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Escherichia coli Reporter Strains Allow for the In Vivo Evaluation of Recombinant Elongation Factor Protein (EF-P)

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Abstract: Background: Elongation factor protein (EF-P) in bacteria helps ribosomes to incorporate contiguous proline residues (xPro) into proteins. In this way, EF-P rescues ribosomes from stalling at these xPro motifs. Whereas EF-P deficiency is lethal for some species, others show reduced virulence or generally lower growth rates, such as *Escherichia coli* (*E. coli*). EF-P needs to be post-translationally modified to gain full functionality. Methods: We constructed *E. coli* K-12 mutant strains with deletion of the *serA* gene leading to an auxotrophy for L-serine. Then, we engineered a 6xPro motif in the recombinant *serA* gene, which was then chromosomally inserted under its native promoter. Furthermore, mutant strains which were deleted for *efp* and/or *epmA* (encoding the EF-P modification protein EpmA) were engineered. Results: Δ*efp*, Δ*epmA*, and Δ*efp*/Δ*epmA* double mutants showed already significantly reduced growth rates in minimal media. Δ*serA* derivatives of these strains were complemented by the wt *serA* gene but not by 6xPro-*serA*. Δ*serA* mutants with intact *efp* were complemented by all *serA*-constructs. Chromosomal expression of the recombinant *efp* gene from *E. coli* or from the pathogen, *Staphylococcus aureus* (*S. aureus*), restored growth, even without *epmA* expression. Conclusions: We provide a novel synthetic reporter system for in vivo evaluation of EF-P deficiency. In addition, we demonstrated that both EF-P-*E. coli* and EF-P-*S. aureus* restored the growth of a 6xPro-*ser*A: Δ*efp*, Δ*epmA* strain, which is evidence that modification of EF-P might be dispensable for rescuing of ribosomes stalled during translation of proline repeats.

Keywords: elongation factor protein EF-P; polyproline stretch; ribosome stalling; *serA* gene; efp; epmA; polyPro-serA reporter strain

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1. Introduction

During protein synthesis, ribosomes become stalled on polyproline-containing sequences due to the cyclic pyrrolidine structure of proline. Polypeptides with a high proline content are thus poorly translated, especially if proline occurs in motifs consisting of two, three, or more contiguous residues [1–4]. Specific proteins can rescue ribosomes from stalling at these motifs. In Archaea and eukaryotes, this is achieved via the archaeal/eukaryotic translation initiation factor a/eIF-5A, and in bacteria, via the homologous elongation factor protein, EF-P, respectively [5]. All living organisms express either a/eIF5A or EF-P [6–8]. EF-P helps to incorporate contiguous proline residues into proteins and, in vitro, can even facilitate the incorporation of structurally diverse non-canonical amino acid residues, as shown recently [9]. Only one gene encodes EF-P in bacteria [5], which is a small (21 kDa) protein containing three β-barrel domains (I to III), reminiscent of the L-shape of tRNA [10–12]. The N-terminal domains of eIF5A and domain I of EF-P show the same fold, as do the C-terminal domain of eIF5A and domains II and III of EF-P [10,11].

For full functionality, a/eIF5A and EF-P need to be modified post-translationally [13]. These are unique modifications. In many prokaryotes, a specific lysine residue is modified at the tip of a flexible loop in the N-terminal domain of aIF5A or in domain I of EF-P. A post-translational modification occurs on a conserved lysine residue of EF-P in many bacteria and is referred to as β-lysinylation of amino acid residue Lys34 (K34). Lysinylation is only found in about 25% of analyzed bacterial species [7,13–15]. This modification occurs in three sequential steps, requiring the enzymes EpmA, EpmB, and EpmC [14], formerly termed YjeK, YjeA, and YfcM [5] in *E. coli*. Different modifications, e.g., rhamnosylation of EF-P at Arg32, have been found in *Pseudomonas aeruginosa* (*P. aeruginosa*) [13,14], and a 5-aminopentanol moiety is attached to Lys32 in the Gram-positive bacteria *Bacillus subtilis* [16,17] and in *S. aureus* [18]. Enzymes for the known EF-P lysinylation, rhamnosylation, or aminopentanolylation pathways can be identified in only about 35% of the available bacterial genomes. It is unclear as to how the remaining majority of bacteria activate their corresponding EF-Ps [19]. As pathogenic species, such as *P. aeruginosa, Bacillus cereus*, or *S. aureus*, also have their cognate EF-P modification systems, an understanding of the individual post-translational modification systems is of great interest. This could lead to targets for species-specific antimicrobial compounds, which are unlike broad-range antibiotics.

In bacteria, the effects of EF-P deficiency vary. Whereas an EF-P deficiency is lethal in *Neisseria* strains, *Bacillus efp*-mutants showed only marked defects in sporulation or swarming motility [17,20,21]. *P. aeruginosa efp*-mutants displayed reduced virulence and increased sensitivity towards several antibiotics [22]. For *E. coli*, divergent reports have been given, e.g., the temperature sensitivity of growth [23] or the slower growth of an *efp*-deletion mutant only [22,24]. By using transposon-mediated differential hybridization, the homologous *efp* gene of *S. aureus* was deemed to be essential for growth [25]. Recombinant expression of EF-P proteins was successfully accomplished in *E. coli* strains (*Thermus thermophilus*: [10,12], *P. aeruginosa* [11,14], *Shewanella oneidensis* [14]).

