Improved soluble expression of the gene encoding amylolytic enzyme Amo45 by fusion with the mobile-loop-region of co-chaperonin GroES in Escherichia coli

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Abstract

The gene encoding the amylolytic enzyme Amo45, originating from a metagenomic project, was retrieved by a consensus primer-based approach for glycoside hydrolase (GH) family 57 enzymes. Family 57 contains mainly uncharacterized proteins similar to archaeal thermoactive amylolpullulanases. For characterization of these family members soluble, active enzymes have to be produced in sufficient amounts. Heterologous expression of amo45 in E.coli resulted in low yields of protein, most of which was found in inclusion bodies. To improve protein production and to increase the amount of soluble protein, two different modifications of the gene were applied. The first was fusion to an N-terminal His-tag sequence which increased the yield of protein, but still resulted in high amounts of inclusion bodies. Co-expression with chaperones enhanced the amount of soluble protein 4-fold. An alternative modification was the attachment of a peptide consisting of the amino acid sequence of the mobile-loop of the co-chaperonin GroES of E.coli. This sequence improved the soluble protein production 5-fold compared to His₆-Amo45 and additional expression of chaperones was unnecessary.

Keywords: Metagenome mining, glycoside hydrolase family 57, expression in E.coli, chaperones, Fusion with the mobile-loop of GroES

Introduction

The starch industry depends on biocatalytic processes to modify and fractionate starch. There is a constant need for new and better enzymes for starch and carbohydrate processing. The target is highly thermostable and highly active enzymes as well as provision of new primary products such as oligosaccharides of defined size, composition and degree of branching, new types of linkages, cyclic or more complex polysaccharides, and secondary sugar derivatives such as substituted starches and polyols. Most industrial enzymes, for example, starch-modifying enzymes of the α-amylase family have been obtained so far from organisms isolated from the natural environment. Only a small fraction of the bacterial population in soil and other habitats can be propagated in the laboratory which means there is a huge and largely unexplored wealth of genes in the environment. Metagenomics is an approach to extract DNA and prospective genes instead of isolating microorganisms from habitats and screen for useful genes. The mining of DNA from a geothermal habit from Iceland resulted in novel amylolytic enzymes of the glycoside hydrolase family 13 by applying a consensus primer-based approach for this glycoside hydrolase (GH) family (Labes et al. 2008). Besides GH13 family, amylolytic enzymes were also grouped in some other GH families, for example, family 70 and 77 (Cantarel et al. 2009). Recently new members of amylolytic enzymes, especially branching enzymes, were classified in GH family 57 (Palomo et al. 2010). As these enzymes exhibit interesting features like branching activities, a similar approach of mining environmental DNA
was used for α-amylases of the family GH57 and is described in this paper. Applying the consensus primer-based oligonucleotides approach on a metagenomic library derived from thermophilic microorganisms resulted, among others, in the gene amo45.

In order to characterize the gene product, over-expression was necessary. Here we chose E.coli as it is the best known and most commonly used host for expression of recombinant proteins. Despite many advantages, for example, number of vectors with strong promoters which facilitates high yields of protein production, some proteins are difficult to produce in large amounts and in an active form. This is caused by high or low GC content of the genes, rare codons in the host, instability of the mRNA or protein, and inclusion body formation.

To overcome such problems, codon optimization according to codon usage (Grantham et al. 1980; Ikemura 1981; Altenbuchner & Mattes 2005), co-expression of additional rare tRNA genes like argU, ileY, ilx, leuW, proL, and metT by using additional plasmid (Stratagene) or genetic-engineered strains (Novagen), fusion to maltose-binding protein (MBP) or to the N-utilization substance protein A (NusA) (Waugh 2005), and chaperone co-expression (Thomas et al. 1997; Nishihara et al. 1998) have mainly been utilized.

