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Running Head:

Multiprotein complex production in *E. coli*: The SecYEG-SecDFYajC-YidC holotranslocon

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Summary

A modular approach for balanced overexpression of recombinant multiprotein complexes in *E. coli* is described, with the prokaryotic protein secretase/insertase complex, the SecYEG-SecDFYajC-YidC holotranslocon (HTL) used as an example. This procedure has been implemented here in the ACEMBL system. The protocol details the design principles of the mono- or polycistronic DNA constructs, the expression and purification of functional HTL and its association with translating ribosome nascent chain (RNC) complexes into a RNC-HTL supercomplex.

1. Introduction

About a third of the proteome in living cells is integrated into or transported across membranes, catalyzed by the ubiquitous Sec machinery which is conserved in all kingdoms of life. In prokaryotes, the Sec machinery contains the core translocon, SecYEG. Regulatory subunits including SecDF, YajC and YidC can associate with the core translocon giving rise to the SecYEG-SecDFYajC-YidC holotranslocon complex *(1-4)*. All subunits in this heptameric holotranslocon are transmembrane proteins (*3,5*).

Heterologous expression has been instrumental to advance protein research in the life sciences, and *E. coli* has largely dominated the field of recombinant expression, a trend which remains unbroken to date, although mammalian and insect cell based eukaryotic expression systems have been making their impressive mark more recently *(6-9)*. *E. coli* remains unsurpassed as a simple and cost effective expression host for protein production, and a very large number of plasmids and host strains are conveniently available to the research community for recombinant expression in this prokaryotic system.

2

In living cells, the catalysts of biological activity are typically not single, isolated proteins, but protein complexes which can contain many different subunits *(10,11)*. Many complexes are characterized by low abundance and heterogeneity in their native hosts, which is refractory to their extraction from native source material, and necessitates recombinant overexpression to purify them in sufficient amounts for detailed mechanistic studies. The SecYEG-SecDFYajC-YidC holotranslocon transmembrane protein complex, HTL, has been discovered in *E. coli* membranes, where it catalyzes the transport of membrane-bound or periplasmic proteins as they emerge from the actively translating ribosome *(3-5, 12-14)*. A particular challenge in expressing functional complexes such as HTL can be encountered when many heterologous subunits are co-produced from individual expression cassettes, resulting in imbalanced expression levels of the individual proteins and impedes proper complex assembly. Certain subunits can be expressed either more weakly of strongly, and, occasionally, one subunit may dominate the recombinant overexpression experiment to a degree that it becomes detrimental to overall yield. As a consequence, a complex containing all subunits at their physiological stoichiometric ratios cannot be obtained. Moreover, the HTL consists of assembly blocks which by themselves can form stable entities (SecYEG, SecDF and YidC), further complicating HTL production and purification as a homogeneous, functional assembly, prenecessitating elaborate promoter and expression cassette design, tag placement and systematic trial-and-error to achieve success *(13-15)*. Originally, only six subunits (SecYEG, SecDF and YidC) were thought to be present in HTL and thus included in the overexpression experiments *(15)*. Later, a seventh subunit, YajC, was identified and added to the overexpression setup to yield complete, functional and stable heptameric HTL *(12-14)*.

In order to meet these challenges and to produce and purify HTL in the quality and quantity required for detailed study, we developed ACEMBL, a modular overexpression

system for protein complex expression in *E. coli* (**Fig. 1**) *(15-17)*. ACEMBL affords the means to assemble mono- or polycistronic expression cassettes to optimally balance expression levels and achieve properly assembled complexes with correct subunit stoichiometry. ACEMBL consists of small, custom designed expression plasmids containing all elements required for expressing recombinant proteins in *E. coli* as an expression host (**Fig. 1**). Two families of plasmids exist, Acceptors and Donors (**Fig. 1**). Acceptors and Donors can each contain one or several genes of interest, arranged in (i) single gene expression cassettes consisting of a promoter, a gene and a transcriptional terminator, or (ii) in the form of polycistrons where several genes are transcribed from the same promoter, or a combination of both *(15)*. Donors and Acceptors, each containing one or several genes of interest, are conveniently fused by Cre recombinase to yield Donor-Acceptor fusions which contain all genes that need to be co-produced. Promoters, genes and terminators are in BioBrick design *(18)* and exchangeable in the individual Donors and Acceptors, a feature which enabled us to test the best combinations for producing functional HTL in parallel by trial and error within a reasonable timeframe until a suitable HTL expression construct was identified (**Fig. 2**). ACEMBL can be implemented in a robotics environment *(15, 16)*, however, for most applications in our laboratory (including HTL) production, the manual approach, optionally with a multichannel pipette, is sufficient to achieve success. We have described earlier the conceptual use of ACEMBL to generate HTL expression constructs *(13-15)*. In the present contribution, we describe the principle of HTL multigene expression construct assembly which can be used as a blueprint for other transmembrane or soluble complexes. We highlight important considerations for the production and purification of functional HTL (**Fig. 3**), and describe the assembly of HTL with actively transcribing ribosome nascent chain complexes (RNCs) into a RNC-HTL supercomplex (**Fig. 4**).

