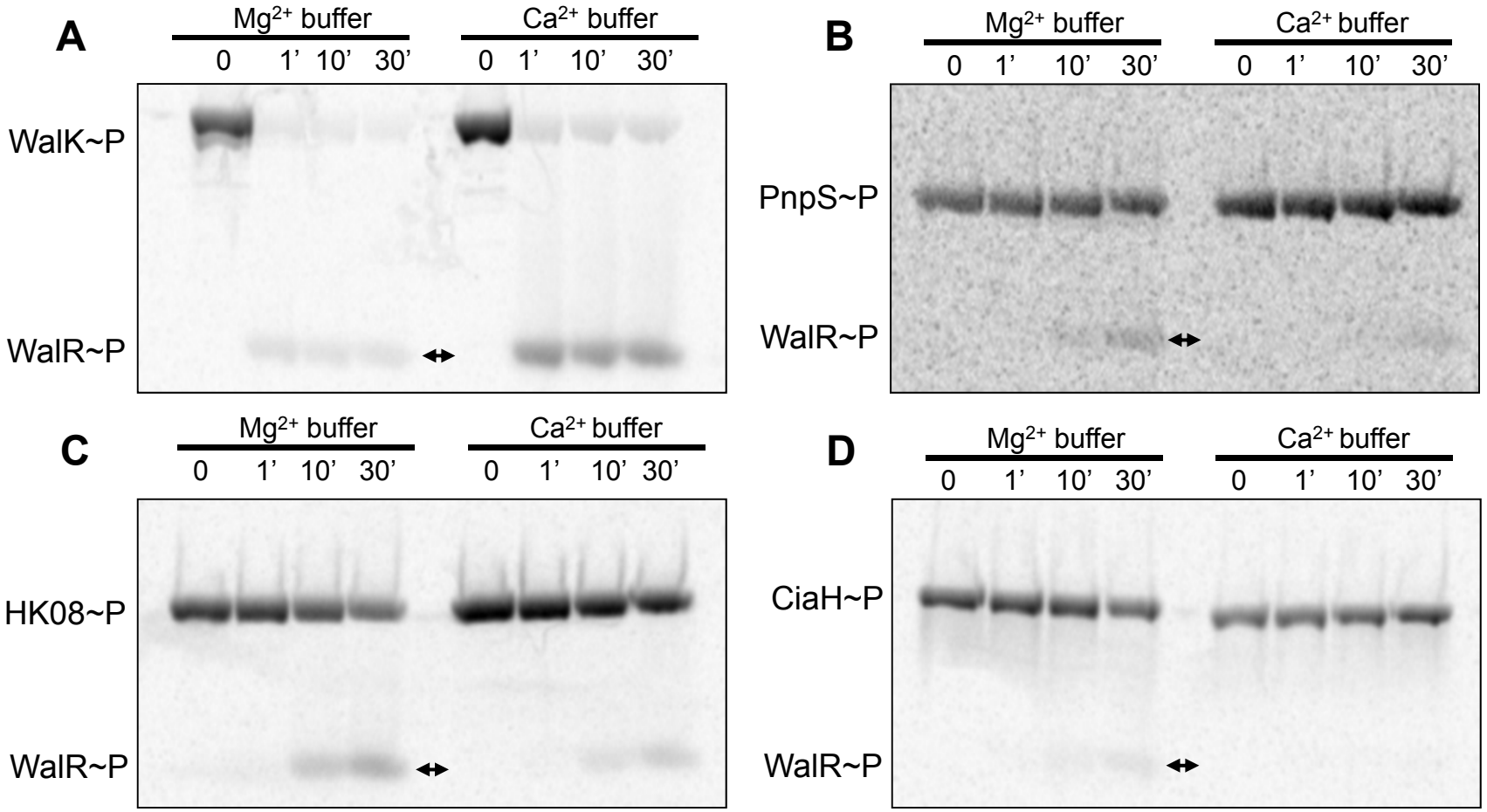

A
Mg2+ buffer
Ca2+ buffer
0 1' 10' 30'
0 1' 10' 30'
WalK~P
WalR~P
B
Mg2+ buffer
Ca2+ buffer
0 1' 10' 30'
0 1' 10' 30'
PnpS~P
WalR~P
C
Mg2+ buffer
Ca2+ buffer
0 1' 10' 30'
0 1' 10' 30'
HK08~P
WalR~P
D
Mg2+ buffer
Ca2+ buffer
0 1' 10' 30'
0 1' 10' 30'
CiaH~P
WalR~P

