Video Article

The Multifaceted Benefits of Protein Co-expression in *Escherichia coli*

Alessandra Stefan[1,2], Alessandro Ceccarelli[1], Emanuele Conte[1], Alejandro Montón Silva[1], Alejandro Hochkoeppler[1,2]

[1]Department of Pharmacy and Biotechnology, University of Bologna

[2]CSGI, Department of Chemistry, University of Firenze

Correspondence to: Alejandro Hochkoeppler at a.hochkoeppler@unibo.it

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Abstract

We report here that the expression of protein complexes *in vivo* in *Escherichia coli* can be more convenient than traditional reconstitution experiments *in vitro*. In particular, we show that the poor solubility of *Escherichia coli* DNA polymerase III ε subunit (featuring 3'-5' exonuclease activity) is highly improved when the same protein is co-expressed with the α and θ subunits (featuring DNA polymerase activity and stabilizing ε, respectively). We also show that protein co-expression in *E. coli* can be used to efficiently test the competence of subunits from different bacterial species to associate in a functional protein complex. We indeed show that the α subunit of *Deinococcus radiodurans* DNA polymerase III can be co-expressed *in vivo* with the ε subunit of *E. coli*. In addition, we report on the use of protein co-expression to modulate mutation frequency in *E. coli*. By expressing the wild-type ε subunit under the control of the *araBAD* promoter (arabinose-inducible), and co-expressing the mutagenic D12A variant of the same protein, under the control of the *lac* promoter (inducible by isopropyl-thio-β-D-galactopyranoside, IPTG), we were able to alter the *E. coli* mutation frequency using appropriate concentrations of the inducers arabinose and IPTG. Finally, we discuss recent advances and future challenges of protein co-expression in *E. coli*.

Video Link

The video component of this article can be found at https://www.jove.com/video/52431/

Introduction

The introduction of overexpression technologies boosted either the biochemical studies of low-copy number enzymes and the industrial production of pharmacologically active proteins (*e.g.,* insulin). Since the advent of these technologies, significant advances have been achieved to increase the yield and quality of recombinant proteins. In addition, prokaryotic[1,2] and eukaryotic[3] overexpression systems were developed over the years, offering useful alternatives to the "work horse" of protein biotechnology, *i.e., Escherichia coli*. In particular, the availability of alternative platforms to *E. coli* led to the production of recombinant peptides or proteins bearing post-translational modifications. However, it should be mentioned that *E. coli* still represents the organism of choice for recombinant protein production. This is due to several factors, among which the most relevant can be considered: i) the availability of quite a number of overexpression systems (expression vectors and strains) for *E. coli*[1,2]; ii) the short generation times, and high biomass yields, of *E. coli* in a variety of rich and synthetic media; iii) the facile manipulation either at the biochemical and at the genetic level of this microorganism; iv) the isolation of strains capable of the production of toxic proteins[4]; v) the construction of strains featuring homogeneous induction at the population level[5,6]. In addition, it was recently shown that expression systems suitable for the production in *E. coli* of post-translationally modified proteins can be devised and constructed[2].

At present, protein overexpression is mainly used to obtain monomeric or homo-oligomeric proteins, whose hypersynthesis can be performed with a single gene cloned into an appropriate plasmid. However, attention was recently paid to the construction of *E. coli* protein co-expression systems, challenging the production, *in vivo*, of hetero-oligomeric complexes[2]. Interestingly, early experiments of protein co-expression addressed the inter-species assembly of large and small subunits of cyanobacterial ribulose-1,5-bisphosphate carboxylase/oxygenase[7,8], and the association of truncated and full-length forms of HIV-1 reverse transcriptase[9]. These pioneering studies demonstrated that protein co-expression represents a powerful alternative to traditional *in vitro* reconstitution. In addition, protein co-expression in *E. coli* was used to produce different proteins bearing post-translational modifications[2], to obtain proteins containing unnatural amino acids[2], and to increase the yield of overexpressed membrane proteins[2]. Moreover, the potential of protein co-expression as a tool to confer to *E. coli* competence in protein secretion is under active investigation[2].

Two main strategies of protein co-expression in *E. coli* can be pursued: i) the use of a single plasmid to host the different genes to be overexpressed; ii) the use of multiple plasmids in single cells to co-express the target proteins. In the first case, the criteria for the choice of plasmid do not differ from those of traditional single protein overexpression experiments, although particular plasmids containing tandem promoter/operator elements were constructed for co-expression[10]. This first approach is therefore quite simple. However, it should be mentioned that the use of a single plasmid to co-express different proteins faces two major difficulties: i) the molecular mass of the vector increases with the number of hosted genes, limiting the number of co-expressed proteins; ii) when multiple genes are cloned under the control of a single promoter, polarity can decrease the expression of the genes distal from the promoter. The use of dual or multiple plasmids in single *E. coli* cells has to

accomplish the compatibility of the vectors of choice, therefore imposing constraints to the eligible combinations of plasmids. However, this second co-expression strategy features the advantage of containing the molecular mass of vectors, and limits polarity. We recently constructed a protein co-expression system designed to facilitate the shuttling of genes between the co-expression plasmids[11]. In particular, we constructed the pGOOD vectors series, the relevant features of which are: i) a p15A origin of replication, to provide compatibility of the pGOOD plasmids with the commercial vectors containing the ColE1 origin (*e.g.,* the pBAD series[12]); ii) a tetracycline-resistance cassette; iii) the presence of *lac*-derived regulatory elements, *i.e.,* the Promoter-Operator(O_1) couple and the *lacI*^{q} gene. Using an appropriate pBAD-pGOOD couple, we were able to overexpress the catalytic core of *E. coli* DNA polymerase III, composed of three different subunits, *i.e.,* α (the 5'-3' polymerase), ε (the 3'-5' exonuclease) and θ (stabilizing ε)[13]. In particular, we demonstrated that the co-expression of the αεθ complex was strictly dependent on the addition to the *E. coli* culture medium of both IPTG and arabinose, triggering overexpression from pGOOD and pBAD, respectively (**Figure 1A**).