Reporter systems have been constructed with an engineered tetra-proline (4Pro) motif in GFP for the *P. aeruginosa* system to study the influence of the various modification systems [26]; similarly, in *B. subtilis*, a fluorescent reporter system has been described [17]. For *E. coli*, a P_{cadBA}-dependent *lacZ* reporter was successfully introduced in an *efp* mutant strain. This allowed for complementation assays of several EF-P variants in this strain through the monitoring of β-galactosidase (LacZ) activity [14]. *E. coli* reporter strains used to visualize EF-P activity via GFP activity have also been described [24]. To our knowledge, no positive selection system has been given so far for *E. coli efp* mutants where the in vivo function of recombinant Efp protein was assessed. Here, we report a novel synthetic *E. coli* reporter system which allows for the in vivo functional expression of *efp* genes. We combined the essential *serA* gene for L-serine biosynthesis [27] with a 6xPro motif and showed that this leads to growth impairment of *E. coli* mutants carrying a chromosomal *efp* gene deletion. We show further that this growth defect can be complemented via the expression of either the homologous recombinant *efp* gene from *E. coli* or the recombinant gene from *S. aureus* (efp_{Sa}). Interestingly, this complementation also works without a cognate modification (Epm) system.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids, and DNA Primers

All strains are derived from *E. coli* K-12, wild-type strain W3110 (lab name: LJ110 [28]). The pedigree of the strains constructed in the present study is given in Table 1, as well as the list of plasmids used and created in this work. Table 2 contains the list of DNA primers used in this study. Oligonucleotides were custom-synthesized by Biomers (Ulm, Germany).

Table 1. Strains and plasmids used in this work. * gene *efp* from *E. coli*; ** gene *efp* from *S. aureus*.

Strains		
Name	**Relevant Genotype**	**Reference**
LJ110	*E. coli* K-12 W3110, wild-type, prototroph	[28]
NT1352	LJ110 Δ*ser*A	This work
NT1367	LJ110 Δ*ser*A Δ*efp*	This work
NT1368	LJ110 Δ*ser*A *fuc*::P_{serA}-*ser*A Δ*efp*	This work
NT1373	LJ110 Δ*ser*A *fuc*::P_{serA} 6Pro-*ser*A	This work
NT1379	LJ110 Δ*efp* Δ*ser*A *fuc*::P_{serA} 6Pro-*ser*A	This work
NT1397	LJ110 Δ*efp* Δ*ser*A *fuc*::P_{serA} 6Pro-serA Δ*epm*A	This work
NT 1411	LJ110 Δ*efp* Δ*ser*A fuc::P_{serA} 6Pro-serA xyl::P_{tac}-*efp-Ec* *	This work
NT 1413	LJ110 Δefp Δ*serA* fuc::P_{serA} 6Pro-serA xyl::P_{tac}-*efp-Sa* **	This work
NT 1415	LJ110 Δ*efp* Δ*ser*A *fuc*::P_{serA} 6Pro-serA Δ*epm*A xyl::P_{tac}-*efp-Ec* *	This work
NT 1417	LJ110 Δ*efp* Δ*ser*A *fuc*::P_{serA} 6Pro-serA Δ*epm*A xyl::P_{tac}-*efp-Sa* **	This work
NT1433	LJ110 Δ*epm*A	This work
NT1435	LJ110 Δ*efp*	This work
NT1437	LJ110 Δ*efp* Δ*epm*A	This work
Plasmids		
pTarget F	*pMB1 aadA* sgRNA	[29]
pCas	*repA101*(Ts)*kan* P_{cas}-*cas9* P_{araB}-*λRed lacI*^{q} P_{lac}-sgRNA-*pMB1*	[29]
pTarget–cat	*cat*^{R}-cassette into the *Bcl*I site of the pTargetF plasmid with disruption of Sm^{r} gene	This work
pTarget-cat_{sg}-*fuc*	*pMB1 aadA* sgRNA-*fuc*	This work
pTarget-cat_{sg}-*serA*	*pMB1 aadA* sgRNA-*serA*	This work
pTarget-cat_{sg}-*efp*	*pMB1 aadA* sgRNA-*efp*	This work
pTarget-cat_{sg}-*epmA*	*pMB1 aadA* sgRNA-*serA*	This work
pJem2	*rhaR rhaS rhaP* $_{BAD}$ *–T7le-eGFP , mob, kan*	[30]
pJNNmod	*ColE1* P_{tac},*lacI*^{q},bla	[31]
pSerA	*ColE, lacI*^{q}, P_{serA}-*ser*A	This work
p6Pro-serA	*ColE1, lacI*^{q}, P_{serA}-6Pro-*ser*A	This work
p4Pro-serA	*ColE1, lacI*^{q}, P_{serA}-4Pro-*ser*A	This work
p6Pro-stop-serA	*ColE1, lacI*^{q}, P_{serA}-4Pro-UAA-*ser*A	This work
pJNN-efp-Ec *	*ColE1 lacI*^{q}P_{tac}-*efp (E. coli)*	This work
pJNN-efp-Sa **	*ColE1 lacI*^{q}P_{tac}-*efp* **	This work
pJem2-efp-Ec *	*rhaR rhaS* P_{rha} *–efp-Ec *, mob, kan*	This work
pJem2-efp-Sa **	*rhaR rhaS* P_{rha} *–efp-Sa *, mob, kan*	This work

Table 2. List of DNA primers. Bases in italics denote target sequences for Cas9 protein.

