SUPPLEMENTAL INFORMATION

Rhodobacterales use a unique L-threonine kinase for the assembly of the nucleotide loop of coenzyme B_{12}

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Running title: BluE, a unique L-Thr kinase found in *Rhodobacterales*

SUPPLEMENTAL TABLES

Table S1. Locus tag list. List of BluE and PduX homologues and their corresponding locus tags, phylum Class, order, genera, species and strain names used in the phylogenetic analysis.

BluE homologues

Locus Tag	Organism	Phylum	Class	Order
ACMU_15650	*Actibacterium mucosum* KCTC 23349	Proteobacteria	Alphaproteobacteria	Rhodobacterales
U879_17450	*Defluviimonas* sp. 20V17	Proteobacteria	Alphaproteobacteria	Rhodobacterales
IQ03DRAFT_04856	*Gemmobacter caeni* CGMCC 1.7745	Proteobacteria	Alphaproteobacteria	Rhodobacterales
Ga0057541_01298	*Maribius* sp. MOLA 401	Proteobacteria	Alphaproteobacteria	Rhodobacterales
B161DRAFT_02277	*Meganema perideroedes* DSM 15528	Proteobacteria	Alphaproteobacteria	Rhizobiales
U745DRAFT_3163	*Oceanicola* sp. MCTG156(1a)	Proteobacteria	Alphaproteobacteria	Rhodobacterales
RCAP_rcc02055	*Rhodobacter capsulatus* SB1003	Proteobacteria	Alphaproteobacteria	Rhodobacterales
RSP_0788	*Rhodobacter sphaeroides* 2.4.1	Proteobacteria	Alphaproteobacteria	Rhodobacterales
OCH239_08945	*Roseivivax halodurans* JCM 10272	Proteobacteria	Alphaproteobacteria	Rhodobacterales
RGAI101_1337	*Roseobacter* sp. GAI101	Proteobacteria	Alphaproteobacteria	Rhodobacterales
SSE37_12289	*Sagittula stellata* E-37	Proteobacteria	Alphaproteobacteria	Rhodobacterales
ATO10_12729	*Shimia* sp. 22II-S11-Z10	Proteobacteria	Alphaproteobacteria	Rhodobacterales
PM01_02495	*Sulfitobacter pontiacus* 3SOLIMAR09	Proteobacteria	Alphaproteobacteria	Rhodobacterales
TP2_17155	*Thioclava pacifica* DSM 10166	Proteobacteria	Alphaproteobacteria	Rhodobacterales

PduX homologues

Locus Tag	Organism	Phylum	Class	Order
Acear_0846	*Acetohalobium arabaticum* Z-7288	Firmicutes	Clostridia	Halanaerobiales
Bresa_02388	*Brenneria salicis* Dye EX2, ATCC 15712	Proteobacteria	Gammaproteobacteria	Enterobacteriales
CFNIH1_21285	*Citrobacter freundii* CFNIH1	Proteobacteria	Gammaproteobacteria	Enterobacteriales
CDM120_03509	*Clostridium difficile* M120	Firmicutes	Clostridia	Clostridiales
DSY4058	*Desulfitobacterium hafniense* Y51	Firmicutes	Clostridia	Clostridiales
Desca_0591	*Desulfotomaculum carboxydivorans*	Firmicutes	Clostridia	Clostridiales
ETAF_1814	*Edwardsiella tarda* FL6-60	Proteobacteria	Gammaproteobacteria	Enterobacteriales
Entcl_1739	*Enterobacter cloacae* SCF1	Proteobacteria	Gammaproteobacteria	Enterobacteriales
CrFGI571_1619	*Enterobacteriaceae bacterium* FGI 57	Proteobacteria	Gammaproteobacteria	Enterobacteriales
ELI_0756	*Eubacterium limosum* KIST612	Firmicutes	Clostridia	Clostridiales
Halha_1700	*Halobacteroides halobius* MD-1	Firmicutes	Clostridia	Halanaerobiales
HR38_27680	*Klebsiella oxytoca* M1	Proteobacteria	Gammaproteobacteria	Enterobacteriales

Ga0057525_01204	*Listeria monocytogenes* Lm60	Firmicutes	Bacilli	Bacillales
Mahau_0759	*Mahella australiensis* 50-1 BON, DSM 15567	Firmicutes	Clostridia	Thermoanaerobacterales
Moth_1103	*Moorella thermoacetica* ATCC 39073	Firmicutes	Clostridia	Thermoanaerobacterales
MU9_1018	*Morganella morganii* morganii KT	Proteobacteria	Gammaproteobacteria	Enterobacteriales
plu2966	*Photorhabdus luminescens laumondii* TTO1	Proteobacteria	Gammaproteobacteria	Enterobacteriales
STM2058	*Salmonella enterica* sv. Typhimurium LT2	Proteobacteria	Gammaproteobacteria	Enterobacteriales
EBL_c14750	*Shimwellia blattae* DSM 4481	Proteobacteria	Gammaproteobacteria	Enterobacteriales
TEPIRE1_11390	*Tepidanaerobacter acetatoxydans* Re1	Firmicutes	Clostridia	Thermoanaerobacterales
Tph_c05150	*Thermacetogenium phaeum* PB, DSM 12270	Firmicutes	Clostridia	Thermoanaerobacterales
TherJR_1311	*Thermincola potens JR*	Firmicutes	Clostridia	Clostridiales
Tlie_0163	*Thermovirga lienii* Cas60314, DSM 17291	Synergistetes	Synergistia	Synergistales
Tola_1706	*Tolumonas auensis* TA 4, DSM 9187	Proteobacteria	Gammaproteobacteria	Aeromonadales
Vpar_0909	*Veillonella parvula* Te3, DSM 2008	Firmicutes	Negativicutes	Selenomonadales
XNC1_1145	*Xenorhabdus nematophila* ATCC 19061	Proteobacteria	Gammaproteobacteria	Enterobacteriales
Ga0058816_01570	*Yersinia kristensenii* 33639	Proteobacteria	Gammaproteobacteria	Enterobacteriales