In the present report, we illustrate how protein co-expression can efficiently solve difficulties linked to the poor solubility of a protein complex subunit. In addition, we show how in *vivo* protein complementation tests can be performed, and we finally report on the use of protein co-expression to tune mutation frequency in *E. coli*. To this aim, we used pGOOD-pBAD couples suitable to illustrate relevant examples of each case study.

Protocol

1. Isolation of *E. coli* Co-transformants

1. Prepare electro-competent cells of the appropriate *E. coli* strain to be transformed. Transfer to 1 ml of LB medium (Tryptone, Yeast Extract, NaCl at 10, 5, and 10 g/L, respectively) a single colony of the strain of choice, and incubate at 37 °C under shaking conditions of 180 rpm. Dilute the pre-culture 1:500 in 25 ml of fresh LB medium and incubate the culture at 37 °C.
2. At log-phase (0.6 O.D.) centrifuge the cells suspension at 5,000 x g for 20 min), and resuspend the pellet in 10%, v/v ice-cold sterile glycerol-water in half of the original culture volume. Repeat this step 4 times, halving each time the resuspension volume. Finally, resuspend the pellet in glycerol/water and divide the cells suspension in 50 µl aliquots. Store the aliquots at -80 °C up to 6 months.
3. Dissolve the desired plasmid in sterile water supplemented with 0.5 mM EDTA. Thaw on ice an aliquot of electro-competent cells and mix with an appropriate amount (2.5-5 ng) of vector. Dispense the mixture into a 0.1 cm cuvette suitable for electroporation, and apply 1.8 kV.
4. Immediately transfer the electroporated cells into 1 ml of SOC medium (LB medium supplemented with 0.2% w/v glucose, 10 mM $MgCl_2$, 2.5 mM KCl), incubate for 1 hr under shaking, and finally transfer 100 µl aliquots to Petri dishes containing LB agar with the appropriate antibiotic. Incubate O/N at 37 °C.
5. Purify the transformants by streaking single colonies on Petri dishes.
6. Prepare electro-competent cells of the primary transformants and repeat steps 1.1 to 1.5 to transform with further plasmids.
7. Prepare glycerol stocks of the co-transformants. Transfer a single colony in LB supplemented with the appropriate antibiotics, incubate at 37 °C under shaking, and at log-phase (0.6 O.D.) centrifuge the cells suspension at 5,000 x g for 20 min. Resuspend the pellet in LB-antibiotics medium containing 15% v/v glycerol. Dispense in aliquots and store at – 80 °C.

2. Co-expression of α and ε Subunits of *E. coli* DNA Polymerase III

1. Transfer with a sterile loop a small amount of the appropriate strain glycerol stock (*e.g.,* TOP10/pGOOD-ε243 and TOP10/pGOOD-ε243-θ/pBADα1160, see **Figure 1**) into a Petri dish containing LB medium and antibiotics. Let the cells suspension dry onto the Petri dish, and streak the cells droplet. Incubate at 37 °C O/N.
2. Transfer, using a sterile toothpick, a single colony to 1 ml of LB-antibiotics medium. Incubate 8 hr at 37 °C. Dilute the pre-culture 1:500 in fresh medium and incubate at 30 °C for 15 hr.
3. Add IPTG, arabinose, or arabinose and IPTG, 1 mM each. Incubate at 30 °C for 2.5 hr. Collect the cells, and store the pellets at -20 °C.
4. Thaw pellets on ice and resuspend in lysis buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 2.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 8). Gently homogenize the cells suspension with a cold glass potter.
5. Sonicate at 15 W for 15 sec, followed by 15 sec cooling interval (4 cycles).
6. Centrifuge the total protein extracts at 10,000 x g for 20 min, at 4 °C to recover the soluble fraction.
7. Analyze by SDS-PAGE (12.5% acrylamide) aliquots of each soluble protein extract. To this aim, transfer 20 µl of each soluble protein extract to Eppendorf tubes containing 60 µl of H_2O and 20 µl of loading buffer (250 mM Tris-HCl pH 6.8, 10 mM β-mercaptoethanol, 10% (w/v) SDS, 50% (v/v) glycerol, 0.25% (w/v) bromophenol blue) and boil for 5 min. Load 18 µl of each sample and perform the electrophoresis at 140 V for about 1.5 hr.

3. Co-expression of α Subunit of *Deinococcus radiodurans* DNA Polymerase III and ε Subunit of *E. coli* DNA Polymerase III

1. Prepare a pre-culture of the *E. coli* strain TOP10/pBAD-α*Dr*/pGOOD-ε243 in 1 ml of LB-ampicillin-tetracycline medium and incubate 8 hr at 37 °C. Dilute 1:1,000 in fresh medium and incubate O/N at 37 °C.
2. Dilute 1:100 into 200 ml of fresh medium, incubate at 37 °C for 3 hr, then induce for 3 hr with arabinose and IPTG, 1 mM each. Harvest the cells, and store the pellet at -20 °C.
3. Resuspend the pellet in 50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF. Homogenize and disrupt the cells as described in 2.4 and 2.5.
4. Immediately centrifuge the cells lysate at 10,000 x g for 20 min. Discard the pellet. Filter the supernatant with a Büchner funnel equipped with 3 layers of paper filter. Apply gentle vacuum. To avoid excessive foaming, keep the vacuum Erlenmeyer flask on ice during filtration.
5. Determine protein concentration according to Bradford[14].

4. Gel Filtration Chromatography

1. Equilibrate a water-jacketed 16x70 (200) gel filtration column with 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8. Load onto the column the soluble protein extract. For optimal resolution, use a 1 ml sample loop. Perform the chromatography at 0.6 ml/min. Keep column temperature at 4 °C throughout.
2. Collect 0.9 ml fractions and analyze them by SDS-PAGE. Transfer 20 µl of each relevant fraction to Eppendorf tubes containing 60 µl of H_2O and 20 µl of loading buffer (250 mM Tris-HCl pH 6.8, 10 mM β-mercaptoethanol, 10% (w/v) SDS, 50% (v/v) glycerol, 0.25% (w/v) bromophenol blue) and boil for 5 min. Load 18 µl of each sample and perform the electrophoresis at 140 V for about 1.5 hr.
3. Determine the 3'-5' exonuclease and the DNA polymerase activity of each fraction in 96-well microplates. Assay the exonuclease activity using thymidine 5'-monophosphate p-nitrophenyl ester (*p*NP-TMP) as substrate[15]. Estimate DNA polymerase activity using the PPX (Pyrophosphatase, Purine nucleoside phosphorylase, Xanthine oxidase) enzyme-coupled assay[16] and 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) as the electron acceptor[16].

