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Supplemental Information

A Conserved Proline Triplet in Val-tRNA Synthetase and the Origin of Elongation Factor P

Agata L. Starosta, Jürgen Lassak, Lauri Peil, Gemma C. Atkinson, Christopher J. Woolstenhulme, Kai Virumäe, Allen Buskirk, Tanel Tenson, Jaanus Remme, Kirsten Jung, and Daniel N. Wilson

Supplemental Figures

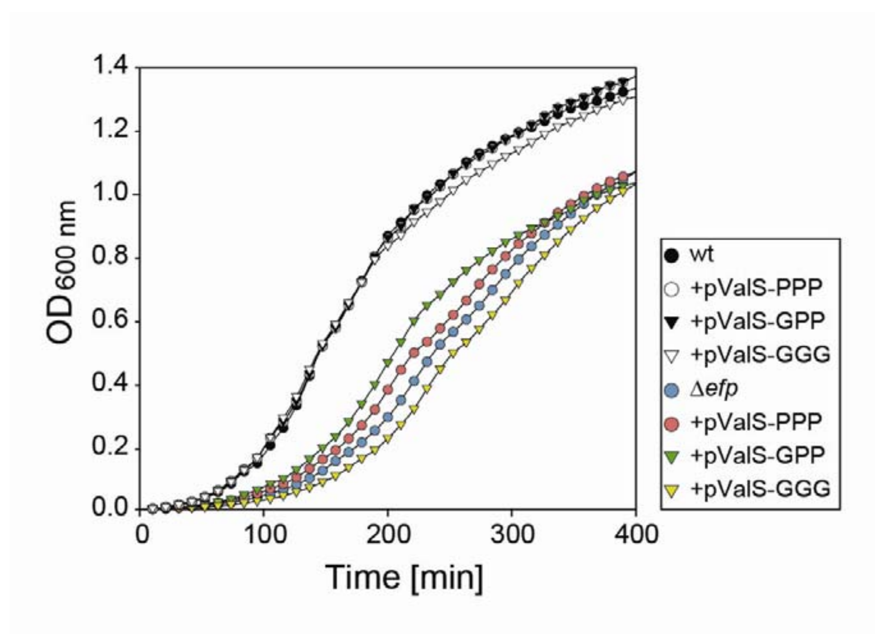

Figure S1, Related to Figure 1, Expression of ValS can improve growth of *E. coli* strains lacking EF-P. Growth curves (OD_{600nm}) of *E. coli* strain BW25113 without plasmid (wt) or containing plasmids pValS-PPP, pValS-GPP or pValS-GGG, compared with *E. coli* strain BW25113 lacking *efp* (JW4106,(Baba et al., 2006)) without plasmid (Δ*efp*) or containing plasmids pValS-PPP, pValS-GPP or pValS-GGG. As expected, overexpression of the inactive ValS-GGG mutant did improve the growth of the Δ*efp* strain, but rather impaired growth further. In contrast, overexpression of the wildtype ValS and the ValS-GPP slightly improved the growth of the Δ*efp* strain, but not to the levels of the wildtype strain. The more efficient rescue by ValS-GPP compared to wildtype ValS may result because ValS contains PPP and therefore requires EF-P for expression, whereas the ValS mutant, although less active than wildtype ValS, contains GPP conferring less dependence on EF-P for expression (Peil et al., 2013). The lack of complete rescue by ValS overexpression is expected given that many *E. coli* proteins, both essential and non-essential, are dependent on EF-P for expression and their loss leads to growth defects in *E. coli* (Baba et al., 2006).