Table S2. Strains and plasmids used in this study. *S. enterica* strains are derivatives of subsp. *enterica* serovar Typhimurium strain LT2. Strains and plasmids were constructed during the course of this work unless stated otherwise.

Strain/Plasmid	Relevant genotype	Reference Source
Salmonella enterica		
JE7088	Δ*metE2702 ara-9*	Laboratory collection
Derivatives of JE7088		
JE11685	/ pBAD24 *bla*$^+$	
JE11686	/ pBAD30 *bla*$^+$	
JE12914	/ pPDU15 *bla*$^+$	
JE12656	Δ*pduX516*	
JE12686	Δ*pduX516* / pPDU15 *bla*$^+$	
JE13063	Δ*pduX516* / pBAD30 *bla*$^+$	
JE15873	Δ*pduX516* / pRcBLUE3*bla*$^+$	
JE16285	Δ*pduX516* / pRsBLUE3 *bla*$^+$	
JE21278	Δ*pduX516* / pRsBLUE7 *bla*$^+$	
JE21979	Δ*pduX516* / pBAD24 *bla*$^+$	
JE12941	Δ*cobD1371*	
JE14935	Δ*cobD1371* / pBAD24 *bla*$^+$	

Escherichia coli		
BL21 (λDE3)	F$^-$ / *ompT, hsdS$_B$, (rB$^-$, mB$^-$), dcm, gal,* λ (DE3)	
C41 (λDE3)	F$^-$ / *ompT, hsdS$_B$, (rB$^-$ mB$^-$) dcm, gal* λ (DE3); BL21 derivative optimized for protein overexpression, including at least one uncharacterized mutation	(Miroux & Walker, 1996)
C43 (λDE3)	F$^-$ / *ompT gal hsdS$_B$ (rB$^-$mB$^-$) [dcm] [lon]*C41 derivative, including at least two uncharacterized mutations	(Miroux & Walker, 1996)
BL21 (λDE3) RIL	RIL Codon Plus plasmid (RIL) *argU,*$^+$ *ileY*$^+$, *leuW*$^+$ tRNAs *cat*$^+$	(Miroux & Walker, 1996)
BL21/pLysSRARE2	*lysS*$^+$ *cat*$^+$ *argU*$^+$, *argW*$^+$, *ileX*$^+$, *glyT*$^+$, *leuW*$^+$, *proL*$^+$, *metT*$^+$, *thrT*$^+$, *tyrU*$^+$, *thrU*$^+$ tRNAs	Strategene
Rosetta2(DE3)/pLysS	F$^-$ / *ompT hsdS8 (rB$^-$ mB$^-$) gal dcm lacY1 / lysS*$^+$ *cat*$^+$	
Lemo21 (λDE3)	*fhuA2 [lon] ompT gal* λ (DE3) *[dcm]* Δ*hsdS* Δ*lacI*::P*lacUV5*::T7 i21 Δ*nin5* (*cat*$^+$) pLemo = pACYC184 plasmid - P*rhaBAD-lysY*$^+$, *cat*$^+$	Novagen NEB
DH5α	F$^-$ / *endA1 hsdR17(rk$^-$, mk$^+$) glnV44 thi-1 recA1 gyrA96* (Nx^R) *relA1 U169 deoR (Φ80-*d*lacZ* M15 Δ*(lacZYA-argF) phoAsupE44 relA1*	(Woodcock *et al.*, 1989)
***Rhodobacter sphaeroides* 2.4.1**		
JE8777	*cobB*$^+$ *bluE*$^+$	T. Donohue
JE24356	*cobB*$^+$ *bluE*$^+$/ pBBR1MCS-2	
JE24357	Δ*cobB* / pBBR1MCS-2	
JE24358	Δ*bluE* / pBBR1MCS-2	
JE24359	Δ*cobB* Δ*bluE* / pBBR1MCS-2	
JE24360	Δ*bluE* / p*Rs*BLUE10	
JE24361	Δ*cobB* Δ*bluE* / p*Rs*BLUE10	
Plasmids		
pPDU15	*S. enterica pduX*$^+$ in pBAD30 *bla*$^+$	
pRcBLUE3	*R. capsulatus bluE*$^+$ in pBAD24 *bla*$^+$	
pRsBLUE3	*R. sphaeroides bluE*$^+$ in pBAD24 *bla*$^+$	
pPDU23	*S. enterica pduX*$^+$ in pTEV5 *bla*$^+$	
pRsBLUE4	*R. sphaeroides bluE*$^+$ in pTEV5 *bla*$^+$	
pRsBLUE7	*R. sphaeroides bluE06* (encodes *Rs*CobD^{G99A}) in pTEV5 *bla*$^+$	
pRsBLUE9	*bluE* deletion plasmid for *R. sphaeroides*	
pRsBLUE10	*R. sphaeroides bluE* in pBBR1MCS-2	
pTA925-PduX-His6	*S. enterica pduX*$^+$ in pTA925 *kan*$^+$	(Fan & Bobik, 2008), T. Bobik
pTA925-His8-PduX	*S. enterica pduX*$^+$ in pTA925 *kan*$^+$	(Fan *et al.*, 2009), T. Bobik
pBAD24	Cloning / complementation vector *bla*$^+$	(Guzman *et al.*, 1995)
pBAD30	Cloning / complementation vector *bla*$^+$	(Guzman *et al.*, 1995)
pTEV5	Cloning / overexpression vector, *N*-terminal rTEV protease-cleavable His$_6$ tag *bla*$^+$	(Rocco *et al.*, 2008)
pK18mobsacB	Suicide vector for allelic exchange *kan*$^+$ *sacB*$^+$	(Schafer *et al.*, 1994)
pBBR1MCS-2	Cloning / complementation vector *kan*$^+$	(Kovach *et al.*, 1995)