5. Mutation Analysis of Populations Co-expressing the Wild-type ε Subunit of *E. coli* DNA Polymerase III and the Mutagenic εD12A Variant

1. Transfer a single colony of *E. coli* TOP10 containing the pBAD-ε and the pGOOD1-εD12A[11] vectors to 1 ml of LB-antibiotics medium. Incubate O/N at 37 °C.
2. Dilute the pre-culture 1:250 in 3 flasks containing fresh medium (10 ml), add the appropriate inducers (arabinose, IPTG, or arabinose and IPTG, 1 mM each) and incubate at 37 °C for 8 hr. Prepare in parallel non-induced cultures.
3. Collect aliquots of 1 ml, then dilute 1:500 in new flasks containing fresh medium (10 ml) supplemented or not with the appropriate inducers. Incubate O/N at 37 °C.
4. Repeat steps 5.2 and 5.3 and collect aliquots of 1 ml.
5. Determine the number of generations occurred in each culture. Transfer on LB plates, 100 µl of appropriate serial dilutions of inoculum and culture. Incubate O/N at 37 °C. Count the colonies on the LB plates, and calculate the log of the number of cells present in the inoculum ($\log_I$) and in the culture at the end of growth ($\log_C$). To determine the number of generations, use the formula: ($\log_C$– $\log_I$)/0.301.
6. Centrifuge at 5,000 x g for 20 min and resuspend the cells in 1 ml of 50 mM Tris-HCl pH 7.6, 50 mM NaCl. To permeabilize the cells, add 2-3 drops of chloroform and vortex for 20 sec.
7. Determine the β-glucosidase activity of each aliquot in a 96-well microplate, using *p*-nitrophenyl-β-D-glucopyranoside (PNPGluc) as substrate. Add to each well 100 µl of permeabilized cells and 100 µl of substrate (16 mg/ml stock solution in H_2O). Take care to avoid air bubbles in the wells. Read the Absorbance at 420 nm, using a microplate reader and the appropriate filter.

Representative Results

The ε subunit of *E. coli* DNA polymerase III consists of 243 amino acids and features poor solubility[17,18], unless the residues 187-243 are deleted[17]. However, we have previously shown[11] that the co-expression of full-length α, ε, and θ subunits yields soluble DNA polymerase III catalytic core (**Figure 1**). In particular, using the pBAD-pGOOD co-expression system, we demonstrated that: i) the overexpression of α and ε subunits can be independently controlled by arabinose and IPTG, respectively (**Figure 1A**); ii) full-length ε subunit can be detected in soluble protein extracts isolated from *E. coli* cells overexpressing α, ε, and θ and subjected to gel-filtration (**Figure 1B**). In this case, exonuclease activity was distributed into 3 major peaks (**Figure 1B**, filled circles), and the peak centered at fraction 23 was shown to contain the αεθ catalytic core (**Figures 1C** and **1D**). The stability of ε is greatly increased upon binding to α and θ. When we previously overexpressed full-length ε in *E. coli*, a low amount of free ε was detected by western blotting in soluble protein extracts[19] (**Figure 2A**). It is interesting to note that, under the same conditions, we always detected higher amounts of free C-ter truncated forms of ε (ε-234, ε-228, ε-213, or ε-186), among which ε-186 performed better[19] (**Figure 2A**). We did also show that the proteolysis of ε C-terminal domain is prevented by the binding to α and θ subunits[19]. The effect, if any, of co-expression on solubility of ε can be easily tested. As reported in **Figure 2B**, soluble protein extracts isolated from cells overexpressing α, ε, or α and ε can be analyzed by SDS-PAGE, and conveniently compared with total protein extracts. In this case, the electrophoretic analysis indicates that, when overexpressed alone, ε subunit features poor solubility (compare lanes 1 and 2), while the co-expression of α greatly enhances the concentration of ε in soluble protein extracts (compare lanes 3 and 4).

Co-expression can also be used to perform inter-species complementation tests. Accordingly, we thought it might be of interest to evaluate the interaction between subunits belonging to the replicative machineries of $Gram^+$ and $Gram^-$ bacteria. *Deinococcus radiodurans* is a $Gram^+$ bacterium, featuring a large α subunit (1,335 amino acids, 150 kDa) and a putative ε subunit containing 197 amino acids. Interestingly, *D. radiodurans* ε subunit is devoid of the C-terminal domain present in its *E. coli* counterpart. Nevertheless, the identity and homology between *E. coli* and *D. radiodurans* ε subunits are equal to 29% and 59%, respectively (considering the region 1-178, *D. radiodurans* coordinates). To assay the competence of *D. radiodurans* polymerase III α subunit (α*Dr*) in binding *E. coli* ε subunit, we have cloned into the pBAD vector a synthetic gene coding for α*Dr* and we have co-transformed *E. coli* with pBAD-α*Dr* and pGOOD-ε. The simultaneous addition of IPTG and arabinose to the culture medium triggers the co-expression of α*Dr* and ε (**Figure 3A**). The association of these 2 proteins was tested by gel filtration. Cells co-expressing the DNA polymerase III α*Dr* and ε subunits were collected by centrifugation (5,000 x g, 20 min), suspended in lysis buffer, disrupted by sonication, and the soluble proteins were immediately loaded onto a Superdex 200 column (**Figure 3B**). As **Figure 3C** shows, when the collected fractions were subjected to 3'-5' exonuclease and to DNA polymerase activity assays, the activity peaks were detected at significantly shifted positions, suggesting that α*Dr* is not competent in binding *E. coli* ε subunit. This was confirmed when aliquots of fractions 32, 36, and 52 were concentrated and analyzed by SDS-PAGE. Fraction 36 was found to contain α*Dr* and to be devoid of *E. coli* ε subunit, while fraction 52 contained *E. coli* ε but was devoid of α*Dr* (**Figure 3D**).