4

2. Materials

We strongly recommend carrying out the design of all expression constructs *in silico* using a DNA cloning software of choice (i.e. VectorNTI, ApE, others). Gene synthesis has become very affordable, and we prefer synthetic genes for generating the individual genes of interest, in which internal restriction sites for subcloning into the ACEMBL Donor and Acceptor plasmids are eliminated by design. We also recommend codon optimization for expression of the genes of interest, which is a service provided at no cost from synthetic DNA suppliers, and typically includes removal of potentially problematic RNA secondary structure elements in the transcripts. If synthetic genes are used, we further recommend to additionally eliminate any restriction sites that are part of the so-called multiplication modules in the plasmids *(15, 16)*. This allows for maximum flexibility of gene assembly for co-expression (e.g. if purification tag placement needs to be revised in later iterations). The modular concept of ACEMBL furthermore allows transferring expression cassettes between various plasmids *(15)*.

All reagents are prepared using ultrapure water (Millipore Milli-Q system or equivalent; conductivity of 18.2 MΩ·cm at 25°C) and analytical grade reagents. Buffers, antibiotics and enzymes are stored at -20°C.

2.1 Generation of Donor and Acceptor plasmids for HTL subassemblies

1. Restriction endonucleases and reaction buffers (New England Biolabs, NEB)
2. T4 DNA ligase and buffer (NEB)
3. Gel extraction kit (i.e. Qiagen, Germany)
4. Plasmid purification kit (i.e. Qiagen, Germany)
5. Regular *E. coli* competent cells (TOP10, HB101, or comparable).
6. *E. coli* competent cells containing *pir* gene (for Donor plasmids)

7. Antibiotics ampicillin, chloramphenicol, spectinomycin (for concentrations see Ref. 15)

8. Agar for pouring plates

9. Media (LB, TB, SOC) for growing minicultures

2.2 Materials for generating multigene Donor-Acceptor fusions expressing complete HTL

1. *E. coli* competent cells (TOP10, HB101, or comparable)

2. Cre recombinase enzyme (New England Biolabs, NEB)

3. Antibiotics ampicillin, chloramphenicol, spectinomycin (for concentrations see Ref. 15)

4. Agar for pouring plates

5. Media (LB, TB, SOC) for growing minicultures

2.3 Materials for producing HTL

1. *E. coli* competent cells C43(DE3)

2. ACEMBL:holo-translocon (HTL) multigene expression plasmid encoding for YidC, SecYEG, SecDF and YajC fitted with appriopriate affinity purification tags *(13, 14),*

3. 2xYT broth with antibiotics ampicillin, chloramphenicol, kanamycin

4. Isopropyl β-D-1-thiogalactopyranoside IPTG (Sigma Aldrich)

5. L-(+)-arabinose (>99%, Sigma Aldrich)

6. Cell disruptor (Constant Systems, Ltd)

7. TSG130 buffer [20mM Tris-Cl (pH 8.0), 130mM NaCl, 10% v/v glycerol]

8. *n*-dodecyl-β-D-maltoside (DDM, Sigma Aldrich)

9. Chelating Ni^{2+}-Sepharose Fast Flow column (GE Healthcare)

10. Superdex 200, 26/60 gel filtration column (GE Healthcare)

11. Q-Sepharose ion exchange column (GE Healthcare)

12. 50-kDa-molecular-weight cutoff centrifugation fliter (Amicon)