pTA925	Cloning / overexpression vector *kan*$^+$	(Johnson *et al.*, 2001), T. Bobik

Table S3. Primer list. List of primers used in this study. Sequences in bold were the restriction enzyme cut site indicated in the primer name.

Name	Sequence
Deletion primers	
pduX_wan5'_b	ATGCGCGCACACTATTCGTACCTGAAAGGTGATAATGTGGTGTAGGCTGGAGCTGCTTC
pduX_wan3'_b	GCCAGTGACCATCTTGAGTAAATGTTGTTTTGGCCAGTGCATATGAATATCCTCCTTAG
RSP0788_delA_EcoRI	NNNNNN**GAATTC** TCGAGCTTGCGCTCAGG
RSP0788_delB	GCCAAGGCCAGCGCGAGCGGC
RSP0788_delC	CGCGCTGGCCTTGGCCCACGGT
RSP0788_delD_SacI	NNNNNN**TCTAGA**GCGTGCTCGCCATCGA
Cloning primers	
pduX_KpnI5'	TAGG**GGTACC**ATGCGCGCACACTATTCGTA
pduX_Hind3'	TAGG**AAGCTT**TCACTGCAGTTTGACCCCG
Rc_BluE_EcoRI5'	AGCT**GAATTC**TTTGGTCGATCGCGGGCGTGAA
Rc_BluE_XbaI3'	ATCG **TCTAGA**ATCGCCACCACCATCATC
RsBluE_Nhe5'	TGAA **GCTAGC**GGCACTGCAATGACGCTG
RsBluE_KpnI5'	TAG**GGTACC**ATGACGCTGGCGGCGGGCCTCA
RsBluE_HindIII3'	TAG**AAGCTT**TCAGGCTCCGCCGATGCGGAA
RSP0788_EcoRI5'	AGT**GAATTC**GGCACTGCAATGACGCTG
RSP0788_XbaI3'	ATC**TCTAGA**GATAGGGCACCCGGTTGAT
RSP0788_HindIII_5'	NNNNNN**AAGCTT**GGCACTGCAATGACGCTG
RSP0788_EcoRI_3'	NNNNNN**GAATTC**TCAGGCTCCGCCGATGC
Site directed mutagenesis	
RsBluE_G99A	GCCGGGCGGCGCAGCGGGCGCCTCGA
Sequencing primers	
pduX_flank5'	GCGTAATGCGACATTTATCCA
pduX_flank3'	TGAGGCGATTCAGGGTATCAT
cobD_flank5'	CGACATTGGCCTCGGTTT
cobD_flank3'	GAAACGCCCTGGCTTAAT
RSP0788_flank 5'	GGTCGAGCATCTGGCCAGCT
RSP0788_flank 3'	GATCCCCGTCGAGAGGTCGA

SUPPLEMENTAL FIGURES

Figure S1. Complementation of *S. enterica pduX* strain. Representative graphs of growth analyses of *S. enterica* cells grown aerobically at 37ºC in NCE minimal medium with A. glycerol (22 mM) and Cby (1 nM) or B. ethanolamine (90 mM), Cby (300 nM), DMB (0.15 mM), and supplemented with arabinose (0.5 mM), ampicillin (0.1 mg mL^{-1}), and $MgSO_4$ (1 mM). Experiments were replicated in three independent experiments, each performed in triplicate. Error bar represent the standard error of the mean. Figure key: Δ*pduX*/p*Rs*BluE (□), Δ*pduX*/p*Se*PduX (▲), Δ*pduX*/p*Rs*BluE^{G99A} (◆), Δ*pduX*/vector (○), *pduX*$^{+}$/vector (△).