Figure S2, Related to Figure 2F-G, Monitoring aminoadenylate formation by ValS and mutants. (A-E) Autoradiograph of thin-layer chromatographic separation of (**A-B**) $\gamma[^{32}P]$-P_i from $\gamma[^{32}P]$-ATP, and (**C-D**) $\alpha[^{32}P]$-AMP and $\alpha[^{32}P]$-ADP from $\alpha[^{32}P]$-ATP, when wildtype ValS (wt), or ValS mutants GPP, PGP, PPG or GGG were incubated with (**A,B,D**) valine or (**C**) no amino acid or (**E**) isoleucine, in the absence (-) and presence (+) of deacylated $tRNA^{Val}$. The origin where the samples were loaded onto the TLC plate correlates with the position of ATP. In (**B**), reactions were treated with pyrophosphatase (PPi-ase) or alkaline phosphatase (AP) as indicated. In (**D**), the migration of AMP and ADP was determined by treatment of $\alpha[^{32}P]$-ATP with increasing concentrations of Apyrase.

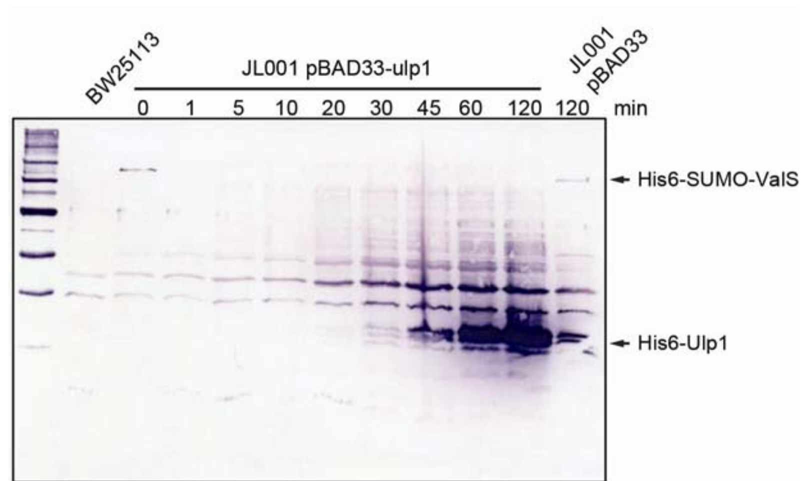

Figure S3, Related to Figure 4B-H, Detection of ValS and Ulp1 by Western blotting. In parallel Ulp1 production and ValS degradation was monitored by Western Blot analysis against the 6x His tags. Cells of the JL001 strain harboring pBAD33-*ulp*1 and pBBR1MCS-5-P_{T7}-SUMO-WFCWS-*valS* were harvested 0min, 1min, 5min, 10min, 20min, 30min, 45min, 60min and 120min after arabinose induction. As control JL001 cells harboring pBAD33 and pBBR1MCS-5-P_{T7}-SUMO-WFCWS-*valS* were analyzed.