2.4. Materials for preparing HTL-ribosome nascent chain supercomplex

1. Purified ribosome nascent chain (RNC) complexes comprising a 108-amino acid long FtsQ nascent chain with a signal sequence (detailed preparation protocol see Ref *Schaffitzel/Ban.*)

2. DDM solubilized purified HTL.

3.   Methods

The genes encoding for the individual subunits are designed *in silico*, and then inserted into the Donor and Acceptor plasmid of choice. Once designed, mono- or polycistronic expression cassettes can be created by a variety of means including DNA synthesis, restriction/ligation cloning, ligation independent cloning (LIC) or sequence and ligation independent cloning (SLIC) or other methods *(19, 20)*, according to individual user preference. We recommend custom DNA synthesis to facilitate expression cassette construction, in particular if polycistronic expression cassettes are used as in the case of HTL.

3.1 *In silico* design

1. Group genes into functional units based on a set of criteria (known interaction partners, physiological (sub)assemblies, here YidC, SecYEG, SecDF and YajC). GenBank identifiers for HTL subunits are: SecY, WP_001118868.1; SecE, WP_001275702.1; SecG, AAN82372.1; SecD, ODA87210.1; SecF, AAN78997.1; YidC, WP_000378250.1; YajC, WP_000007629.1.

2. Decide on the number of expression cassettes to be co-expressed and the Donor and Acceptor plasmids you plan to use.

3. Decide on placement of tags and, optionally, on proteolytic sites to remove them (we recommend Tobacco etch virus (TEV) NIa protease and/or precision protease).

4. Decide on the use of polycistrons or individual expression cassettes, and the promoters to be tested. These can be the promoters provided by the standard ACEMBL plasmids (T7, lac), or include different promoters, as in the case of HTL, for example arabinose and trc promoters.

5. Generate the DNA sequence. Decide on DNA assembly strategy (SLIC, restriction/ligation, PCR assembly, others). Add custom restriction sites to 5' and 3' ends which are compatible with the polylinkers in the Donor and Acceptor plasmids in case you use restriction/ligation-based cloning.

6. Create all DNA sequences *in silico* and validate by simulating the reading frame in the *in silico* plasmid sequences.

3.2 Preparation of Donor and Acceptor plasmids

1. Choose from ACEMBL system components pACE1, pACE2, pDK, pDC, pDS to generate the expressing construct of choice. If promoters other than those already present on the original plasmids (T7, lac) are to be used, we recommend to create the 'empty' expression cassette first in the format promoter-polylinker-terminator, substitute the original expression cassette, and keep the resulting new Donor or Acceptor plasmid, fitted with the preferred promoter, in stock for future expressions of different complexes. All ACEMBL plasmids contain a LoxP site for generating Donor-Acceptor fusions by Cre recombinase. In the case of the HTL expression, an arabinose promoter (controlling a YidC-SecDF polycistron) and two trc promoters (controlling the SecYEG polycistron or the YajC gene, respectively), resulted in the construct yielding properly assembled complex *(13, 14)*.

2. Digest several micrograms Donor or Acceptor plasmid by the restriction enzymes selected by *in silico* design according to manufacturers' recommendation.

3. Analyze the digestions by agarose gel electrophoresis to confirm that the digestions are complete.

4. Purify digested plasmid by using commercial gel extraction kits (for example Qiagen gel extraction kit). Elute the extracted DNA in the minimal volume defined by the manufacturer. Determine the concentration of the extracted DNA spectrophotometrically (e.g. Thermo Scientific NanoDrop 2000). Store in frozen aliquots.

3.3 Inserting genes into digested Donor or Acceptor

1. Digest several micrograms of the DNA (generated by DNA synthesis, SLIC, PCR assembly or other method of choice) encoding for the desired individual gene or polycistron (the 'insert') with the enzymes identified by *in silico* design, according to the manufacturers' recommendation. For polycistron generation, follow the guidelines and protocols in Ref. 15)

2. Purify digested insert DNA by using a commercial gel extraction kit. Elute the extracted DNA in the minimal volume defined by the manufacturer. Determine the concentration of the extracted DNA spectrophotometrically (e.g. Thermo Scientific NanoDrop 2000).