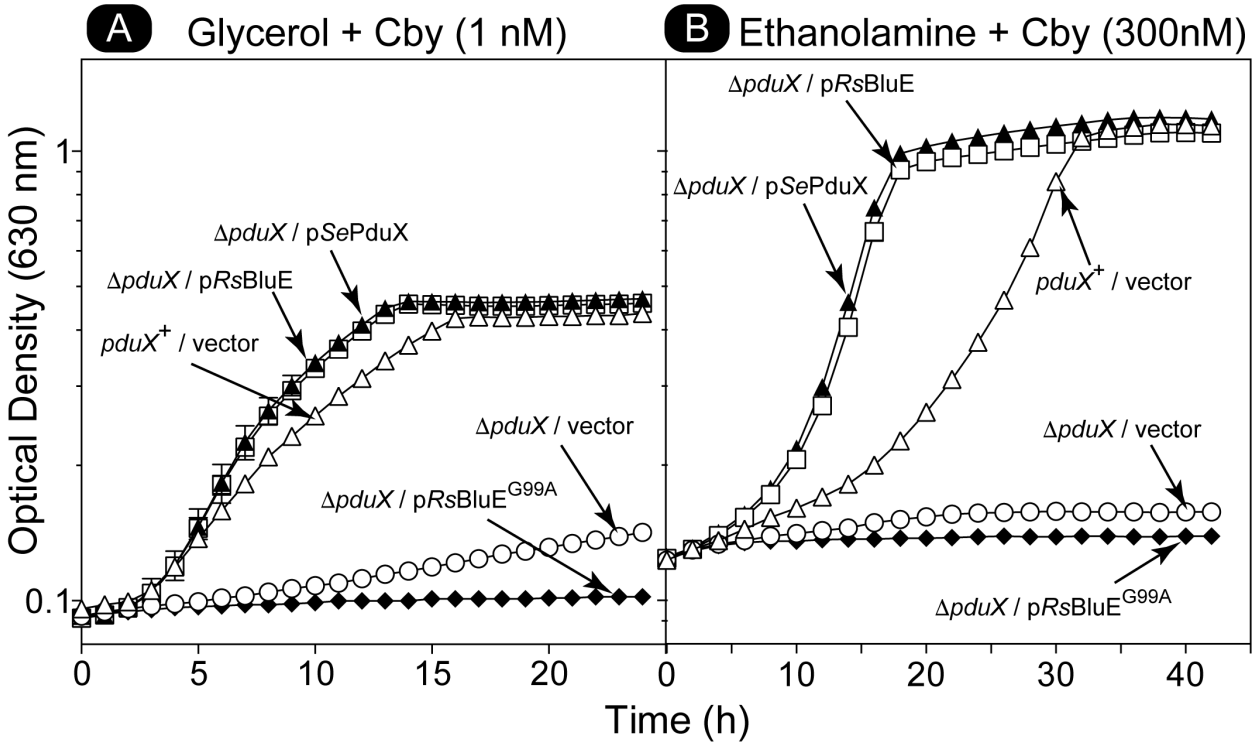


Figure S2. Pigmentation scan of *R. sphaeroides bluE*$^+$ and Δ*bluE* cells supplemented with or without cyanocobalamin (CNCbl). Cells were grown aerobically in Sistrom's Medium (5 mL) with succinate (10 mM) for 3 days at 30°C without the addition of CNCbl (panel A). The absence of BluE function resulted in a 'blush' phenotype (panel B) that could be complemented by ectopically expressing the *bluE*$^+$ allele squares, panel A), or by supplementing the medium with CNCbl (15 nM). Cultures (5 mL) were centrifuged at 6,000 x *g* for 5 min . Cells were re-suspended in 0.5 mL of Sistrom's medium and absorbance readings were taken at 10 nm increments. Sistrom's medium was used as a blank for absorbance readings. Differences in pigments were noted by observation of readings taken at 800 and 875 nm. Results were obtained in technical triplicate and error bars represent standard error of the mean (SEM). Pellets shown in panels B and C were were obtained using a microcentrifuge.

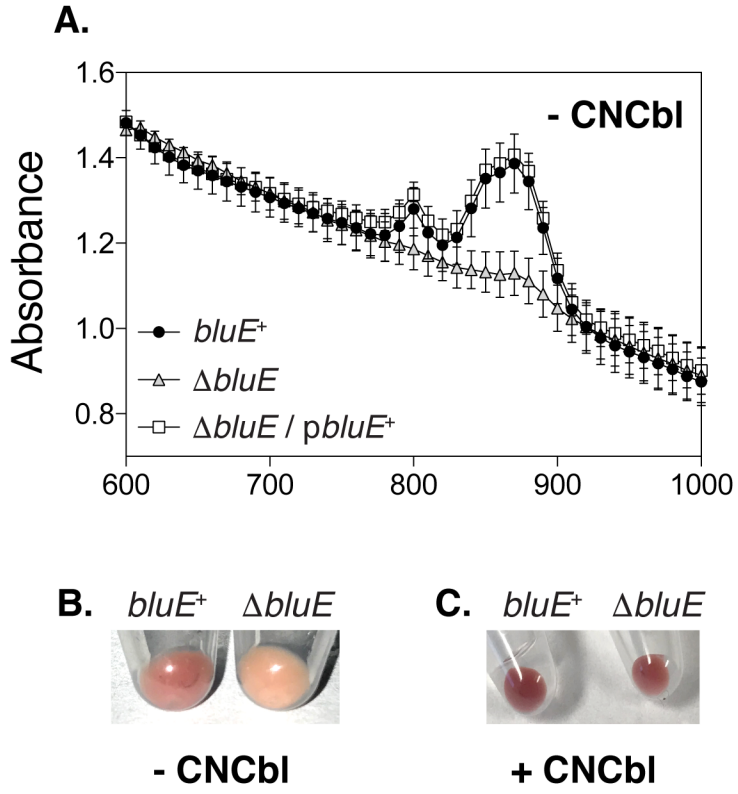


Figure S3. Standard curve for ATP to ADP conversion. Standard curve used to calculate the percent conversion of ADP to ATP using the ADP-Glo™ Kinase/ATPase Assay kit (Promega). Luminescence was measured at 560 nm. RLU; relative light units.