Replication fidelity primarily relies on the ability of DNA polymerases to discriminate against erroneous base pairings during DNA extension. Nevertheless, mismatched deoxynucleotides can be incorporated at 10^{-4} frequency[20], and the action of 3'-5' exonucleases is essential to proofread the newly synthesized DNA strand. Moreover, it was estimated that this DNA editing action increases replication fidelity by 3 orders

of magnitude[20]. Accordingly, mutator strains can be constructed by impairing DNA proofreading, *e.g.,* by conditionally expressing a mutagenic variant of *E. coli* DNA polymerase III ε subunit. This can be obtained constructing a variant of ε competent in binding α but devoid of its proofreading activity, and controlling the production of mutagenic ε by appropriate expression systems. Using this strategy, it was shown that *E. coli* mutation frequency increases under conditions inducing mutagenic variants of ε[11,21]. However, no fine tuning of the mutation frequency was accomplished by this means. Protein co-expression can be used to obtain a better control of mutator strains. To this aim, we co-transformed *E. coli* with pBAD-ε and pGOOD1-εD12A, in order to induce the expression of the wild-type and the mutagenic D12A variant of ε with arabinose and IPTG, respectively. As a phenotypic test, we determined the appearance of β-glucosidase activity, which is cryptic (Bgl^-) in wild-type *E. coli*[22,23]. Spontaneous mutants able to utilize β-glucosides as carbon sources (Bgl^+) can be enriched or isolated using liquid[23] or solid media[24], respectively. When cells were induced to express the D12A mutagenic variant, β-glucosidase activity was acquired in ca. 20 generations (**Figure 4A**). It should be noted that a similar trend was observed in non-induced cells (**Figure 4A**), most likely because the transcriptional control exerted on pGOOD1 is rather leaky[11]. Nevertheless, when the wild-type ε subunit was induced, alone or in conjunction with the D12A variant, β-glucosidase activity was acquired by *E. coli* at moderate levels (**Figure 4A**). The β-glucosidase activity of Bgl^+ mutants was reported to range between 3 and 40 μM/min[23]. The level determined here in *E. coli* populations subjected to the expression of εD12A is much lower, but it should be considered that we did not attempt to isolate Bgl^+ mutants on solid media.

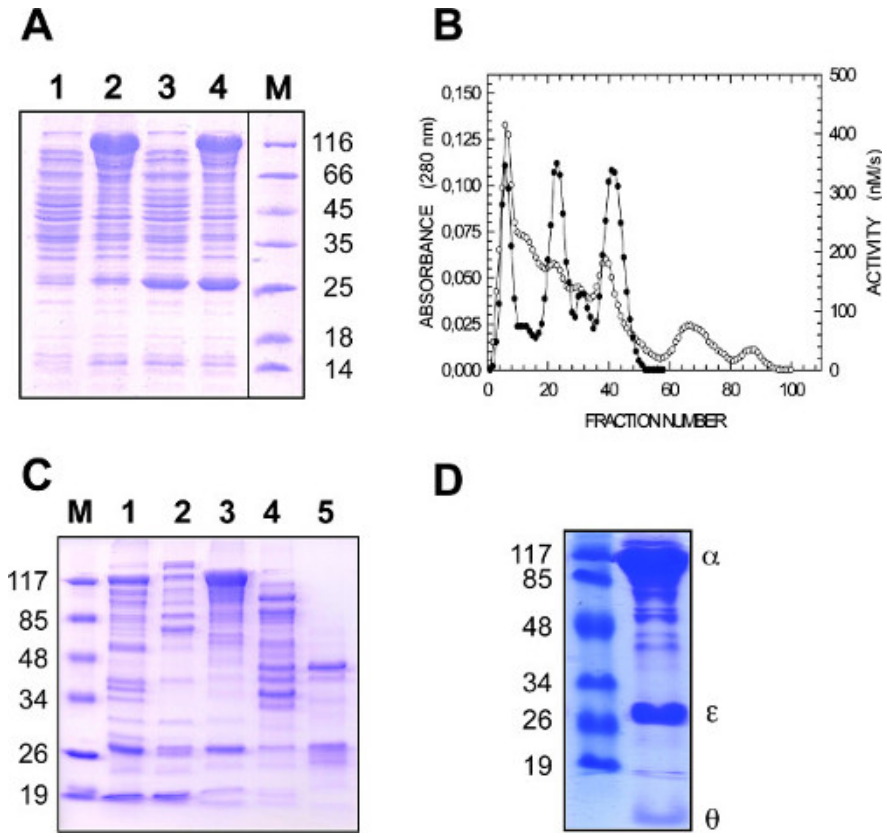

Figure 1. Co-expression of *E. coli* DNA polymerase III catalytic core. (**A**) SDS-PAGE of total proteins extracted from *E. coli* TOP10 / pBAD-α1160 / pGOOD-ε243 grown in the absence of inducers, in the presence of arabinose only, of IPTG only, or in medium supplemented with both arabinose and IPTG (lanes 1-4, respectively). (**B**) Absorbance (empty circles) and exonuclease activity (filled circles) of fractions isolated by subjecting to gel-filtration soluble proteins extracted from *E. coli* TOP10 overexpressing α, ε, and θ subunits of *E. coli* DNA Pol-III. (**C**) SDS-PAGE of fractions 6, 12, 23, 31, and 41 (lanes 1-5, respectively) reported in Panel B. (**D**) SDS-PAGE of a concentrated aliquot of fraction 23, whose exonuclease activity and electrophoretic pattern are reported in Panels B and C, respectively. Reprinted with permission from *Biotechnology Letters*, **33**, Conte *et al.*: "pGOODs: new plasmids for the co-expression of proteins in *Escherichia coli*", 1815-1821 (2011). Please click here to view a larger version of this figure.