Supplemental Table

Table S1 Strains, plasmids and oligonucleotides

Strains and plasmids	Relevant genotype or description	Source or reference
***Escherichia coli* strains**		
DH5αλpir	*recA1 gyrA* (*lacIZYA-argF*) (80d *lac* [*lacZ*] M15) *pir* RK6	(Macinga et al., 1995)
BW25113	Δ*(araD-araB)567*, Δ*lacZ4787*(::rrnB-3), lambda-, *rph-1*, Δ*(rhaD-rhaB)568*, *hsdR514*	(Datsenko and Wanner, 2000)
JL001	BW25113 Δ*valS*::*kan* pBBR1MCS-5-P_{T7}-SUMO-WFCWS-*valS*; Kan^r Gm^r	This study
Plasmids		
pRedET	Red/ET expression plasmid Tet^r	Gene Bridges
pFRT-PGK-gb2-neo-FRT	PCR-template (plasmid DNA) for generating a FRT-flanked PGK-gb2-neo cassette; Kan^r	Gene Bridges
pTB145	$lacI^q$ P_{T7}::*h-ulp1(403-621)* pBR/colE1; Amp^r	(Bendezu et al., 2009)
pTB146	$lacI^q$ P_{T7}::*h-sumo* pBR/colE1; Amp^r	(Bendezu et al., 2009)
pBAD33	*ori*-p15a *araC* P_{BAD}; Cm^r	(Guzman et al., 1995)
pUlp1 (pBAD33-*ulp*1)	*ulp*1 in pBAD33; Cm^r	This study
pBBR1MCS-5	oriT mobRK2 pBBR origin of replication lacZα; Gm^r	(Kovach et al., 1995)
pBBR1MCS-5-P_{T7}-SUMO-WFCWS-*valS*	SUMO-WFCWS-*valS* in pBBR1MCS-5	This study
pET21b-ValS (wt + mutants)	*valS* cloned into pET21b (Novagen/Merck) expression plasmid	This study
pValS (pQE70-ValS) (wt + mutants)	*valS* cloned into pQE70 (Qiagen) expression plasmid	This study
Oligonucleotides		
Construction of *valS*::*kan* inframe deletion		
D*valS*-us	GCT ATA AAG CCT ACC GCG TGG CTG GTT TCA ACC TGA ATA CGG CAA CCT GGA AAT AAA ATT AAC CCT CAC TAA AGG GCG	This study
D*valS*-ds	GAG ATA AAA AAG GCC GGA GCA TGC TCC GGC CTT CGT TTT CAT CAC TGT GTT TTG ATA ATA CGA CTC ACT ATA GGG CTC	This study
valS-chk-Fw	CGC AAA AGC GTA GCA GCA GC	This study
valS-chk-Rev	TGG GCG ATT TGG ATG ACC TTC C	This study
Construction of pBBR1MCS-5-P_{T7}-SUMO-WFCWS-*valS*		
T7 Promoter Primer	TAA TAC GAC TCA CTA TAG GG	
SUMO-WFCWS-ValS-OL-Rev	GCT CCA GCA AAA CCA ACC ACC AAT CTG TTC TCT GTG AGC	This study
SUMO-WFCWS-ValS-OL-Fw	TGG TTT TGC TGG AGC GAA AAG ACA TAT AAC CCA CAA GAT ATC GA	This study
XmaI-ValS-Rev	GCA GTC ACC CGG GTT ACA GCG CGG CGA TAA CAG C	This study
ValS-636-Seq-Fw	GGT AAA GAT TAT CTG GTG GTC GCG	This study
ValS-1378-Seq-Fw	TCG GTG CTG ATG TTG TCC TGC	This study
ValS-2091-Seq-Fw	CGA TAT CGC CGC AGG CAT TC	This study
Construction of pBAD33-*ulp*1		
SacI-His6-ulp1-Fw	GCT GCG GAG CTC AAT AAT TTT GTT TAA CTT TAA GAA GGA GAT ATA CC	This study
XmaI-ulp1-Rev	GCG TCC CGG GTT ATT TTA AAG CGT CGG TTA AAA TCA AAT GG	This study
pBAD-Fw	GGC GTC CAC ACT TTG CTA TGC	(Lassak et al., 2010)
pBAD-Rev	CAG TTC CCT ACT CTC GCA TG	(Lassak et al., 2010)
Construction of ET21b-*valS* and pQE70-*valS*: wt and mutants		
FOR valS pQE70	GAA TTC ATT AAA GAG GAG AAA TTA AGC ATG CTT GAA AAG ACA TAT AAC CCA CAA G	This study
REV valS pQE70	CAG GAG TCC AAG CTC AGC TAA TTA AGC TTT TAC AGC GCG GCG ATA ACA GCC TG	This study
FOR pET21b-valS	GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG GAA AAG ACA TAT AAC CCA CAA G	This study
REV pET21b-valS	GGC CGC AAG CTT GTC GAC GGA GCT CCC CAG CGC GGC GAT AAC AG CCT G	This study
FOR GGG valS	GAA AGT TTC TGC ATC ATG ATC GGT GGT GGT AAC GTC	This study