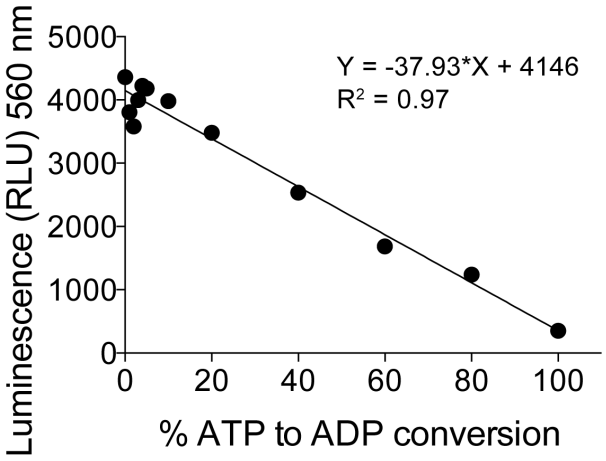


Figure S4. SDS-PAGE gel of partially purified detergent-free H_6-tagged proteins. SDS-PAGE gels showing partially purified proteins. Each gel shows a protein size maker in lane 1, followed by whole cell extracts in lane 2, and in lane 3 enriched fractions of partially purified His_6-tagged proteins eluted from a Ni-NTA resin with 200 mM imidazole. A. *Rs*BluE, B. *Se*PduX, and C. extracts from cells expressing the empty overexpression vector pTEV5. Dashed line indicates the location where the gel images were spliced together for simple and clear presentation. For whole cell extracts, protein concentration was calculated using a Bradford Assay and 20 μg of protein was loaded to each lane.

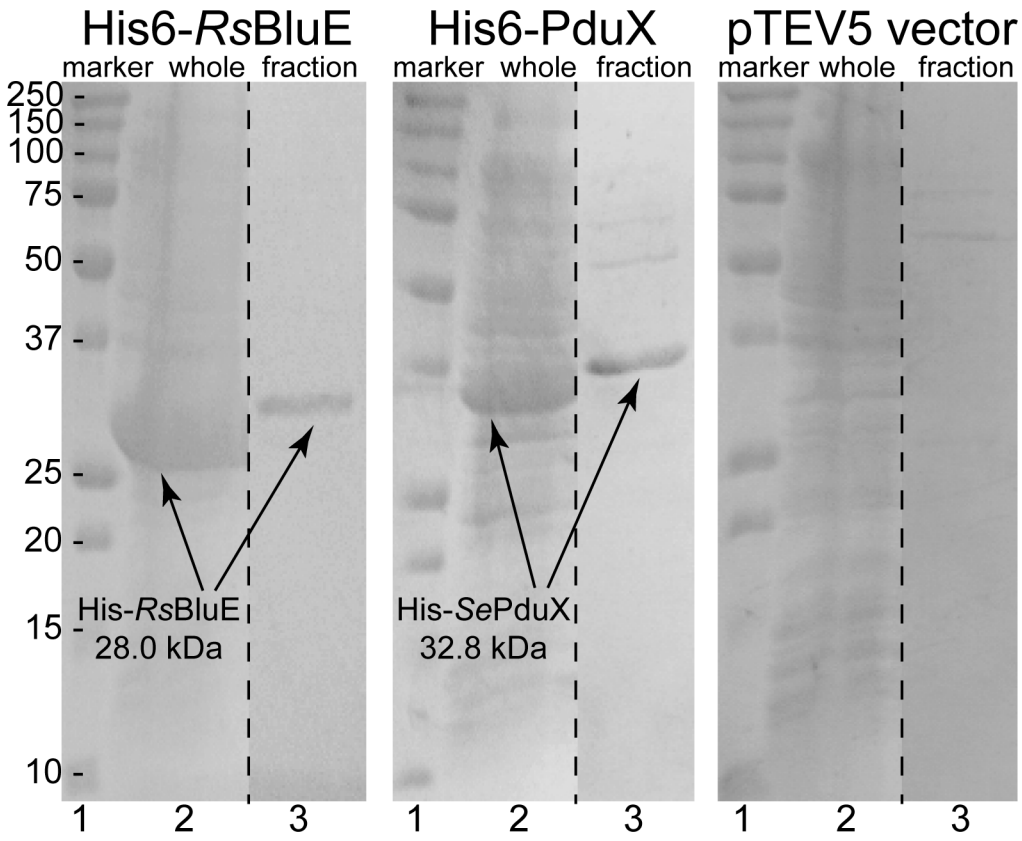


Figure S5. SDS-PAGE gel comparing overexpression and solubility of detergent free *N*- and *C*-terminal His-tagged *Se*PduX and *Rs*BluE proteins. SDS-page gel showing the soluble and insoluble fractions of previously published plasmids (Fan *et al.*, 2009, Fan & Bobik, 2008) encoding *Se*PduX with a non-cleavable *C*-terminal His_6-tag (pTA925-PduX-His6 (Fan & Bobik, 2008), lanes 2, 3) and a non-cleavable *N*-terminal His_8-tag (pTA925-His8-PduX (Fan *et al.*, 2009), lanes 4, 5), and plasmids generated during the course of this work encoding rTEV protease-cleavable *N*-terminal His_6-tagged *Se*PduX (pPDU23, lane 6, 7), and *Rs*BluE (pRsBLUE4, lanes 8, 9), and protein expression levels when these inducible plasmids are not induced with IPTG (lanes 10). Proteins were purified as described in *Materials and Methods*. Lane 1 shows the protein size marker. The theoretical molecular weights for *Rs*BluE and *Se*PduX without the His-tags or rTEV cleavage sites are shown in Kilodaltons (kDa). Arrows indicate the location of the overexpressed proteins of interest. For whole cell extracts, protein concentration was calculated using a Bradford Assay and 20 μg of protein was loaded to each lane.