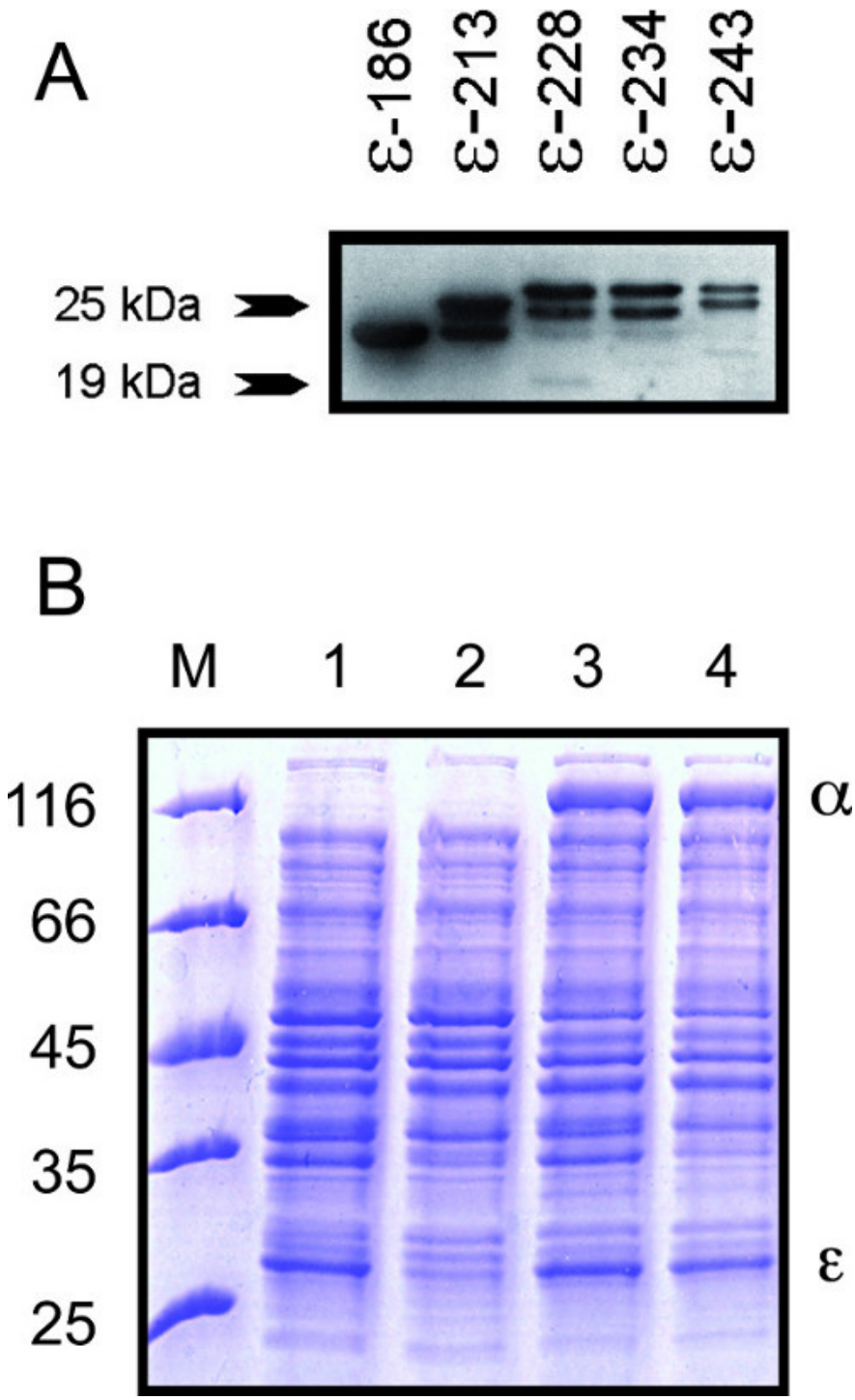

Figure 2. Proteolysis of *E. coli* DNA polymerase III ε subunit. (**A**) Degradation of monomeric as revealed by western blotting of proteins extracted from *E. coli* TOP10 overexpressing full-length or truncated forms of ε; arrowheads indicate the position of 25 and 19 kDa pre-stained molecular markers. Reprinted with permission from Elsevier from *Biochimica et Biophysica Acta Proteins and Proteomics*, **1794**, Bressanin *et al*.:"Proteolysis of the proofreading subunit controls the assembly of *Escherichia coli* DNA polymerase III catalytic core", 1606-1615 (2009). (**B**) Co-expression of α and ε subunits of *E. coli* DNA polymerase III catalytic core. SDS-PAGE of total (lanes 1 and 3) and soluble (lanes 2 and 4) protein extracts isolated from cells overexpressing ε subunit (lanes 1 and 2), or α and ε subunits (lanes 3 and 4).