	ACC GGC AGT TTG CA	
REV GGG valS	TGC AAA CTG CCG GTG ACG TTA CCA CCA CCG ATC ATG ATG CAG AAA CTT TC	This study
FOR GPP valS	GAA AGT TTC TGC ATC ATG ATC GGT CCG CCG AAC GTC ACC GGC AGT TTG CA	This study
REV GPP valS	TGC AAA CTG CCG GTG ACG TTC GGC GGA CCG ATC ATG ATG CAG AAA CTT TC	This study
FOR PGP valS	GAA AGT TTC TGC ATC ATG ATC CCG GGT CCG AAC GTC ACC GGC AGT TTG CA	This study
REV PGP valS	TGC AAA CTG CCG GTG ACG TTC GGA CCC GGG ATC ATG ATG CAG AAA CTT TC	This study
FOR PPG valS	GAA AGT TTC TGC ATC ATG ATC CCG CCG GGT AAC GTC ACC GGC AGT TTG CA	This study
REV PPG valS	TGC AAA CTG CCG GTG ACG TTA CCC GGC GGG ATC ATG ATG CAG AAA CTT TC	This study
FOR valS T222P	GATTATCTGGTGGTCGCGACTCCCCGTCCAGAAACCCTGCTG GG	This study
REV valS T222P	CCCAGCAGGGTTTCTGGACGGGGAGTCGCGACCACCAGATA ATC	This study
pQE70-Seq-Fw	CGA GGC CCT TTC GTC TTC ACC	This study
pQE70-Seq-Rev	AGG TCA TTA CTG GAT CTA TCA ACA GG	This study
ValS-APP-OL-Fw	TGC ATC ATG ATC GCG CCG CCG AAC GTC	This study
ValS-APP-OL-Rev	GAC GTT CGG CGG CGC GAT CAT GAT GCA	This study
ValS-CPP-OL-Fw	TGC ATC ATG ATC TGC CCG CCG AAC GTC	This study
ValS-CPP-OL-Rev	GAC GTT CGG CGG GCA GAT CAT GAT GCA	This study
ValS-DPP-OL-Fw	TGC ATC ATG ATC GAT CCG CCG AAC GTC	This study
ValS-DPP-OL-Rev	GAC GTT CGG CGG ATC GAT CAT GAT GCA	This study
ValS-EPP-OL-Fw	TGC ATC ATG ATC GAA CCG CCG AAC GTC	This study
ValS-EPP-OL-Rev	GAC GTT CGG CGG TTC GAT CAT GAT GCA	This study
ValS-FPP-OL-Fw	TGC ATC ATG ATC TTT CCG CCG AAC GTC	This study
ValS-FPP-OL-Rev	GAC GTT CGG CGG AAA GAT CAT GAT GCA	This study
ValS-HPP-OL-Fw	TGC ATC ATG ATC CAT CCG CCG AAC GTC	This study
ValS-HPP-OL-Rev	GAC GTT CGG CGG ATG GAT CAT GAT GCA	This study
ValS-IPP-OL-Fw	TGC ATC ATG ATC ATT CCG CCG AAC GTC	This study
ValS-IPP-OL-Rev	GAC GTT CGG CGG AAT GAT CAT GAT GCA	This study
ValS-KPP-OL-Fw	TGC ATC ATG ATC AAA CCG CCG AAC GTC	This study
ValS-KPP-OL-Rev	GAC GTT CGG CGG TTT GAT CAT GAT GCA	This study
ValS-LPP-OL-Fw	TGC ATC ATG ATC CTG CCG CCG AAC GTC	This study
ValS-LPP-OL-Rev	GAC GTT CGG CGG CAG GAT CAT GAT GCA	This study
ValS-MPP-OL-Fw	TGC ATC ATG ATC ATG CCG CCG AAC GTC	This study
ValS-MPP-OL-Rev	GAC GTT CGG CGG CAT GAT CAT GAT GCA	This study
ValS-NPP-OL-Fw	TGC ATC ATG ATC AAC CCG CCG AAC GTC	This study
ValS-NPP-OL-Rev	GAC GTT CGG CGG GTT GAT CAT GAT GCA	This study
ValS-QPP-OL-Fw	TGC ATC ATG ATC CAG CCG CCG AAC GTC	This study
ValS-QPP-OL-Rev	GAC GTT CGG CGG CTG GAT CAT GAT GCA	This study
ValS-RPP-OL-Fw	TGC ATC ATG ATC CGC CCG CCG AAC GTC	This study
ValS-RPP-OL-Rev	GAC GTT CGG CGG GCG GAT CAT GAT GCA	This study
ValS-SPP-OL-Fw	TGC ATC ATG ATC AGC CCG CCG AAC GTC	This study
ValS-SPP-OL-Rev	GAC GTT CGG CGG GCT GAT CAT GAT GCA	This study
ValS-TPP-OL-Fw	TGC ATC ATG ATC ACC CCG CCG AAC GTC	This study
ValS-TPP-OL-Rev	GAC GTT CGG CGG GGT GAT CAT GAT GCA	This study