Name	DNA Sequence
Targ-univ-rev	actagtattatacctaggactgagctagc
sgRNA-serA	*ggcattctggctgaatcgct*gttttagagctagaaatagcaagttaaaataaggctag
sgRNA-efp	*aaaccggctaccctgtctac*gttttagagctagaaatagcaagttaaaataaggctag
sgRNA-epmA	*gagacacgtttcgttggccc*gttttagagctagaaatagcaagttaaaataaggctag
sgRNA-fuc	*gacgaccgtcaataaccgggg*gttttagagctagaaatagcaagttaaaataaggctag
sgRNA-xyl	*gcccaattcgctattccagc*gttttagagctagaaatagcaagttaaaataaggctag
3′serA-SphI	ttttgcatgcttagtacagcagacgggcgcgaatg
5′P_{serA}-serA-SphI	ttttgcatgcctcttcattaaatttggtgacatgtgtcacg
serA-inv-rev	ttacccaatcctgtcttttgaaatgttgtg
serA-4Pro	atgccaccaccaccagcaaaggtatcgctggagaaagacaag
serA-6Pro	atgccaccaccaccaccaccagcaaaggtatcgctggagaaagacaag
QC-Stop-SerA-I	caggattgggtaaatgtaaccaccaccaccagc
QC-Stop-SerA-II	tggtggtggtggttacatttacccaatcctgtcttttg
efp-Ec-5-NdeI	ttttcatatggcaacgtactatagcaacgattttc
efp-Ec-3′HindIII	ttttaagcttacttcacgcgagagacgtattc
efp-Sa-3′-BamHI	ttttggatccttatcctcttgaaatgtagcttccatcacc
efp-Sa-5′-NdeI	ttttcatatgggcatttcggttaatgattttaaaacagg
L5′del-SerA	cgcgtcagctggtgaaactgggcg
L3′del-SerA-BamHI	ttttggatccagagcaatcgacaattgcctgg
R5′del-SerA-BglII	ttttagatctcccgtctgctgtactaattcccc
R3′del-SerA	gggtaagggaggattgctcctccc
L5′-del-efp	gcgcgatgacaaactcatcttgcg
L3′-del-efp ′-BamHI	ttttggatcctctaacatgattttaagaccagcacg
R5′-del-efp BglII	ttttagatctatgcggttgtggtgcggcctg
R3′-del-efp	tgctgcgccagaaatcgcgttaccg
L5′-del-epmA-L-5	tatcccacagccacgtacttcaggg
L3′-del-epmA-BamHI	ttttggatcctgacaagggcacgaagtctactcgc
R5′-del-epmA-BglII	ttttagatctactgaattaacagcgaagaatggcgtg
R3′-del-epmA	gcagctcccatttcagccatcattaagg
FucP-serA-int-5′	tgctgtgctcactgtttttctttgggcggtagccaataaccttaacgacatg ccccagcaggcgaaaatcctg
FucI-int-3′	ggcgagagtgataaagtctgcgccaacgtggccgatggtcagaa cccccagggttattgtctcatgagcg
xylA-int-5′	gacgaactggtgttgggtaagcgtatggaagagcacttgcgttttgccgcctgct caaggcgcactcccgttctgg
xylB-int-3′	attaaagctgggacattgctcaggccggttaatttcgcggcccaatccagaca ccagggttattgtctcatgagcg
RT-qPCR-serA 5′	caagattaagtttctgctggtagaaggcg
RT-qPCR-serA 3′	tagcgaccagtttttctgcggcgttg
RT-qPCR-ftsZ-5′	tgcatttgcttccgacaacg
RT-qPCR-ftsZ-3′	acgtttgtccatgccgatac

2.2. Construction of Plasmids and Recombinant Strains

Standard methods for plasmid cloning and PCR were used unless stated otherwise [32]. Deletions and gene insertions were performed via the CRISPR-Cas method in combination with λ-red recombineering, as described [29]. Target loci in the genome of *E. coli* were chosen as previously described [33]. All gene modifications were verified by custom DNA sequencing (Eurofins, Ebersberg, Germany). All specific pTarget-cat-sgRNA plasmids (see Table 1) used in this work were constructed through the introduction of a 20 N specific guide sequence in pTarget-cat plasmid using the inverse PCR method. The inverse PCR method was performed using the Targ-univ-rev primer in combination with the corresponding specific primer (sgRNA-serA for pTarget-cat-sgRNA-serA; sgRNA-efp for pTarget-cat-sgRNA-efp; sgRNA-epmA for pTarget-cat-sgRNA-epmA; sgRNA-fuc for pTarget-cat-sgRNA-fuc; and sgRNA-xyl for pTarget-cat-sgRNA-xyl). After inverse PCR, the fragments were digested with *Dpn*I, treated with T4 polynucleotide kinase (NEB GmbH, Frankfurt am Main, Germany) and religated with T4 DNA ligase (NEB GmbH, Frankfurt am Main, Germany). Selected clones after transformation in the DH5α strain [34] were verified via sequencing.

The DNA fragment containing the *ser*A gene and its promoter region was amplified from the chromosomal DNA of the *E. coli* wild-type strain LJ110 using primers 3′serA-SphI and 5′P_{serA}-serA-SphI and cloned into the *Sph*I restriction site of the pJNNmod vector. After cloning of the plasmid using *Xba*I and *Hpa*I restriction enzymes, it was treated with *Klenow* DNA polymerase to obtain blunt ends, and was religated. The resulting plasmid pSerA was used as a basic construct for the creation of p4Pro-serA and p6Pro-serA recombinant plasmids via inverse PCR using serA-inv-rev/serA-4Pro and serA-inv-rev/serA-6Pro primers, respectively. These recombinant plasmids were used as a template for the amplification of the fragment P_{tac}-serA (6Pro-serA/4Pro-serA) cassettes into the *fuc*-locus of chromosomal DNA. These fragments were produced with FucP-serA-int-5′/FucI-int-3′ primers.

The *efp* genes from *E. coli* and *S. aureus* were amplified from chromosomal DNA using efp-Ec-5′NdeI/efp-Ec-3′HindIII and efp-Sa-5′-NdeI/efp-Sa-3′-BamHI primers, respectively. After amplification, these fragments were cloned into the pJNNmod vector (or pJem2 vector) in *Nde*I/*Hind*III enzyme sites. Thus, the obtained recombinant plasmids (pJNNmod-based) were used to amplify the *xyl*A*-P_{tac}-*efp-Ec* (*efp-Sa*)-*xyl*B* fragment (primers: xylA-int-5′/xylB-int′). The fragments were integrated into the chromosome of the reporter strains using the CRISPR-Cas λ-Red method. Deletions of the *ser*A, *efp*, and *epm*A genes from the chromosome of the *E.coli* cells were performed using the CRISPR-Cas method [29]. The flanking regions of HR1 and HR2 were spliced via PCR. For this, the fragments (HR1 and HR2) were amplified, cut with *Bam*HI and *Bgl*II, respectively, then ligated with subsequent reamplification. The created fragments were used for homologous recombination with subsequent removal of the desired gene.