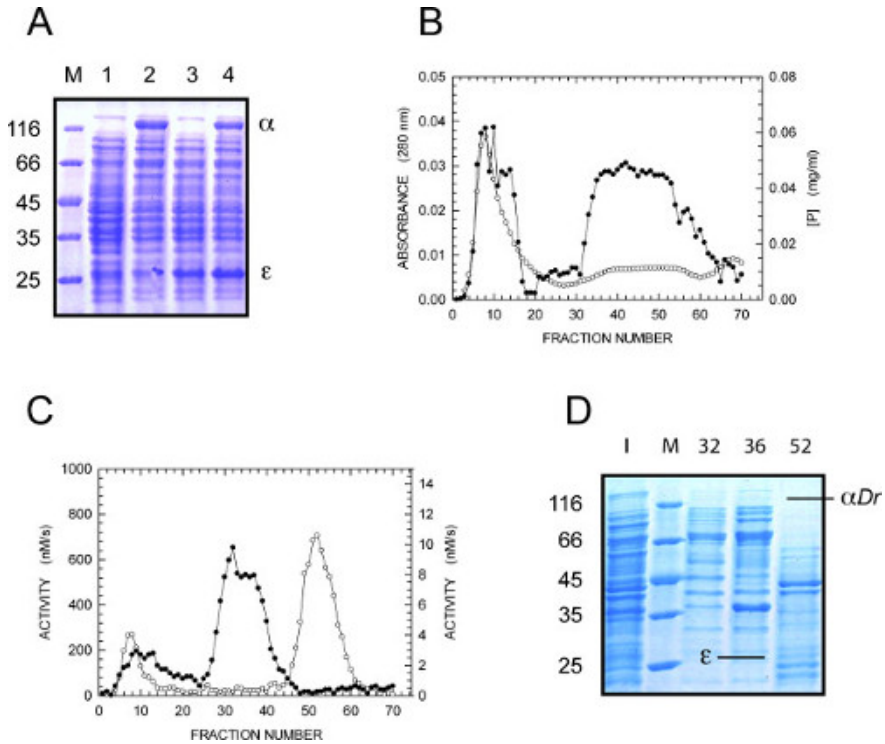

Figure 3. Co-expression of DNA polymerase III α and ε subunits from *Deinococcus radiodurans* and *Escherichia coli*, respectively. (**A**) SDS-PAGE of total protein extracts isolated from cells not induced (lane 1), or induced to overexpress α*Dr*, ε, or α*Dr* and ε (lanes 2-4, respectively). (**B**) Chromatogram (empty circles, left axis) and protein concentration of fractions (filled circles, right axis) eluted form a gel filtration column (Superdex 200) loaded with soluble proteins extracted from *E. coli* overexpressing α*Dr* and ε subunits. (**C**) Proofreading (empty circles, left axis) and DNA polymerase (filled circles, right axis) activities of the fractions reported in Panel B. (**D**) SDS-PAGE of concentrated aliquots of fractions 32, 36, and 52. Please click here to view a larger version of this figure.

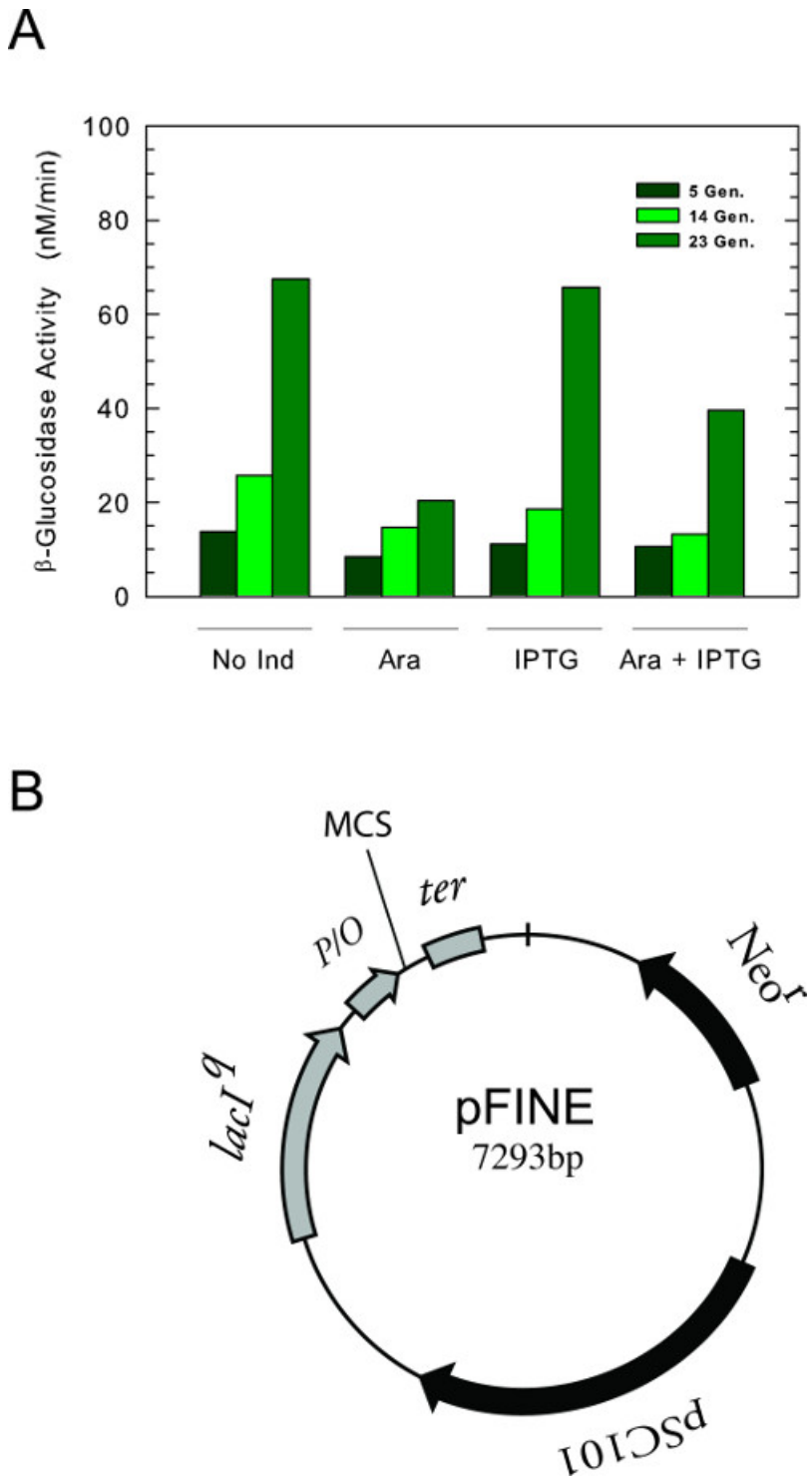

Figure 4. Mutation frequency of *E. coli* cells subjected or not to co-expression of the wild-type and the mutagenic variant D12A of DNA polymerase III ε subunit. The map of the new plasmid pFINE is also shown. (**A**) β-glucosidase activity of *E. coli* TOP10 not induced (No Ind) or subjected to overexpression of wild-type ε subunit (Ara), the mutagenic variant D12A (IPTG), or both wild-type and D12A ε subunits (Ara + IPTG). The β-glucosidase activity is reported as a function of generations. (**B**) Map of the new expression vector pFINE. The multiple cloning site (MCS) of this plasmid is identical to those present in the compatible pBAD and pGOOD vectors, letting facile shuttling of genes among them.