The chaperone family GroEL and its co-chaperone GroES play an essential role in mediating protein folding and preventing protein aggregation. The GroEL/GroES chaperonin system of E.coli has been studied most extensively (Hartl 1996; Xu et al. 1997; Hayer-Hartl 2002; Horwich et al. 2007). While GroEL contains 14 identical 57 kDa-subunits which together assemble to a double-heptameric-ring, GroES contains seven identical 10 kDa-subunits forming a heptameric-ring. The GroEL double-ring consists of an equatorial ATPase domain, an intermediate hinge-domain, and an apical domain exposing hydrophobic surface for substrate binding. The GroES ring complex covers the ends of the GroEL cylinder through its seven mobile loops which fold into a β-hairpin conformation upon binding to GroEL. This has been demonstrated by crystallographic and biochemical studies (Landry et al. 1993; Xu et al. 1997; Richardson et al. 1999). The binding of GroES changes GroEL into a new conformation, in which most of the previously exposed hydrophobic residues of the apical domain are involved in inter-subunit contacts, and have been replaced on the interior wall of GroEL by mostly hydrophilic residues. The consequence is the movement of the substrate into the GroES–GroEL cavity (Xu et al. 1997; Horwich et al. 2007).

Since the mobile loop of GroES mediates GroES–GroEL interaction, a peptide containing the amino acid sequence of the mobile loop of GroES was used to improve the expression of a highly toxic protein Bax under overexpression of GroEL in E.coli when attached to the N-terminus of the gene (Donnelly et al. 2001).

The amo45 gene encodes an α-amylase of the GH 57 family. When it was expressed in E.coli, only low amounts of protein were produced and most of it was found precipitated in inclusion bodies. To improve the protein solubility, we fused the mobile-loop encoding sequence of the co-chaperonin groES with the amo45 N-terminal end and constructed an expression vector based on the L-rhamnose inducible regulatory system of E.coli (Wegerer et al. 2008).

Here, we show that fusion with the mobile loop enhances the yield of soluble Amo45 protein.

**Materials and methods**

**Strain and plasmids**

The strain and plasmids used in this study are described in Table I.

**Isolation of the amo45 gene**

The amo45 gene was isolated from a DNA sample obtained from a hot spring in Grensdalur located in the southern part of Iceland after enrichment by addition of starch (Labes et al. 2008). A PCR-based screening using degenerate primers targeted against sequences encoding conserved amino acid sequences for enzymes of GH family 57 was used to retrieve a fragment of the amo45 gene. The primers (GH57-ECY-f, GH57-GWFF-r, Table II) were designed according to the CODEHOP strategy (Rose et al. 1998) as previously described for enzymes of the GH family13 CODEHOP primers (Labes et al. 2008; Turner et al. 2005). The PCR was carried out with TEG DNA polymerase (Matis production) with a PTC-0225 MJ Research thermal cycler as previously described (Labes et al. 2008). The resulting amplification products were separated on agarose gels and purified using GFX spin columns (GE Healthcare Life Sciences). The fragments with sizes of 800–1100 bps were selected and cloned into TOPO TA-sequencing vectors by the TA-cloning method (Invitrogen). Twenty-four clones from each band were sequenced with M13 forward and reverse primers on ABI 3700 DNA sequencers, using a BigDye Terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA). Similarity searches using BLAST were performed on the NCBI server (http://www.ncbi.nlm.nih.gov) and
multiple sequence alignments were done using ClustalX software.

The method for the retrieval of full-length genes has been described (Labes et al. 2008; Turner et al. 2005). In short, the flanking regions, upstream and downstream the fragments obtained with the degenerate primers, were amplified from the corresponding genomic DNA in a series of nested PCR reactions. In the first reaction, one gene-specific, 5′-biotin-labeled primer and one arbitrary primer (Arb1 or Arb2), targeting the unknown flanking sequence were used and a nested gene-specific primer downstream of the previous one and a primer (Arb3) targeting the 5′ consensus sequence of the previously used arbitrary primer was used in the second PCR. Table II lists the primers used for the retrieval of the amo45 gene. The resulting PCR products were cloned and sequenced as described above and the sequences were assembled with the existing amo45 gene fragment.

### Construction of expression vectors

Recombinant DNA techniques, for example, plasmid preparation, agarose gel electrophoresis were performed by conventional methods (Sambrook et al. 1989).

