Strategies for the bacterial expression (and purification) of 'complicated' proteins

(you are encouraged to interrupt for questions in English or German)

https://www.embl.de/pepcore/pepcore_services/protein_expression/ecoli/optimisation_expression_levels/

http://wolfson.huji.ac.il/purification/Expression_Systems.html
Follow-up Questions:

1. What is a Strategy? Does A GENERAL STRATEGY exist?
2. What are complicated Proteins?
3. What means Expression? Where does expression start?
   What kind of expression product do we want?
   What do we want to do with the expression product?
4. What is Purification? Is there anything like a PURE protein?

The answers define the choice of experimental approach
Follow-up Questions:

• What is a Strategy? Does A GENERAL STRATEGY exist?

Answer:

No general strategy exists.

There is NO „cookbook“ with recipes!
What means Expression?

One gets an Expressionproduct; so it is a Production-process

A) General Strategic Decisions:

• Homologous Expression: = Expression of the Protein in a Host from which the Gene was derived

• Heterologous Expression: = Expression of the Protein in a Host differing from its Origin.

B) General Strategic Decisions:

• Bacterial Expression

• Expression in Eukaryotic Cells (mammalian, yeast, insect)

here we deal with bacterial expression
• What is a Complicated Protein?
What is a „Complicated Protein“?

Please, name an „Uncomplicated“ Protein!

**Answer:**

In a sense, all proteins are potentially „complicated“ when it comes to expression and purification.

There is **NO** „uncomplicated‘ protein“.
Where does expression start?

Expression starts with the right DNA construct:

4-7 bp upstream of start Met - AUG Shine-Delgarno sequence (AAGAAG) needed for ribosome-binding and translation start (usually present in bacterial expression plasmids)
Synthetic = Designed Genes

Advantages:
- no hassle with gene isolation from bacterial DNA or mammalin cDNA
- adaptation of codon usage to that of the expression host
- balancing AT/GC content in DNA
- rare tRNA triplet codons may be replaced
- ‘personalized’ N- or C-terminal tag sequences
- silent restriction sites may be introduced at critical positions for subsequent plans
- site for digestion by protease can be inserted
• 'Natural‘ or Synthetic DNA - Gene?

**Synthetic = Designed Genes**

**Disadvantages - Dangers:**

- rather costly
- long time-to-delivery (often 6-10 weeks)
- inadvertent intro of secondary structure complications in DNA/mRNA
- Removal of rare-triplets
- Inadvertent introduction of deleterious sequences such as
  - slippery sequences (6 A‘s or more, UUUAAAC) result in frequent frameshift
  - CAGCAG (GlnGln) results in stalled ribosomes
Popular removal of rare-triplets may be a mixed blessing after all:

Why?

Consider how a protein is translated and folded:

translation starts at the N-terminus, folding starts when peptide chain emerges from ribosome
folding takes time

Conclusion: too fast transcription may result in disorderly folding, may result in inclusion bodies

Rare triplets may slowdown translation, allow time for folding

Where??? (see later)
Observations:

Problems of bacterial protein expression often due to

• amount and rate of mRNA transcription and mRNA snarls;

• velocity of translation
  • (too rapid = inclusion body)
  • background translation because of leakiness (add 1% glucose to LB)
Cautious Conclusions:

Remedies for excessive transcription and translation rates

- low copy number plasmid
- temperature
- change culture medium
- place rare codons at strategic DNA positions
Cautious Conclusions:

Remedies for unsatisfactory translation

low copy number plasmid (up to 20/cell)

[intermediate up to about 100; high copy: > 100/cell]

(Definitions differ)

interdependance with temperature
(lower temperature usually result in lower copy number)

interdependance with length of insert: longer inserts decrease copy number
(metabolic burden for cell too large)

Problem of leakiness, i.e. expression without induction.
Remedies for unsatisfactory translation

lower expression temperature.