Discussion

Proteins can be intrinsically disordered, featuring regions whose tertiary structure is not restricted to a limited number of conformations[25]. These disordered proteins are usually prone to aggregation[25], and their isolation and characterization might represent a difficult task. The ε subunit of *E. coli* DNA polymerase III features two distinct domains[26,27], namely: i) the N-ter domain, bearing the 3'-5' exonuclease activity, and competent

in binding the θ subunit; ii) the C-ter domain, responsible for the binding to the α (polymerase) subunit. The C-ter domain of ε is known to confer poor solubility to this protein, promoting its aggregation. It was indeed demonstrated that ε-186, a truncated form of the protein devoid of the C-ter domain, is soluble, and can be purified with high yields[17]. However, when the interaction of ε with α has to be quantitatively studied (*e.g.,* to determine the K_D of the αε complex), the purification of full-length ε is required, implying a difficult biochemical task. In this frame, protein co-expression provides qualitative estimations of the binding of ε to α. We have shown that high yields of the αεθ complex can be obtained *in vivo* by co-expression (**Figure 1**), bypassing the poor solubility of ε subunit. Remarkably, the purification of overexpressed αεθ complex has been reported[28,29]. The co-expression strategy can also be used to test whether or not the insertion of site-specific mutations in one of the interacting partners impairs complex formation. Accordingly, protein co-expression represents a convenient and rapid tool to perform qualitative tests of protein-protein interaction. It should also be noted that our co-expression system relies on 2 different inducers, *i.e.,* arabinose and IPTG. Therefore, appropriate controls can be easily performed when testing the formation of a protein complex, *e.g.,* omitting one inducer from the bacterial growth medium (**Figure 1A**).

We presented here an optimized procedure for the co-expression of the α and ε subunits of *E. coli* DNA polymerase III. When this protocol was designed and tested, the following parameters were identified as critical to the success of co-expression experiments: i) the temperature at which induction is performed; ii) the time-length of induction; iii) the concentration of inducer(s). In particular, we were unable to obtain soluble αεθ complex[11] from cells induced at 37 °C, independently of the induction time. We therefore suggest to test different temperatures for the induction step, and to check the overexpression of proteins as a function of time. In addition, the parallel evaluation of co-expression in different *E. coli* strains can be of help when facing moderate yields of the target proteins.

We have reported here a representative experiment performed to test the binding of proteins from different species, and we have shown that protein co-expression is useful to rapidly test the association of 2 heterologous proteins into a hybrid functional complex. Gel filtration chromatography was used here to evaluate complex formation (**Figure 3**). However, the insertion of an appropriate tag to one of the interacting partners could facilitate this evaluation, letting the use of affinity capture methods. To avoid interference of the tag with protein-protein interactions, the gene coding for one of the interacting partners could be inserted in parallel into pBAD/His and pBAD/*Myc*-His vectors, respectively conferring a hexahistidine motif at the N- and the C-terminus of the target protein.

E. coli mutator strains feature mutation frequencies higher than their wild type counterparts. Mutator strains are of interest in biotechnology[30], *e.g.,* to rapidly evolve a target strain towards novel, desired, phenotypes. We provided here evidence that co-expressing the wild-type and a mutagenic ε subunit of *E. coli* DNA polymerase III, the mutation frequency of the bacterial host can be tuned with sufficient convenience. To further improve this technology, tightly-regulated expression vectors are necessary to repress as much as possible the expression of the strongly mutagenic D12A variant of ε subunit. In particular, it would be interesting to test the mutation frequency in *E. coli* transformed with the pBAD-εD12A and pGOOD1-ε, *i.e.,* the configuration alternative to that presented here. The pBAD vectors are indeed known to be tightly regulated[12], and this feature could help in keeping the basal concentration of εD12A at low levels.

The examples of protein co-expression reported here testify the multifaceted nature of this technique. However, it is important to note that using a multiplicity of compatible plasmids in single *E. coli* cells can greatly expand the challenges which can be taken up by protein co-expression. In recent years, intense work was devoted to expand the repertoire of compatible plasmids to be used for protein co-expression. In particular, a ternary system relying on compatible plasmids bearing the ColE1, p15A, and pSC101 origins of replication was used to co-express either bacterial and mammalian proteins[31]. Recently, a quaternary co-expression system was reported. In this case, 13 heterologous genes were co-expressed in *E. coli* co-transformed with 4 compatible expression vectors, containing the ColE1, p15A, CloDF13, and RSF origins of replication[32]. This co-expression system was successfully used to produce in *E. coli* functionally active soluble hydrogenase I from *Pyrococcus furiosus*[32]. To enlarge our binary system, we constructed a new expression vector, pFINE, containing the pSC101 origin of replication, a neomycin-resistance cassette, and the *lac* regulatory elements (**Figure 4B**). This plasmid contains the same polylinker present in pBAD and pGOOD vectors, thus facilitating gene shuttling among the 3 components of the co-expression system. Although pFINE is a low copy number plasmid, its stability was found to be satisfactory when tested in rich medium. We are currently engaged in further expansion of our ternary co-expression system. To this aim, we constructed a pBAD derivative containing a chloramphenicol-resistance cassette, and we are on the way to exchange the ColE1 origin of replication with one compatible with ColE1, p15A, and pSC101. It is indeed our opinion that the use of multiple plasmids in single *E. coli* cells represents a powerful tool to challenge the expression *in vivo* of protein complexes composed of a multitude of different subunits.

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Durani, V., Sullivan, B. J., Magliery, T. J. Simplifying protein expression with ligation-free, traceless and tag-switching plasmids. *Protein Expr. Purif.* **85**, (1), 9-17 (2012).
2. Hochkoeppler, A. Expanding the landscape of recombinant protein production in Escherichia coli. *Biotechnol. Lett.* **35**, (12), 1971-1981 (2013).
3. Geisse, S., Gram, H., Kleuser, B., Kocher, H. P. Eukaryotic expression systems: a comparison. *Protein Expr. Purif.* **8**, (3), 271-282 (1996).
4. Miroux, B., Walker, J. E. Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J. Mol. Biol.* **260**, (3), 289-298 (1996).