2.3. Growth Measurements

LB agar plates were used for strain propagation with 18 g/L of agar. Antibiotics were added to the following final concentrations: ampicillin-100 mg L^{-1}, chloramphenicol-25 mg L^{-1}, and kanamycin-50 mg L^{-1}. The hydrogen-phosphate-buffered mineral salts (minimal) medium [35] with 10 g L^{-1} of glucose was used with supplementation of L-serine (200 mg L^{-1}, final concentration) when appropriate. Optical density at wave light 600 nm (OD_{600}) was followed with a UV/vis photometer (Cary50, Thermo Fisher Scientific GmbH, Dreieich, Germany) in shake flasks (250 mL with 30 mL of cell culture) on orbital shakers (Infors HT, Einsbach, Germany) at 200 rpm and a temperature of 37 °C. Growth measurements were performed in three biological replicates (independent experiments). Standard deviations are provided as error bars.

2.4. Biochemical Measurements

Total RNA was isolated from cells from the exponential growth phase using the Qiagen Kit (Qiagen, Düsseldorf, Germany). RNA was reverse transcribed using EpiScript™ Reverse Transcriptase (Biozym, Hessisch Oldendorf, Germany) and 6N random primer. Transcript measurements were conducted via RT-qPCR according to the recommendation of suppliers (Biozym, Hessisch Oldendorf, Germany) using an instrument and software from Analytic-Jena (Jena, Germany). For the amplification, the Blue S'Green qPCR Kit (Biozym, Hessisch Oldendorf, Germany) was used. The RT-qPCR was performed in technical triplicate on the biological triplicate. Cycling conditions were 95 °C for 30 s, 52 °C for 10 s, and 72 °C for 20 s, along with a fluorescence measurement. The negative control (NC) was included for each primer pair. The *fts*Z gene, encoding a cell division protein, was used as an internal control to normalize all data.

3. Results and Discussion

3.1. Construction of E. coli Reporter Strains Which Lack Their Own EF-P-System

We aimed to construct *E. coli efp*-deficient mutants with a strong, selectable phenotype. It had been shown before that *efp*-mutants of *E. coli* are viable though compromised [22,24]. Deletion of *efp* or *epmA* in a wild-type *E. coli* K-12 strain (LJ110) was achieved via the CRISPR-Cas method in combination with λ-red recombineering [29]. The single deletion mutants (for a list of strains and primers see Tables 1 and 2) NT1433 (Δ*epmA*) and NT1435 (Δ*efp*) showed both a significantly reduced growth rate (μ) and lower final optical density (fOD) when grown in minimal medium with the carbon source glucose (Table 3) in comparison to the wild-type strain LJ110. This is in accordance with a previous report on a BW25113 Δ*efp* mutant [22]. A combination of the two defects (NT1437) led to a further reduction of growth rate (Table 3). These differences in growth phenotype, however, were not sufficiently strong to be used as a base for recombinant gene expression and selection.

Table 3. Growth rates (μ) and final optical density (fOD) in minimal medium for LJ110 (wt), NT1433 (Δ*epm*A), NT1435 (Δ*efp*), and NT1437 (Δ*epm*A Δ*efp*). The growth experiments in shake flasks were carried out in triplicate on minimal medium supplemented with 10 g/L glucose.

	μ h^{-1}	**fOD**
LJ110	0.58 +/− 0.01	7.14 +/− 0.51
NT1433	0.41 +/− 0.03	4.87 +/− 0.28
NT1435	0.5 +/− 0.03	5.27 +/− 0.16
NT1437	+/− 0.04	4.87 +/− 0.04

3.2. Synthetic Reporter Strains Which Show a Growth Defect in Mineral Media

We therefore aimed for a synthetic reporter strain which allows for parallel investigations of recombinant prokaryotic EF-P expression and could thus constitute a positive selection system. To do so, we first engineered *E. coli* mutant strains, which are L-serine auxotrophs (NT1352). Deletion of *serA* gene rendered this strain strictly dependent on supplementation with L-serine when grown on minimal media (Figure 1). Transformation of the strain NT1352 (Δ*ser*A) with recombinant plasmids containing the cloned *ser*A gene under the control of its own promoter (P_{serA}) or bearing the *ser*A gene fused with 4 or 6 proline-encoding codons at the N terminus (4xPro-SerA, 6xPro-serA) completely restored cell growth on minimal medium plates (see Figure 1). Subsequently, the *serA* gene encoding the enzyme (3-phosphoglycerate dehydrogenase, SerA) variants with a fourfold or sixfold CCA-proline motif in the N-terminus were used for further work.

Deletion of the *efp* gene in the serine auxotroph NT1352 strain led to NT1367. If this strain was transformed with the empty control plasmid (pJNNmod) or a plasmid carrying a stop codon between the 6xPro encoding codons and the residual of the *ser*A gene (4xPro-Stop-serA), it could not grow on minimal medium plates without L-serine

supplementation. If transformed with plasmid-borne copies of 4xPro-*ser*A or 6xPro-*ser*A, only faint growth was observed on agar plates. However, the introduction of the wild-type *ser*A gene led to the restoration of cell growth (Figure 1). The weak-growth phenotype with the 4xPro/6xPro-serA genes might be due to a metabolic burden caused by the high expression of a multi-copy plasmid vector.