The plasmids for amo45 expression were derived from the L-rhamnose inducible expression vector pJOE5751.1. The vector contains a single NdeI site between the rhaP BAD promoter and His₆-eGFP sequence, a single BamHI site between His₆-tag and eGFP and a single HindIII site at the C-terminal end of eGFP. To construct the mobile-loop-eGFP

### Table I. Strains and plasmids used in this study.

| Strain or plasmid | Relevant features | Reference |
|-------------------|------------------|-----------|
| **Plasmids**      |                  |           |
| pGro7             | anaP BAD expression vector containing GroES and GroEL, Cm' | TaKaRa BIO INC. |
| pJOE5751.1        | L-rhamnose-inducible expression vector containing His₆-eGFP, Amp' | Wegerer et al. (2008) |
| pJOE3075          | L-rhamnose-inducible expression vector, Amp' | Stumpp et al. (2000) |
| pLEI45.1          | expression vector containing His₆-amo45, Amp' | This study |
| pLEI88.1          | expression vector containing mobile-loop-eGFP, Amp' | This study |
| pLEI90.1          | expression vector containing mobile-loop-amo45, Amp' | This study |
| **Strain**        |                  |           |
| E.coli K12 JM109  | F’traD36 proA' B’ lacIq Δ(lacZ)M15/Δ(lac-proAB) glnV44e14' gwrA96 recA1 relA1 endA1 thi hsdR17 | Yanisch-Perron et al. (1985) |
| E.coli JM109/pLEI45.1 | Expression strain for His₆-Amo45 | This study |
| E.coli JM109/pLEI45.1/pGro7 | Expression strain for His₆-Amo45 and GroEL-GroES | This study |
| E.coli JM109/pLEI90.1 | Expression strain for mobile-loop-Amo45 | This study |
| E.coli JM109/pLEI90.1/pGro7 | Expression strain for mobile-loop-Amo45 and GroEL-GroES | This study |

### Table II. Primers used in this study.

| Primer       | Nucleotide sequences | Application |
|--------------|----------------------|-------------|
| GH57-ECY-f   | GAGGATCGCAAACAAACTGCaartgytayyt | Screening |
| GH57-GWF-r   | GTGTGTCGCGCAAGAaaranacanwc | Screening |
| amo45-1bio   | GGATCCAGAGGAGTTATGTTGGAAGTATGGTATGGTTG | Retrieval |
| amo45-3      | TGTTGGCCATAATCGCGCCCTTCTCTTTCT | Retrieval |
| amo45-2bio   | GCTCCAGAGCTTCCTCTCAGGGTTGCGGAA | Retrieval |
| amo45-4      | TGGAAAGATGGAGGGATGACCTGTTG | Retrieval |
| amo45-6-bio  | TACACTCCAAAGACATACCGGTTACTG | Retrieval |
| amo45-8      | CCTCATGATCCTTGCTGTTGCGGAA | Retrieval |
| amo45-10-bio | TCCCTTGACGCAAGATCTGATCCCTCT | Retrieval |
| amo45-12     | AACCCGTCGAGGAGATTTCTCA | Retrieval |
| Arb1         | GGCACCCGCGTCGACTAGTACNNNNNNNNGATAT | Retrieval |
| Arb2         | GGCACCCGCGTCGACTAGTACNNNNNNNNNGGCCC | Retrieval |
| Arb3         | GGCACCCGCGTCGACTAGTAC | Retrieval |
| S8481        | TATGGAAATGGTGAAAACCAAAATCTGCTGTTGATCGATTTTCTCTGTTGCGG | synthesis of mobile-loop |
| S8482        | GATCCCGAGGCCGAGCGCAGACGCAGCTGCAAGATCCCGCCGACCAGCAGACTTTTGCATTCACTTCA | synthesis of mobile-loop |
| S7422        | AAAAATGATCAATAATTTATTCAGGTCACATTCTTTA | PCR for amo45 |
| S7423        | AAAAAAGCTTTCTATGCGG | PCR for amo45 |
plasmid pLEI88.1, the oligonucleotides encoding the amino acid sequence (EVETKSAGVLTG-SAAA) of the E. coli GroES mobile-loop and its complement were synthesized by Eurofins MWG Operon with codon optimization for E. coli (Donnelly et al. 2001). The oligonucleotides are described in Table II. Restriction sites for NdeI and a BamHI up- and downstream the mobile-loop sequence were used for integration of the complementary oligonucleotides into pJOE5751.1. Hereby the His\(_6\)-tag sequence was deleted and replaced with the mobile-loop sequence. The host strain for cloning and transformation was E. coli JM109. The sequence of the constructed plasmid pLEI88.1, containing mobile-loop-cGFP, was confirmed by DNA sequencing.