Fig. S15

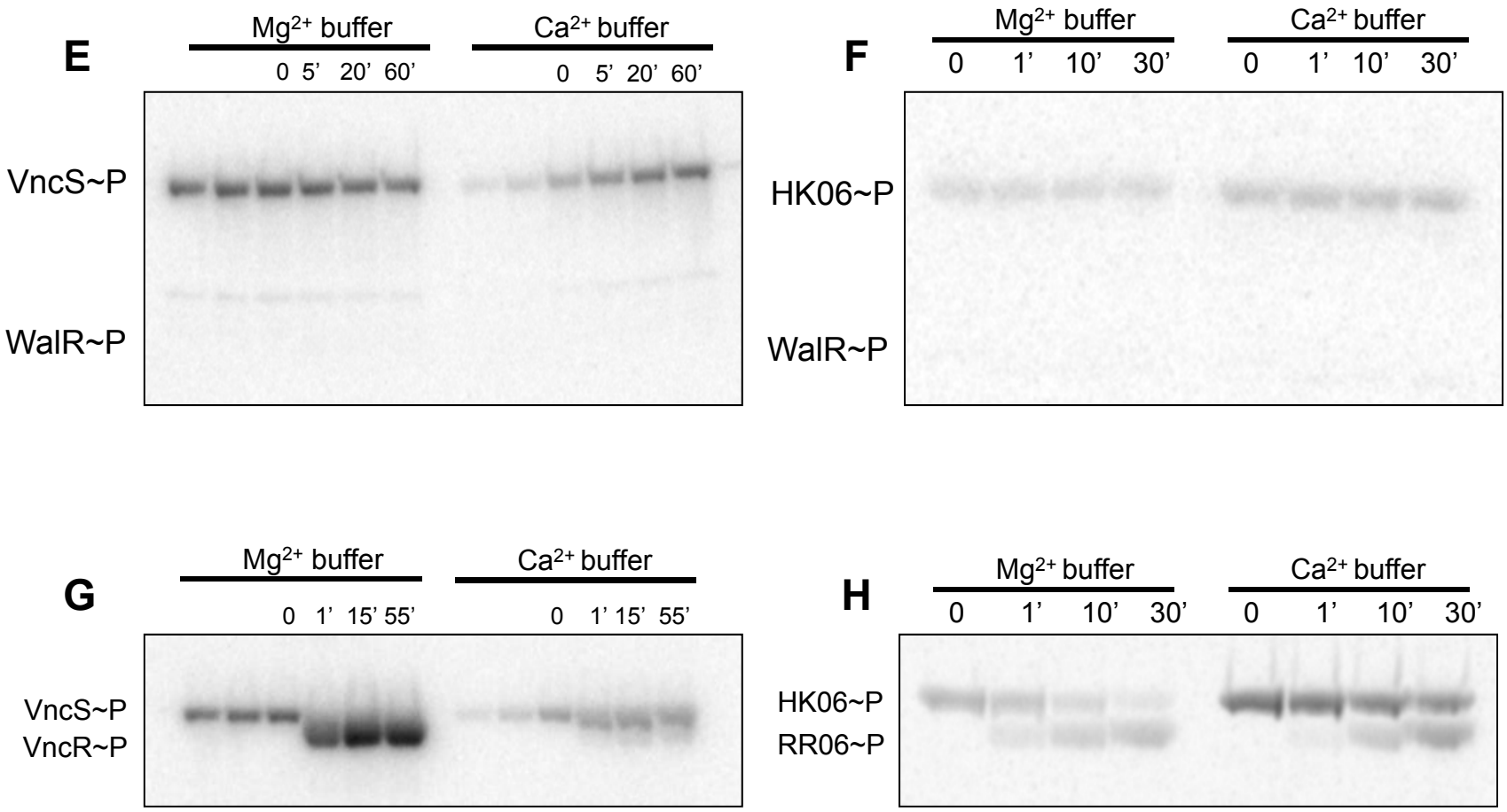
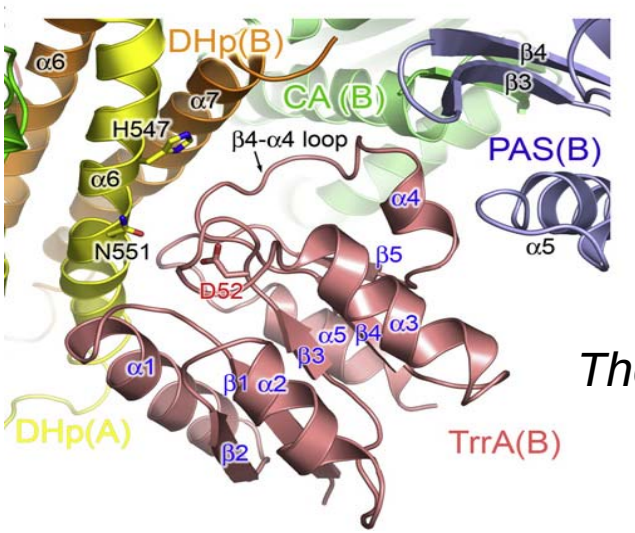