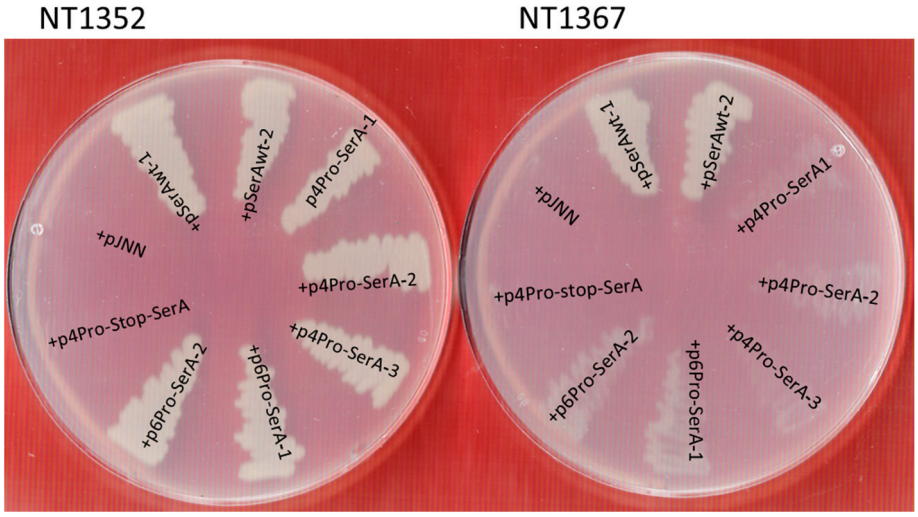

Figure 1. Complementation of strains NT1352 (Δ*serA*) (**left**) and NT1367 (Δ*serA* Δ*efp*) (**right**) strains with various plasmids, containing *serA* (wt), 4-Pro-*serA* or 6Pro-*serA* variants. Growth on minimal medium agar supplemented with 0.4% glucose and ampicillin 100 µg/mL.

To avoid problems with plasmid-borne gene expression, we therefore aimed for a single-copy gene expression. To do so, the *ser*A gene was chromosomally integrated in the NT1352 (Δ*serA*) mutant strain via a CRISPR-Cas targeting method [29]. We chose the chromosomal *fuc* locus (Figure 2) as the integration site as this allowed for easy control of the phenotype on MacConkey agar plates with 1% L-fucose [33]. The altered *serA* genes are under the control of their cognate P_{serA} promoter. Growth in minimal medium without L-serine supplementation was restored in the NT1373 strain (Δ*serA fuc*::P_{serA}-6Pro-*serA*), albeit with a lower growth rate.

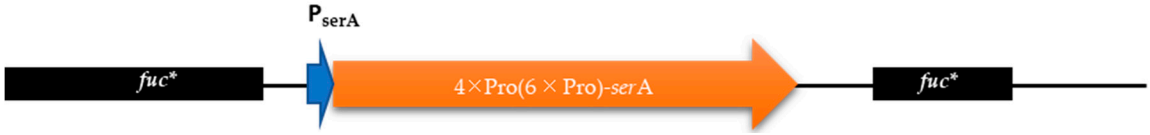

Figure 2. Scheme of integration of various *serA* gene variants (P_{serA}-*serA*, P_{serA}-4xPro-*serA*, or P_{serA}-6xPro-*serA*) in the chromosomal *fuc* locus of *E. coli* K-12.

Also, P_{serA}-4xPro-*ser*A, P_{serA}-6xPro-*ser*A, and P_{serA}-*ser*A were introduced into the *fuc*-locus of strain NT1367 (Δ*efp* Δ*ser*A). So, the obtained strains did not show growth on minimal medium. The control strain NT1368 with an integrated wild-type copy of the *ser*A gene, however, grew well.

Next, we introduced the Δ*epmA* deletion into this strain background. Strain NT1397 (Δ*efp* Δ*ser*A *fuc*::P_{serA}-6xPro-*ser*A Δ*epm*A) was constructed. This was performed with the aim of exacerbating the EF-P-dependency in the absence of L-serine. As expected, a complete growth defect in minimal medium without L-serine supplementation was observed when the *efp* gene was deleted, as well as in combination with Δ*epmA*. Deletion of *epmA* alone led to an intermediate growth phenotype on agar plates or in shake flask cultures (NT1433, Table 3). Interestingly the growth rates of Δ*epmA* and Δ*efp*/Δ*epm*A *E. coli* strains were reduced stronger than of the Δ*efp* strain. It is tempting to speculate that EmpA can have additional function(s) in *E. coli* cells.

This strong and selectable growth phenotype was then used to study whether the defects of the NT1379 strain could be complemented by expression of heterologous *efp* genes such as the one from the pathogenic Gram-positive bacterium, *S. aureus* (*efp*-Sa). This would also allow us to study whether cognate posttranslational modification systems from other bacteria are required for the complementation of the growth defects in the reporter strain (grade of restoration in serine prototrophy).

3.3. Plasmid-Based Expression of Efp Genes

The *efp* genes from *E. coli* and *S. aureus* (*efp*-Ec and *efp*-Sa) were cloned into the pJNNmod vector under the control of the P_{tac}-promoter, as well as into the pJem2 vector (see Table 1) under the control of the rhamnose-inducible promoter P_{rhaA}. Recombinant plasmids were introduced into the NT1379 strain to test their ability to restore growth. The resulting clones were plated on the agar plates with minimal medium supplemented with 0.4% of glucose and 0.2 mM of IPTG for pJNNmod-based variants (0.5% of rhamnose for the pJem2-based plasmid). We observed very weak growth of the NT1379 strain transformed with pJem2-efp-Ec and pJem2-efp-Sa plasmids, respectively. The strains containing the pJNNmod-based recombinant plasmids (pJNN-efp-Sa and pJNN-efp-Ec) did not show any growth on these plates. However, on the plates with pJNN-efp-Sa with prolonged incubation of plates (more than 1 week), some single colonies of the NT1379-pJNN-efp-Sa strain with restored cell growth (*evo*) were selected. The plasmid DNA from evolved ("*evo*") clones were isolated, and the insertion was verified via sequencing. The sequencing of these plasmids, however, did not show any mutation in the *efp-Sa* gene. The NT1379 strain was retransformed with isolated pJNN-efp-Sa-*evo*1 and pJNN-efp-Sa-*evo*2. Although the *efp* gene in these pJNN-efp-Sa-*evo* plasmids did not contain any additional substitutions, the cells showed a serine prototrophic phenotype. To study this phenomenon further, we analyzed the expression of the *efp* gene from the *evo*-derivates in comparison with the expression of this protein located on the primary plasmid, and we analyzed the protein pattern via SDS gel electrophoresis. Expression analysis of *evo* clones via SDS gel electrophoresis showed a significant decrease in the amount of expressed EF-P (*evo*) protein in comparison to the EF-P synthesized from the primary plasmid (see Figure 3), which may be due to a lowered plasmid copy number.