To construct the plasmids for His\(_6\)-amo45 and mobile-loop-amo45 expression, the vectors pJOE5751.1 (His\(_6\)-cGFP) and pLEI88.1 (mobile-loop-cGFP) were cut with the restriction enzymes BamHI and HindIII to eliminate the cGFP gene. The amo45 gene was amplified by PCR with Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific) using primers A Bell and a HindIII digestion site in the primer sequences enabled the insertion of the amo45 PCR product (2030 bp) into pJOE5751.1 and pLEI88.1. The new plasmids for expression of His\(_6\)-amo45 and mobile-loop-amo45 were designated pLEI45.1 and pLEI90.1, respectively.

**Gene expression**

Overnight cultures of E. coli JM109/pLEI45.1, JM109/pLEI45.1/pGro7, JM109/pLEI90.1, and JM109 pLEI90.1/pGro7 were diluted 100-fold in 10 ml LB (Lysogeny Broth) medium containing 100 μg/ml ampicillin and grown at 37°C to an optical density (OD\(_{600}\)) of 0.4. Induction of amo45 expression was achieved by adding L-rhamnose. Expression of groEL-groES genes under control of the arabinose promoter araP\(_{BAD}\) on plasmid pGro7 was achieved by addition of L-arabinose. Induction was done in two different ways, simultaneously by adding both sugars at the same time and in a successive way by adding first arabinose and later rhamnose. In the simultaneous protocol, the overnight culture was diluted 100-fold in LB containing ampicillin and 25 μg ml\(^{-1}\) chloramphenicol and at an OD\(_{600}\) of 0.4, 0.2% rhamnose and 0.05% L-arabinose were added at once and the cells were further incubated at 30°C for 4.5 h. In the successive induction protocol 0.1% arabinose was added at OD\(_{600}\) of 0.3 and the cells incubated for 3 h at 30°C. The cells were harvested, washed by centrifugation, and diluted to OD\(_{600}\) of 0.4. Then 0.2% rhamnose was added and the cells were incubated for another 4.5 h. For better comparison, JM109/pLEI45.1 and pLEI90.1 without pGro7 were treated with arabinose and rhamnose according to the successive induction protocol.

Finally, the cells were harvested by centrifugation and cell pellets corresponding to 10 OD\(_{600}\) units were suspended in 1 ml 0.1 mM potassium buffer (pH 6.5) and lysed by ultrasonication. The lysate was centrifuged for 15 min at 16,000 × g in a bench top centrifuge. The supernatant represented the soluble protein crude extract, while the precipitate, resuspended in the same buffer, represented the insoluble protein fraction.

**SDS-PAGE and enzyme assay**

The soluble and insoluble protein extracts were qualitatively analyzed by 10% SDS-PAGE. The protein concentration was determined with the Bio-Rad protein assay by measuring the absorbance at 595 nm (A\(_{595}\)) with a spectrophotometer (Pharmacia Biotech) using bovine serum albumin as standard. The enzyme activity of Amo45 was determined by measuring the absorbance at A\(_{405}\) resulting from the cleavage of the α-glucosidic linkage of 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNP-G3) by Amo45 and the release of the chromophore (2-chloro-4-nitrophenol) (Winn-Deen et al. 1988; Lorentz 1999). The reaction mixture consisted of 450 μl 0.1 mM potassium buffer (pH6.5), 25 μl enzyme sample containing Amo45 and 25 μl 10 mM CNP-G3 as substrate. The sample and buffer were incubated at first for 5 min at 85°C and then the substrate was added to start the reaction. The reaction was stopped by adding 1 ml 0.4 M Na-borate (pH9.8). The enzyme activity (U mg\(^{-1}\) protein) was defined as the release of 1 μM chromophore per min at 85°C using extinction coefficients of ε\(_{405,pH10}\) 17 × 10\(^3\) M\(^{-1}\) cm\(^{-1}\) for 2-chloro-4-nitrophenol.