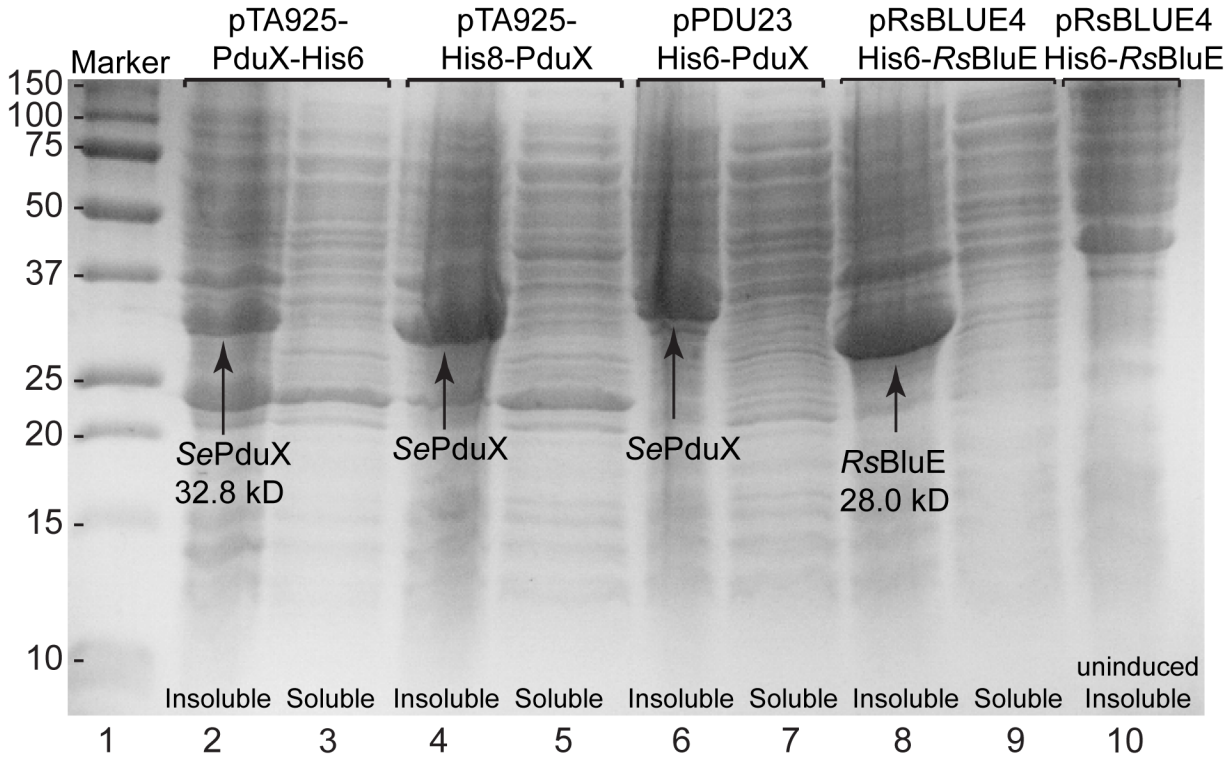


Figure S6. ^{31}P-NMR spectra of *Rs*BluE reaction containing L-Ser and or ATP. Representative ^{31}P-NMR spectra of duplicate experiments used to detect the α, β, or γ phosphates of ATP or ADP, pyrophosphate (PPi), orthophosphate (Pi), and the phosphate group of L-Ser-P. Reaction mixtures containing $MgCl_2$ (1 mM), ATP (3 mM), L-Ser (6 mM), and 10 µL of detergent-free protein (11 µM), eluted directly from the Ni purification column, were incubated at 25°C for 1 h A. L-Ser-P (3 mM) and ATP (3 mM) standards. B. Reaction mixture containing ATP and *Rs*BluE. C. Reaction mixture containing ATP, L-Ser, and *Rs*BluE.