ValS-VPP-OL-Fw	TGC ATC ATG ATC GTG CCG CCG AAC GTC	This study
ValS-VPP-OL-Rev	GAC GTT CGG CGG CAC GAT CAT GAT GCA	This study
ValS-WPP-OL-Fw	TGC ATC ATG ATC TGG CCG CCG AAC GTC	This study
ValS-WPP-OL-Rev	GAC GTT CGG CGG CCA GAT CAT GAT GCA	This study
ValS-YPP-OL-Fw	TGC ATC ATG ATC TAT CCG CCG AAC GTC	This study
ValS-YPP-OL-Rev	GAC GTT CGG CGG ATA GAT CAT GAT GCA	This study

Supplemental Experimental Procedures

Oligonucleotides, plasmids and bacterial strains

Primers, plasmids and strains used in this study are listed in **Table S1**. Strain Δ*valS* was constructed from BW25113 harboring the pBBR1MCS-5-PT7-SUMO-WFCWS-valS by using the pRED®/ET® recombination technology in accordance to the technical protocol of the Quick & Easy *E. coli* Gene Deletion Kit of Gene Bridges (www.genebridges.com). Construction of plasmids encoding *ulp*1 and PT7-SUMO-WFCWS-ValS, respectively, are described in **Table S1**.

Molecular biology methods

Enzymes and kits were used according to the manufacturer´s directions. Genomic DNA was obtained by using the DNeasy blood and tissue kit (Qiagen) and plasmid DNA was isolated using a Hi-Yield plasmid Mini Kit (Suedlabor). DNA fragments were purified from agarose gels by employing a High-Yield PCR cleanup and gel extraction kit (Suedlabor). Restriction endonucleases were purchased from New England Biolabs (NEB). Sequence amplifications by PCR were performed utilizing the Phusion high-fidelity DNA polymerase (Finnzymes) or the Taq DNA polymerase (NEB), respectively. Standard methods were performed according to Sambrook (Sambrook et al., 1989).

Purification of ValS

The *valS* gene was cloned into pET21b using NdeI and SacI restriction sites and then *valS* mutants were generated using site directed mutagenesis strategy. C-term-His-tagged-ValS proteins were expressed in BL21(pLysS) and purified using Protino Ni-NTA agarose beads (Macherey-Nagel) and HiLoad 16/60 Superdex75 preparative gel filtration column (GE Healthcare) using 50 mM Hepes pH 7.8, 50 mM KCl, 100 mM NaCl, 10 mM $MgCl_2$, 2% glycerol, 5 mM β-mercaptoethanol