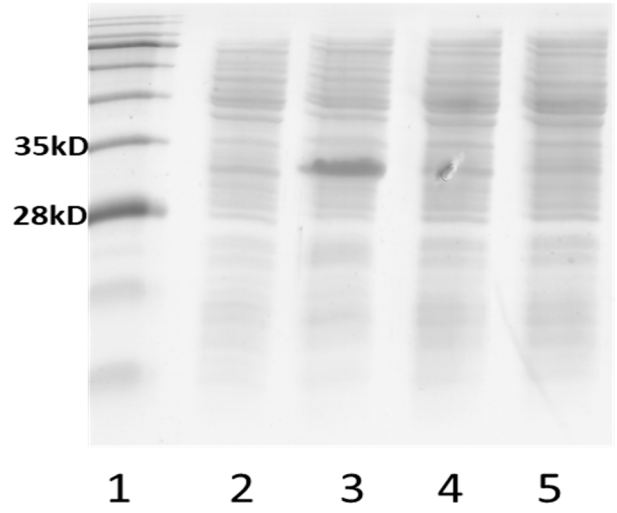

Figure 3. The 12% glycine SDS gel electrophoresis with cell-free extracts (cfe; 25 µg protein/lane) of different strains; lane 1. protein size ladder (Nippon Genetics, Düren, Germany); lane 2: cfe from strain NT1379 with pJNNmod; lane 3: pJNNmod-efp-Sa; lane 4: pJNNmod-Sa-*evo*1; lane 5: pJNNmod-Sa-*evo*2 (line5). Before harvesting, cells were incubated in LB-medium containing ampicillin 100 µg/mL at 37 °C at OD600 ≈ 0.4. IPTG (final concentration of 0.2 mM) was added. After 6 h of expression, the cells were harvested via centrifugation and broken up to obtain the cfe.

The growth experiments in liquid minimal media were carried out with pJem2-based plasmids in the presence of different concentrations of rhamnose (0, 0.1, 0.2, and 1%). The pre-culture was prepared in mineral medium supplemented with 200 µg/mL of L-serine. Then, the cells were washed with mineral medium without serine and inoculated in mineral medium with 1% glucose and appropriate amount of rhamnose. Within 30 h of incubation at 37 °C, we did not observe cell growth carrying an empty vector pJem2 (Figure 4A). In the case of pJem2-efp-Ec and pJem2-efp-Sa, there was a slight increase in optical density (Figure 4B,C).