5. Khlebnikov, A., Datsenko, K. A., Skaug, T., Wanner, B. L., Keasling, J. D. Homogeneous expression of the PBAD promoter in Escherichia coli by constitutive expression of the low-affinity high-capacity AraE transporter. *Microbiology*. **147**, (12), 3241-3247 (2001).
6. Morgan-Kiss, R. M., Wadler, C., Cronan, J. E. Long-term and homogeneous regulation of the Escherichia coliaraBAD promoter by use of a lactose transporter of relaxed specificity. *Proc. Natl. Acad. Sci. USA*. **99**, (11), 7373-7377 (2002).
7. Tabita, F. R., Small, C. L. Expression and assembly of active cyanobacterial ribulose-1,5-bisphosphate carboxylase/oxygenase in Escherichia coli containing stoichiometric amounts of large and small subunits. *Proc. Natl. Acad. Sci. USA*. **82**, (18), 6100-6103 (1985).
8. Van der Vies, S. M., Bradley, D., Gatenby, A. A. Assembly of cyanobacterial and higher plant ribulose bisphosphate carboxylase subunits into functional homologous and heterologous enzyme molecules in Escherichia coli. *EMBO J*. **5**, (10), 2439-2444 (1986).
9. Amann, E., Brosius, J. ATG vectors for regulated high-level expression of cloned genes in Escherichia coli. *Gene*. **40**, (2-3), 183-190 (1985).
10. Scheich, C., Kümmel, D., Soumailakakis, D., Heinemann, U., Büssow, K. Vectors for co-expression of an unrestricted number of proteins. *Nucleic Acids Res*. **35**, (6), e43 (2007).
11. Conte, E., Landolfi, G., Vincelli, G., Stefan, A., Hochkoeppler, A. pGOODs: new plasmids for the co-expression of proteins in Escherichia coli. *Biotechnol. Lett*. **33**, (9), 1815-1821 (2011).
12. Guzman, L., Belin, D., Carson, M., Beckwith, J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol*. **177**, (14), 4121-4130 (1995).
13. McHenry, C. S. DNA replicases from a bacterial perspective. *Annu. Rev. Biochem*. **80**, 403-436 (2011).
14. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **72**, (1-2), 248-254 (1976).
15. Hamdan, S., *et al.* Hydrolysis of the 5'-p-nitrophenyl ester of TMP by the proofreading exonuclease (ε) subunit of Escherichia coli DNA polymerase III. *Biochemistry*. **41**, (16), 5266-5275 (2002).
16. Guillén Suárez, A. S., Stefan, A., Lemma, S., Conte, E., Hochkoeppler, A. A continuous enzyme-coupled assay of phosphate- or pyrophosphate-releasing enzymes. *BioTechniques*. **53**, (2), 99-103 (2012).
17. DeRose, E. F., *et al.* Model for the catalytic domain of the proofreading ε subunit of Escherichia coli DNA polymerase III based on NMR structural data. *Biochemistry*. **41**, (1), 94-110 (2002).
18. Ozawa, K., Jergic, S., Park, A. Y., Dixon, N. E., Otting, G. The proofreading exonuclease subunit ε of Escherichia coli DNA polymerase III is tethered to the polymerase subunit α via a flexible linker. *Nucleic Acids Res*. **36**, (15), 5074-5082 (2008).
19. Bressanin, D., Stefan, A., Dal Piaz, F., Cianchetta, S., Reggiani, L., Hochkoeppler, A. Proteolysis of the proofreading subunit controls the assembly of Escherichia coli DNA polymerase III catalytic core. *Biochim. Biophys. Acta*. **1794**, (11), 1606-1615 (2009).
20. Schaaper, R. M. Base selection, proofreading, and mismatch repair during DNA replication in Escherichia coli. *J. Biol. Chem*. **268**, (32), 23762-23765 (1993).
21. Selifonova, O., Valle, F., Schellenberger, V. Rapid evolution of novel traits in microorganisms. *Appl. Environ. Microbiol*. **67**, (8), 3645-3649 (2001).
22. Reynolds, A. E., Felton, J., Wright, A. Insertion of DNA activates the cryptic bgl operon in E. coli K12. *Nature*. **293**, 625-629 (1981).
23. Schaeffer, S. Inducible system for the utilization of β-glucosides in Escherichia coli. I. Active transport and utilization of β-glucosides. *J. Bacteriol*. **93**, (1), 254-263 (1967).
24. Miller, J. H., Suthar, A., Tai, J., Yeung, A., Truong, C., Stewart, J. L. Direct selection for mutators in Escherichia coli. *J. Bacteriol*. **181**, (5), 1576-1584 (1999).
25. Dunker, A. K., *et al.* The unfoldomics decade: an update of intrinsically disordered proteins. *BMC Genomics*. **9**, Suppl 2. (2008).
26. Taft-Benz, S. A., Schaaper, R. M. The C-terminal domain of DnaQ contains the polymerase binding site. *J. Bacteriol*. **181**, (9), 2693-2695 (1999).
27. Perrino, F. W., Harvey, S., McNeill, S. M. Two functional domains of the ε subunit of DNA polymerase III. *Biochemistry*. **38**, (48), 16001-16009 (1999).
28. Kim, D. R., McHenry, C. S. In vivo assembly of overproduced DNA polymerase III. Overproduction, purification, and characterization of the α, α-ε, and α-ε-θ subunits. *J. Biol. Chem*. **271**, (34), 20681-20689 (1996).
29. Maul, R. W., Ponticelli, S. K. S., Duzen, J. M., Sutton, M. D. Differential binding of Escherichia coli DNA polymerase to the β-sliding clamp. *Mol. Microbiol*. **65**, (3), 811-827 (2007).
30. Greener, A., Callahan, M. XL1-red: highly efficient random mutagenesis strain. *Strategies*. **7**, 32-34 (1994).
31. Chanda, P. K., Edris, W. A., Kennedy, J. D. A set of ligation-independent expression vectors for co-expression of proteins in Escherichia coli. *Protein Expr. Purif*. **47**, (1), 217-224 (2006).
32. Sun, J., Hopkins, R. C., Jenney, F. E. Jr, McTernan, P. M., Adams, M. W. W. Heterologous expression and maturation of an NADP-dependent [NiFe]-hydrogenase: a key enzyme in biofuel production. *PloS One*. **5**, (5), (2010).