$tRNA^{Val}$ charging assay

Charging reactions were performed as described (Splan et al., 2008). Briefly, each reaction (50 µL) contained 500 pmol [^{14}C]-Valine (Perkin Elmer) or [^{14}C]-Threonine (Hartman Analytics), 100 µmol ATP, 7.5 pmol ValS in 100 mM Hepes pH 7.4,

20 mM KCl, 15 mM $MgCl_2$, 10 mM DTT and 50 pmol $tRNA^{Val}$ (Sigma Aldrich). Each reaction was incubated at 37°C for 1-30 min and then quenched with 5 µL of 3 M sodium acetate pH 5.0. Excess of free amino acid was removed using Amicon Ultra 3.5K (Merck-Millipore) (3x wash with cold buffer) and then the radioactivity of tRNA samples was measured using scintillation counting.

Monitoring of PPi and AMP formation

Assays to monitor aminoadenylate formation were performed as previously described (Splan et al., 2008). Briefly, reactions contained 100 µmol amino acid, 600 pmol ATP, 2 µCi α[^{32}P]-ATP or γ[^{32}P]-ATP (Hartmann Analytic), 15 pmol ValS in 100 mM Hepes pH 7.4, 20 mM KCl, 15 mM $MgCl_2$, 10 mM DTT and 400 pmol $tRNA^{Val}$ (where indicated) in 50 µL. Reactions were incubated at 37°C for 5 min and then quenched with 5 µL of 3 M sodium acetate pH 5.0. Where indicated, 0.1 U of Inorganic Pyrophosphatase from *E. coli* (NEB) or 5 mU of Apyrase (NEB) (supplemented with Apyrase buffer) was added. ATP/ADP/AMP standards were prepared using dilutions of Apyrase (0/0.05/0.5/5 mU). Thin-layer chromatography was performed using 20 x 10 cm TLC PEI Cellulose F plates (Merck-Millipore), which were pre-equilibrated in water. Samples (0.5 µL) were spotted 1 cm from the bottom edge of the plate and the nucleotides separated by running in tanks containing 0.15 M formic acid-0.15 M LiCl. Plates were then dried and exposed to Amersham Hyperfilm MP (GE Healthcare) for >1h.

Western blotting analysis

To monitor SUMO-ValS degradation and Ulp1 production, exponentially grown cells were harvested at certain times after arabinose induction. Cells were resuspended in water yielding an OD_{600nm} of 10. 10 µl of the cell suspension was mixed with an equivalent volume of Laemmli buffer and boiled for 5min before separation by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) using 12.5% gels (Laemmli, 1970). SUMO-ValS and Ulp1 were detected by Western immunoblotting according to standard protocols (Sambrook et al., 1989) using mouse monoclonal anti 6xHis tag antibody coupled to alkaline phosphatase (Abcam).

ValS-XPP library

pQE70-ValS-XPP library was constructed as essentially described in van den Ent & Loewe (van den Ent and Lowe, 2006) after generation of a mutant fragment of valS by overlap extension PCR (Lassak et al., 2010). valS::kan PT7-SUMO-valS was transformed with pBAD33 or pBAD33-ulp1 and a pQE70-ValS-XPP variant. Overnight (ON) cultures of transformants were grown to exponential phase in LB under repressing conditions (+0.2% Glucose). Subsequently cells were washed and diluted to OD 600nm= 0.05 into fresh medium lacking Glucose but containing 0.2% arabinose. Growth was recorded by measuring OD600nm after 1.5h incubation at 37°C.

Complementation analysis

JW4106 (Δ*efp*) was transformed with pQE70-vectors containing either a copy of *efp* or ValS-PPP, ValS-GPP or ValS-GGG. ON cultures of transformants were grown to exponential phase in LB under repressing conditions (+0.2% Glucose). Subsequently, cells were washed and diluted into fresh medium lacking Glucose but containing 1mM IPTG. Doubling times were calculated from exponentially grown cells.

Supplemental References

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