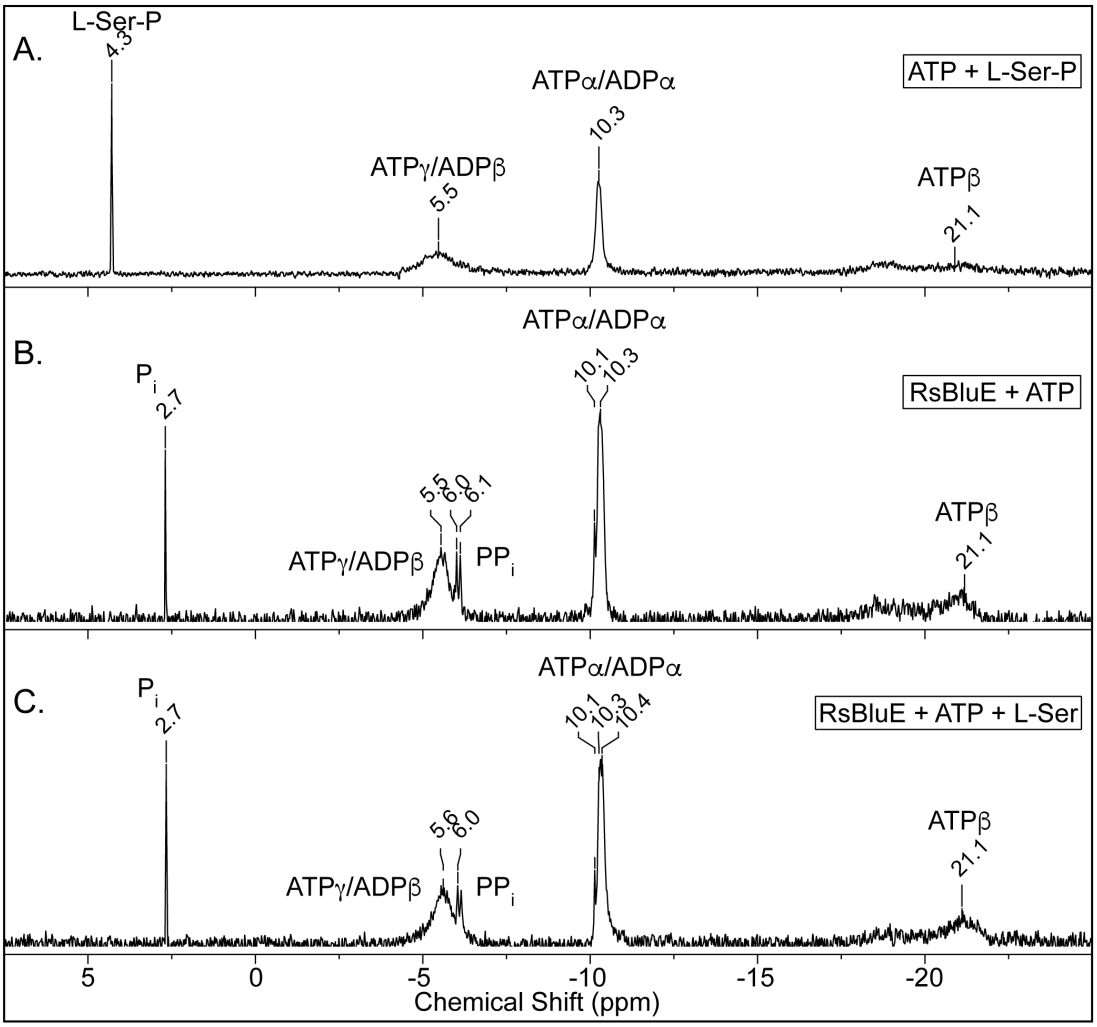


Figure S7. ATPase activity as a function of substrate. *R. sphaeroides* BluE (*Rs*BluE) specific activity as a function of ATP, L-Thr, or L-Ser concentration (panels A, C, E, respectively). Panels B, D, and F show specific activities of *S. enterica* PduX (*Se*PduX) as a function of ATP, L-Thr, or L-Ser concentration, respectively. Specific activity is reported in µmol of ATP per min per mg of protein with the mean standard error of triplicate reactions represented by the error bars. Activity was measured by the NADH-consuming assay described in *Material and Methods*. Assays were performed with purified, sarkosyl-solubilized *Rs*BluE and *Se*PduX (3 µM) enzymes. For ATPase specific activity L-Thr concentration was held at 50 mM while the ATP concentration was varied (5 - 100 mM). To measure the effect of L-Thr and L-Ser on ATPase activity, the ATP concentration was held at 50 mM while the concentration L-Thr or L-Ser was varied (0 - 100 mM). Independent experiments were performed in duplicate. Error bars represent the standard deviation of triplicate reactions.