Additional measure:

inspect DNA sequence and locate rare triplet codons

slow-down of translation increases chance of individual domain folding
Remedies for unsatisfactory translation

☐ change culture medium:

usual medium is LB medium (lysogeny broth; often termed Luria-broth, Lennox-broth, Luria-Bertani medium)

changes involve going to

Terrific Broth or Super Broth (extremely rich)

OR

Minimal Medium (M9)
Possibilities to overcome expression in inclusion bodies

- lower temperature of pre-culture and induced culture
- got to M9 medium
- high salt stress (300 - 500 mM NaCl) +1 mM Glycinbetain as an osmoprotectant
We have experience with:

expression plasmids
pQE plasmids; pET plasmids; pRSET; pACYC)

concentration of inductor (IPTG; from almost nothing, e.g. 5 µM to rarely 1000 µM; mostly we stay in the 50 to 200 µM range)

temperature of expression (we stay in the 16°C to 25°C range)

length of expression; depends on expression temperature;
examples: 16°C, overnight; 25°C 3 hrs.

Usually, bacteria can be harvested and stored at -80°C.

Once bacteria are broken, stable storage of fractions may turn out to be problematic
Tagging a Protein

Questions:

- Purpose of tagging?
- Which tag shall be employed?
- N-terminal versus C-terminal tagging?
Tagging a Protein

Questions:

- Purpose of tagging?

  Identification by Western-blotting; Purification; Solubilization
Tagging a Protein

Questions:

- Which tag shall be employed?

**Small tags** (His$_6$ (~10aa); Myk (~10 aa); Flag (~10 aa); HA-tag (Human influenza hemagglutinin (~10 aa); S-tag (~15aa);

**Large tags** (Glutathion-S-transferase (~220 aa BIG); MBP-Tag (Maltose-binding-Protein (~400 aa BIG))
Tagging a Protein

Questions:

☐ N-terminal versus C-terminal tagging? At both ends?

N-terminal: often better tolerated
  on Westerns full-length and C-terminal degradation products visible
  purification includes all tagged proteins, proteolytic fragments

C-terminal: on Western full-length and N-terminal degradation products visible

Both end-tagging for special applications, e.g. in pDUET plasmids.

Tag-removal via pre-designed protease restriction sites
Most complicated proteins for expression:

Membrane Proteins
As soon as the membrane proteins shall be soluble **AND** functional things tend to get even more difficult!!!

**Classic approach:** express protein, purify membranes (vigorously), make protein soluble with detergent (which ones?), centrifuge 1hr at 100 000xg, take sup and purify (e.g. via affinity tag).

Based on our experience, this is easy to suggest, very difficult to do carry through.
Novel Strategies:

- Attach peptides to or within membrane protein which solubilize or facilitate solubilization (T4L, Mistic, YaiN, YbeL)
- Attach known well-expressed membrane domain for targeting purposes
- Cell-free expression of membrane proteins in presence of detergent.
Domain organization:

Six transmembrane spans with short linkers

C-terminal catalytic domain
T4L = Lysozyme of the T4 phage; 160 aa, hydrophilic. Insertion into different loops between transmembrane spans. When outside, enzymatically inactive mutant is needed to avoid hydrolysis of cell wall.

**Examples from the bench:**
TsR = Chemotaxisrezeptor für Serin aus E. coli mit 2 Transmembranspannen.

Example:

Protease-restriction site (TEV; Thrombin, Factor Xa, Proteinase K)
Cassette approach with Yain and YbeL from *E. coli*:

approx. 100 aa long, hydrophilic, predicted to be non-globular α-helical
YbeL-6TM-AC
  6TM-AC-YaiN
YaiN-6TM-AC
  6TM-AC-YbeL
YbeL-6TM-AC-YaiN
YbeL-6TM-AC-YbeL
YaiN-6TM-AC-YbeL
YaiN-6TM-AC-YaiN

6TM-AC (control)

In pET28a(+) E. coli BL21 or C43
Cell-free expression of membrane proteins

No detergent added
MP precipitate during synthesis

Detergent added, MP in detergent micells

Preformed liposomes added,
MP insert into liposomes
Fig. 1. Different modes for CF expression of MPs. MPs can be expressed as precipitate (Mode A), solubilized in an appropriate detergent and further reconstituted into proteo-liposomes, or they can be soluble expressed in the presence of detergent (Mode B), purified and reconstituted into proteo-liposomes. EM, electron microscopy.
Lit. References:


Cell-free:
Klammt, C. et al. (2005) FEBS J. 272:6024

YaiN/YbeL:

Mistic:
Thanks for your attention!