Fig. S15 (continued)

A



From Yamada, S., *et al.*, *Cell Structure* **17**:1333-1344, 2009.

Thermotoga maritima

B

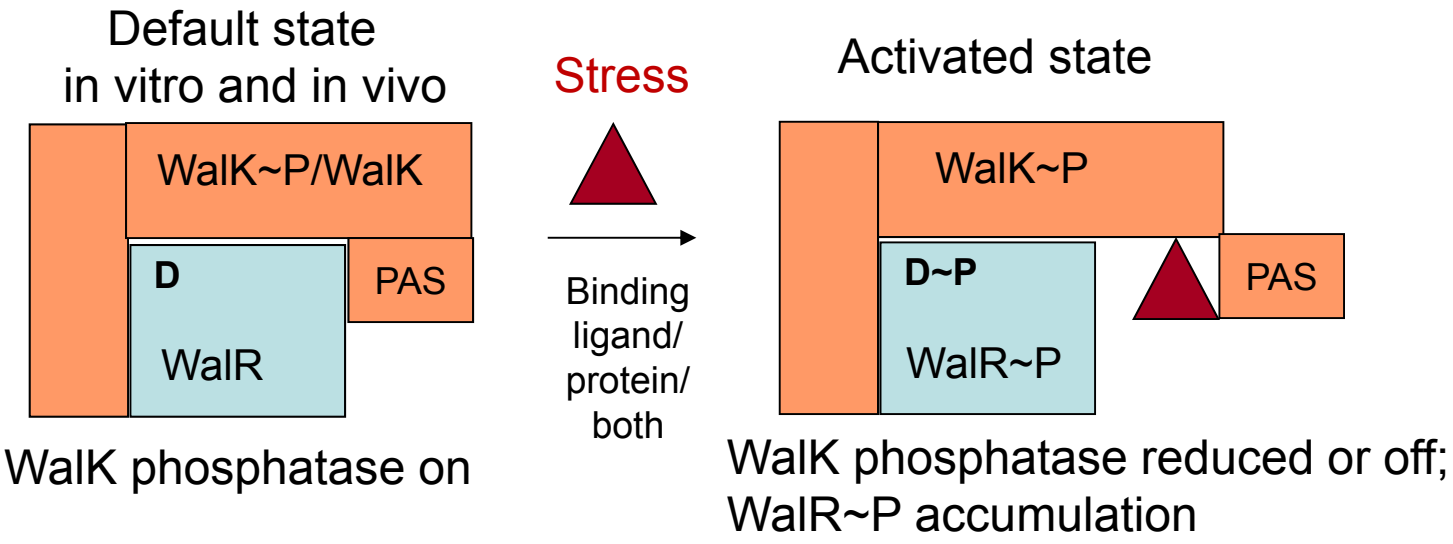


Fig. S16