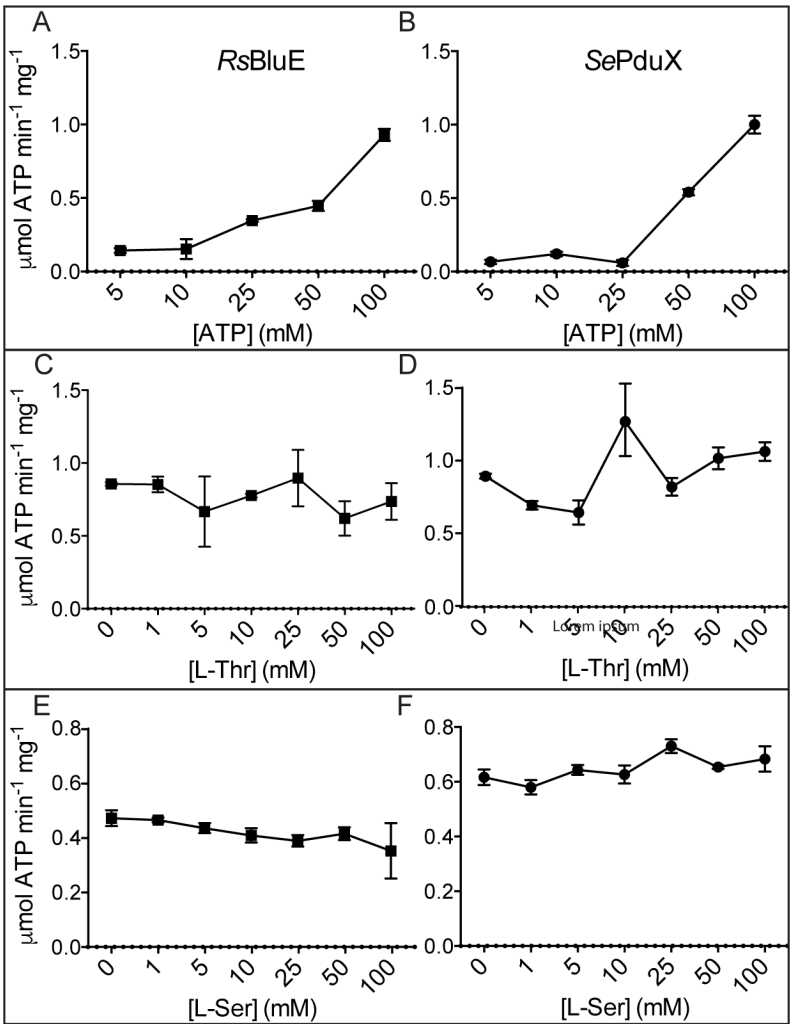
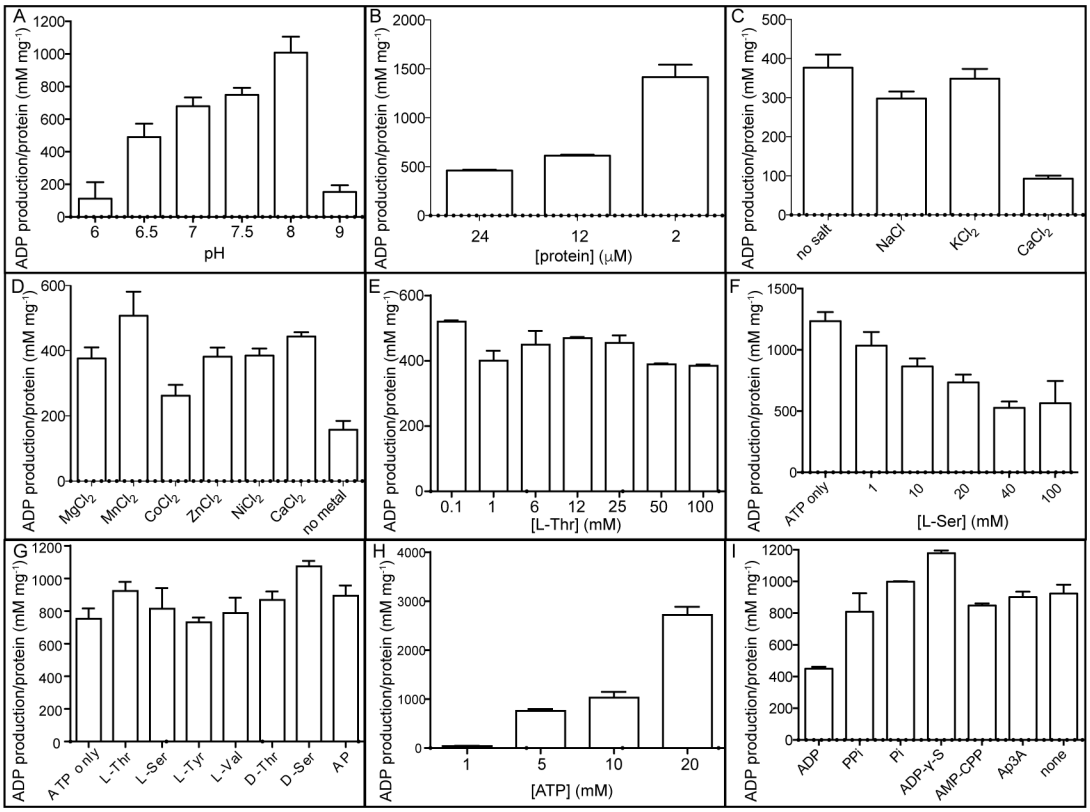


Figure S8. Optimization of *Rs*BluE reaction conditions. ATPase activity assay measured with ADP-Glo™ Kinase/ATPase Assay kit, expressed as mM of ATP per mg of protein, with the standard error of the mean of triplicate reactions represented by the error bars. Unless otherwise indicated, each reaction mixture contained HEPES buffer (50 mM, pH 7.0 at 25°C), $MgCl_2$ (1 mM), ATP (0.1 mM), L-Thr (10 mM), and Sarkosyl-solubilized protein (12 µM) incubated at 25°C for 1 h. A. *Rs*BluE activity as a function of pH. B. *Rs*BluE activity as a function of protein concentration (2 - 24 µM). C. *Rs*BluE activity in the presence of added salts (100 mM). D. *Rs*BluE activity as a function of added divalent metals (1 mM). E. *Rs*BluE activity as a function of L-Thr concentration (0.1 - 100 mM). F. *Rs*BluE activity as a function of L-Ser concentration (0 - 100 mM). G. *Rs*BluE activity as a function of added L- and D- amino acids (10 mM). H. *Rs*BluE activity as a function of ATP concentration (1 - 20 mM) with L-Thr (50 mM). I. *Rs*BluE activity in the presence of ATPase/kinase inhibitors ADP (20 mM), sodium pyrophosphate (PPi, 10 mM), sodium *ortho*-phosphate (Pi, 10 mM), adenosine 5′-[γ-thio]triphosphate (ADP-γ-S, 0.2 mM), α,β-methyleneadenosine 5'-triphosphate (AMP-CPP, 10 mM), and P1,P3-di(adenosine-5') triphosphate ammonium salt (Ap3A, 10 mM).



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