**Results**

**Retrieval of the amo45 gene**

A set of degenerate CODEHOP primers targeted against sequences encoding conserved regions in enzymes of the GH family 57 was used to amplify gene fragments from environmental DNA prepared from hot spring samples (65°C, pH6) enriched with starch. BlastX analysis of one of the sequenced fragments indicated similarities with a protein from the hyperthermophilic bacterium Thermocrinis albus, annotated as GH of family 57. The complete gene, designated amo45, was retrieved in one step for the 5’ part and three steps for the 3’part. The predicted protein, Amo45 showed a high degree of homology (98% identity, eight amino acid residues exchanges)
to the uncharacterized GH family protein of *Thermoocrinus albus* DSM14484 (Genbank accession number YP_003474157.1) which was annotated during genome sequencing of *Thermoocrinus albus* DSM 14484. NCBI conserved domain search in both protein sequences revealed for the amino acid residues 1–288 an N-terminal putative catalytic domain of the GH family 57 (cd10797), followed by a domain of unknown function (DUF3536) (spanning amino acid residues 296–483) and an extended C-terminal region (amino acid residues 483–673) with no hits for conserved domains. Within the N-terminal putative GH57 catalytic domain of both proteins three amino acid exchanges were found whereas the catalytic amino acids His5, His7, and Glu139 were conserved in both proteins.

**Cloning and expression of the amo45 gene**

In a first attempt at heterologous expression of the native *amo45* in *E. coli*, the gene was inserted into the L-rhamnose inducible expression vector pJOE3075 (Stumpp et al. 2000). The protein production in *E. coli* JM109 was low and most of the protein was found in the insoluble fraction. Even expression at lower temperatures down to 22°C and/or using *E. coli* TOP10 as host did not prevent the formation of inclusion bodies (data not shown). To improve the expression, the gene was inserted in another L-rhamnose inducible expression vector pJOE5751.1 and thereby N-terminally fused to a His$_6$-tag sequence of the vector to allow His$_6$-Amo45 production. Rhamnose induction of JM109/pLEI45.1, harboring His$_6$-amo45, resulted in amounts of protein which were easily detected on SDS-PAGE. Again, most protein was in the insoluble fraction (Figure 1). The amount of soluble enzyme was sufficient to test its amylolytic activity. No activity was detected for the tested polymeric substrates starch, pullulan and amyllopectin (data not shown) and only cleavage of the α-glucosidic linkage of 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNP-G3) was found. Preliminary characterization of the His$_6$-Amo45 enzyme revealed a temperature optimum at 85°C. Crude extract of JM109/pLEI45.1 exhibited a specific enzyme activity of His$_6$-Amo45 of about 12 mU mg$^{-1}$ (Figure 3). To improve the solubility of His$_6$-Amo45 the effect of coexpression of chaperonin GroES–GroEL on the folding of His$_6$-Amo45 was studied. The genes *groEL* and *groES* were provided on the L-arabinose inducible vector pGro7. When the cells of JM109/pLEI45.1/pGro7 were simultaneously induced with rhamnose and arabinose, the specific enzyme activity of His$_6$-Amo45 in crude cell extract increased slightly to 15 mU mg$^{-1}$. A prominent protein band at a size of approximately 57 kDa on SDS-PAGE indicated a dominant production of GroEL (Figure 1). To exclude a competitive expression of His$_6$-amo45 and chaperones, the pGro7 was first induced for 3 h

![Figure 1. Expression of His$_6$-amo45 with and without co-expression of groEL–groES, analyzed by SDS-PAGE. Lanes 1–4: Crude extract of JM109 pLEI45.1 (His$_6$-amo45). Lanes 5–10: Crude extract of JM109 pLEI45.1/pGro7. The −, ++ and +/+ indicate uninduced, with arabinose and rhamnose simultaneously induced (4.5 h), and successively induced (3 h + 4.5 h) cell cultures, respectively. The M, S, and I labeled the molecular weight marker, soluble, and insoluble fractions, respectively.](image-url)
before the His$_6$-Amo45 production was started (see Material and Methods). This resulted in a much less-dominant protein band at 57 kDa. On the other hand, the chaperone co-expression increased the His$_6$-Amo45 enzyme activity 4-fold to 46 mU mg$^{-1}$ in the crude cell extract compared to the absence of GroEL–GroES overproduction. Obviously, a higher amount of GroEL–GroES in the cells improved the folding of His$_6$-Amo45.