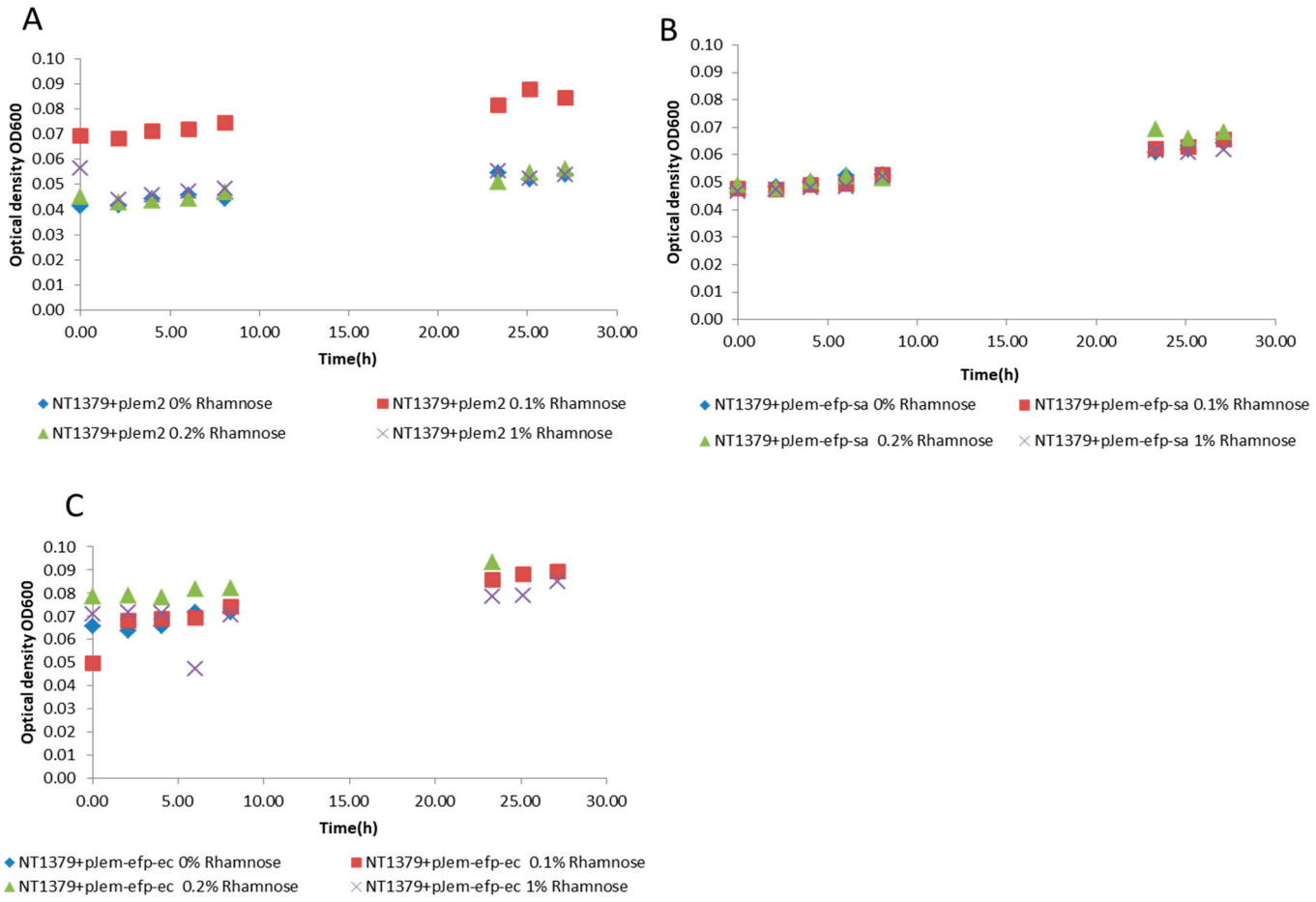

Figure 4. Growth of strain NT1379 with various pJem2-based plasmids. Shake-flask-based growth (mineral salt medium, 1% of glucose, 50 µg/L of kanamycin, various concentrations of L-rhamnose as inducer). (**A**) Growth of cells transformed with pJem2 vector; (**B**) pJem2-efp-Sa plasmid; (**C**) pJem2-efp-Ec.

3.4. Complementation of Growth Defects by Chromosomally Integrated Recombinant Efp Genes

So far, these results have indicated that the use of plasmid-encoded genes for EF-P protein is not optimal for studying the functionality of the recombinant EF-P proteins since it was difficult to assess the effects of copy number, induction efficiency, and plasmid stability on cell growth. We therefore decided to use constructs with chromosomally integrated *efp* genes. *E. coli* strains with chromosomally integrated *efp* genes under the control of a P_{tac} promoter (IPTG inducible) were created via the CRISPR-Cas9 method [29]. This time, gene cassettes were introduced into the *xyl* (xylose degradation) locus of the chromosome of strain NT1379. The introduction of P_{tac}-*efp-Ec* gave strain NT1411 (*efpEc*), whereas insertion of P_{tac}-*efpSa* yielded strain NT1413. We also created the corresponding strains NT1415 and NT1417, respectively (see Table 1), with the deletion of the *epmA* gene. This was conducted to test whether the EF-P protein from *S. aureus* needs the modification system of *E. coli* (EpmA).

We then tested whether these single-copy genes could restore growth in our reporter gene system. Growth in shake flasks (minimal medium) was followed at 37 °C. As can be seen from Figure 5, the control strains NT1379 and NT1397 were unable to grow, whereas the wild-type strain LJ110 displayed typical growth with a final OD of ca. 4.6. The basic reporter strain NT1373 (Δ*serA* fuc::PserA-6xPro-*serA*), which is *efp* wild-type, apparently grew slower than LJ110, however. This may be due to the lower gene expression of the modified *serA* gene (see transcript analyses).

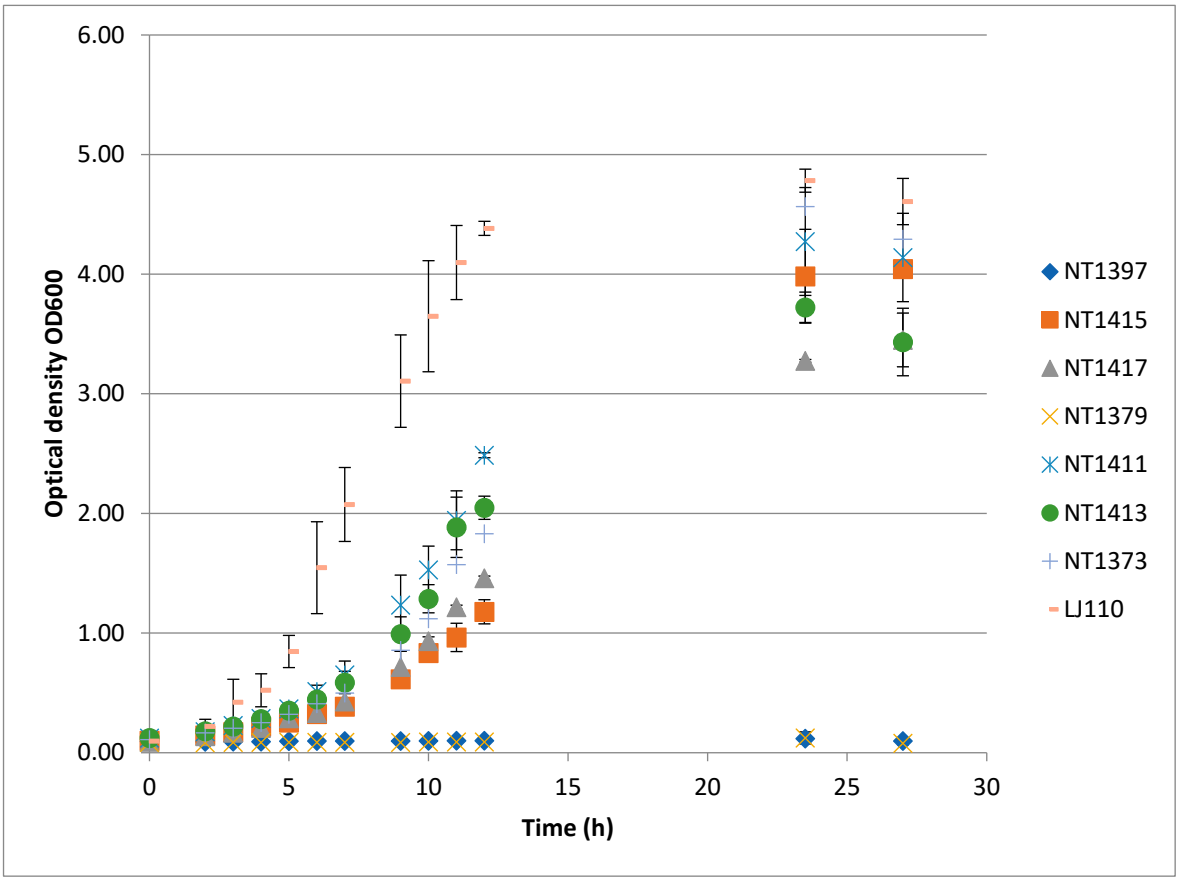

Figure 5. Growth of the strains in minimal medium supplemented with 1% glucose and 0.2 mM of IPTG.