**Construction and expression of mobile-loop-amo45**

The amo45 gene was introduced into the constructed expression vector pLEI88.1 to replace the eGFP and to generate pLEI90.1 (mobile-loop-amo45). Induction of JM109 pLEI90.1 with rhamnose resulted in a specific enzyme activity of about 60 mU mg$^{-1}$. This is in accordance with SDS-PAGE analysis (Figure 2) which showed a lower amount of insoluble protein. Unfortunately a higher amount of soluble protein could not be demonstrated due to overlap with an *E. coli* protein. By simultaneous expressing of groEL–groES and mobile-loop-amo45 in JM109/ pLEI90.1/pGro7, the specific enzyme activity decreased to 35 mU mg$^{-1}$ (Figure 3). It was assumed that this effect might be due to the dominant chaperone production (Figure 2). Therefore, groEL–groES and mobile-loop-amo45 were induced one after the other and the specific activity returned to 62 mU mg$^{-1}$. Thus, under these conditions additional expression of groEL–groES had no beneficial effect on the folding of mobile-loop-Amo45. Additionally, all of the non-induced samples showed a specific enzyme activity of about 1–2 mU mg$^{-1}$ and clearly indicated that the production of the Amo45-variants only occurred after induction with rhamnose.

**Discussion**

Metagenomic techniques opened the way to the vast and unexploited gene pool of uncultured microorganisms. Nevertheless, the process from identifying a gene in environmental habitats to the production of highly active enzymes is quite often long and tedious. Hurdles to overcome are incomplete genes, DNA sequencing errors, erroneous PCR, wrongly determined translational starts, low expression levels, lack of expected enzyme activities deduced from sequence comparisons, lack of special cofactors and last but not least, the correct folding of the protein during overexpression in the new host. Some of the problems can be solved by providing a strong promoter and optimized translation initiation region and maybe by resynthesizing the gene optimized for the host codon usage. In the case of amo45, protein production was considerably increased in *E. coli* by providing the rhamnose-inducible promoter and an optimized translation initiation region, which was achieved by fusion of amo45 with the His$_6$-tag sequence of the vector. To improve the correct folding of the protein, genes encoding various chaperones were co-expressed. No positive effect was seen with DnaK, DnaJ, and GrpE (data not shown) in contrast to GroEL–

![Figure 2](image_url). Expression of mobile-loop-amo45 with and without co-expression of groEL-groES, analyzed by SDS-PAGE. Lanes 1–4: Crude extract of JM109 pLEI90.1 (mobile-loop-amo45). Lanes 5–10: Crude extract of JM109 pLEI90.1 pGro7. The $-, ++$ and $+/+$ indicate uninduced, with arabinose and rhamnose simultaneously induced (4.5 h), and successively induced (3 h + 4.5 h) cell cultures, respectively. The M, S, and I labeled the molecular weight marker, soluble and insoluble fractions, respectively.
GroEL, which clearly assisted in the heterologous soluble expression of His$_6$-Amo45. However, it was important to induce groEL–groES and His$_6$-amo45 expression in a two-step protocol and not simultaneously. Obviously, the extremely high expression rate of the chaperones is in competition to the expression of His$_6$-amo45 and reduces optimal protein production.

Instead of co-expression of the groEL–groES genes we fused the mobile-loop sequence originating from groES to the N-terminal end of amo45. Despite the different N-terminal regions of mobile-loop-amo45 and His$_6$-amo45 the overall Amo45 protein production was about the same. On the other hand, this new N-terminal extension increased the amount of soluble Amo45 by 5-fold relative to that of His$_6$-tagged Amo45, when expressed under the same conditions. Most likely, the mobile loop, originating from GroES, increased the affinity of nascent peptide sequence of Amo45 for GroEL and hence the assistance of folding of Amo45 by GroEL improved. However, as pointed out by Donnelly et al. (2001) it seems also possible that this peptide extension facilitates initial steps in the folding process. Previously it had been shown that the mobile loop of GroES improved the expression of a gene encoding the highly toxic protein Bax when groEL–groES was expressed simultaneously (Donnelly et al. 2001). Without co-expression of groEL–groES, the mobile-loop-Bax production increased just slightly. This is in contrast to our results, where simultaneous co-expression with chaperones had a negative effect on the mobile-loop-amo45 expression. However expression of His$_6$-amo45 under successive co-expression of groEL–groES increased the amount of soluble protein. Obviously, under the conditions used here for the mobile-loop-amo45 expression, there is already sufficient GroEL–GroES in the cell to fully support folding of Amo45 and any high production of additional proteins is detrimental.

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