Interestingly, all strains with chromosomally inserted P_{tac}-*efp* grew and reached final OD values of >3 in 27 h of cultivation and were in the range of the control strain, NT1373. Thus, growth was restored. The strains, however (including NT1373), all showed a prolonged growth lag compared to the wild-type strain. Strain NT1411 (P_{tac}-*efp-Ec*) reached a final OD of >4. Next in rank was NT1415, with only marginally reduced growth yield. This can be interpreted as the EpmA modification system not being absolutely essential for EF-P function in *E. coli*.

Strain NT1413 with P_{tac}-*efp*Sa grew well and reached a final OD of ca. 3.4 (see Table 4). The corresponding strain with Δ*epmA* (NT1417) gave the same final OD value, although its growth rate was lower. The *efp* gene from the *S. aureus* (Gram-positive) is thus able to complement the growth defect exerted by the deletion of the *E. coli efp* gene in our reporter system. The EpmA protein of *E. coli* is not necessary for the complementation.

Table 4. Growth rate (μ) and final optical density (fOD) for the strains with chromosomally integrated *efp* gene. Cells were inoculated at a starting OD of ~0.07. The NT1411 and NT1415 strains contain the *efp* gene from *E.coli* under the control of the P_{tac} promoter. The NT1413 and NT1417 strains carry a chromosomal copy of the *efp* gene from *S. aureus* integrated under the control of the P_{tac} promoter. n.a. = not applicable, as no increase in OD could be determined.

	μ h^{-1}	**fOD**
LJ110	0.52 +/− 0.03	4.6 +/− 0.19
NT1373	0.24 +/− 0.01	4.29 +/− 0.42
NT1397	n.a.	0.09 +/−0.001
NT1415	0.21 +/− 0.02	4.04 +/− 0.05
NT1417	0.25 +/− 0.01	3.45 +/− 0.22
NT1379	n.a.	0.08 +/− 0.01
NT1411	0.26 +/− 0.001	4.14 +/− 0.36
NT1413	0.29 +/− 0.02	3.43 +/− 0.28

The average values were calculated based on the data obtained from three independent experiments.

3.5. Transcript Analyses of Recombinant Strains

The *ser*A gene was transferred to the *fuc*-locus with the original promoter region (P_{serA}) in strains NT1373, NT1411, NT1413, NT1415, and NT1417. We wanted to know whether the expression of the 6xPro-serA gene was in the same range as the transcription of the wt *serA* gene. Therefore, a qRT-PCR analysis was performed. The transcript values were normalized to the chromosomal *ftsZ* gene. Differences in the expression of the *serA* variants became apparent in the strains with *fuc*-localized variants in comparison to the strains with the wild-type *ser*A gene (see Table 5). Thus, the lack of full restoration of growth in the *efp* strains can be best compared with strain NT 1373 (ΔserA fuc:P_{serA}-6Pro-*serA*).

Table 5. The relative expression ratio of the *ser*A gene in various strains. The values were normalized with the *fts*Z gene of *E.coli*. RNA samples were taken from cells in the exponential growth phase (for details, see Section 2).

	RQ
LJ110	1.76 +/− 0.13
NT1373	1.11 +/− 0.07
NT1411	1.12 +/− 0.04
NT1413	1.10 +/− 0.03
NT1415	0.92 +/− 0.03
NT1417	1.22 +/− 0.01

The complete restoration of cell growth (to the level of NT1373 strain) after the introduction of the *efp* gene from *S. aureus* into the *efp-Ec*-deficient strain (NT1413) and into the isogenic strain with deleted *epm*A (NT1417) allows us to draw a conclusion about the functionality of the EF-P-Sa translational factor in *E. coli* cells. Moreover, recombinant EF-P-Sa (as well as EF-P-Ec) does not appear to require post-translational modification via the EpmA of *E. coli*. The gene for the EF-P protein from *S. aureus* was able to complement the *efp* deletion of the reporter strain and restored the ability of the strain to grow on minimal medium. This is in accordance with the notion that the post-translational EF-P modification is not strictly required but rather may instead play a more nuanced role in EF-P activity [8].

In conclusion, we can state that recombinant *serA* genes with integrated 4x or 6xPro residues can be used as excellent reporter genes with which to study EF-P-dependency in

an *E. coli* K-12 host strain which is *serA*-deficient. Without a functional *efp* gene, growth in mineral media is not restored, highlighting the role of EF-P in rescuing ribosomes from stalling at poly-Pro residues in translation. The novel synthetic reporter system can be used successfully for the expression of the homologous *efp* gene from *E. coli* K-12. Additionally, the *efp* gene from the pathogenic Gram-positive bacterium *S. aureus* complemented the growth defect regarding serine auxotrophy. In both cases, it is advisable to use chromosomally integrated *efp* genes to avoid troubles pertaining to plasmid-based expression. Interestingly, the EpmA function (post-translational ß-lysinylation of EF-P) is not necessary for this complementation as the *epmA* mutants of *E. coli* did not prevent the activity of the recombinant EF-Ps.

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