3. Set up ligation reactions by mixing purified insert and digested and gel extracted Donor or Acceptor, respectively (see **Subheading 3.2**) in 10-20 µL reaction volume with T4 DNA ligase and perform ligation reactions at 25ºC overnight. Optionally, analyze the ligation reaction by agarose gel electrophoresis to evaluate the ligation efficiency.

4. Transform *E. coli* competent cells (TOP10 or HB101 for Acceptors, *pir* gene containing cells for Donors; see **Note 1**) with ligation reaction mixture. Incubate the transformation reaction in a 37°C shaker for several hours and plate on agar plates in a dilution series to ensure optimal colony separation.

5. Pick colonies, grow minicultures and purify plasmids using standard procedures.

6. Identify positive clones by restriction digestion and DNA sequencing of the insert.

3.4 Cre-LoxP fusion of Donors and Acceptor

1. Prepare 20 ul reaction mixture for Cre reaction by combining 100 ng each of Donor and Acceptor plasmids. Add Cre enzyme and Cre buffer according to the recommendations of the supplier. Incubate at 30°C. Transform regular *E. coli* competent cells (TOP10, HB101, or comparable) with Cre reaction mixcture following standard transformation protocols. Incubate the transformation reaction in a 37°C shaker for one or several hours.

2. Plate the transformation reaction on agar plates containing the proper antibiotics combination (for HTL: ampicillin, chloaramphenicol, kanamycin). Incubate at 37°C over night.

3. Pick colonies and inoculate 25 mL aliquots of LB medium supplemented with corresponding antibiotics. After overnight incubation, prepare Acceptor-Donor fusion plasmid using standard kits (Qiagen). Predict Acceptor-Donor fusion plasmid sequence by using web-based Cre-ACEMBLER software *(21)*. Check plasmids by restriction digestion using appropriate enzymes identified by restriction pattern prediction (see **Note 2**).

3.5 Producing recombinant HTL

1. Grow freshly transformed *E. coli* C43(DE3) with ACEMBL plasmid encoding for HTL (Fig. 2) in 2xYT broth with antibiotics to $OD_{600} = 0.8$.

2. Induce for 3h by adding 1 mM IPTG and 0.2% (wt/vol) arabinose.

3. Pellet cells by centrifugation and break cells at 25 kpsi with cell disruptor in TSG130 buffer.

4. Collect membrane fraction containing HTL and solubilize by rotation in TSG130 containing 2% (wt/vol) DDM for 1h at 4°C.

5. Clarify DDM-soluble fraction by centrifugation and purify by using Ni^{2+} metal affinity chromatography (column pre-equilibrated in TSG130 containing 0.1% DDM).

6. Wash column thoroughly (10 column volumes) with buffer containing 30 mM imidazole.

7. Elute bound HTL with buffer containing 500 mM imidazole.

8. Pool peak fractions and purify immediately using a S200 size exclusion column, placed in-line with a Q-Sepharose column equilibrated in TSG130 + 0.05% DDM.

9. Pool peak fractions and concentrate (50kDa cutoff Amicon filter).

10. Concentrate to 5-10mg/ml (molar extinction coefficient: ε_{HTL} = 497,000 M-1cm-1 (*13*).

3.6 Assembling RNC-HTL supercomplex

1. Mix DDM-solubilized HTL and purified RNC[FtsQ] in a 25:1 molar ratio.

2. Isolate resulting RNC[FtsQ]-HTL supercomplex by sedimentation centrifugation through a sucrose cushion to separate unbound HTL from ribosome associated HTL as described earlier for the core translocon, SecYEG, bound to RNC (*21).

4. Notes

1. Donors and their derivatives can only be propagated in cells that express the *pir* gene (such as BW23473, BW23474, or PIR1 and PIR2, Invitrogen) due to the conditional origin present on these plasmids *(23)*. In contrast, Acceptors and their derivatives contain regular ColE1 origin of replication and can be propagated in regular *E. coli* strains (TOP10, HB101, or comparable).

2. Cre-ACEMBLER application software can be downloaded from https://github.com/christianbecke/Cre-ACEMBLER/downloads/.

   Accompanying information can be downloaded from www.embl.fr/multibac/multiexpression_technologies/cre-acembler/.

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Competing financial interest statement

The authors declare no competing financial interest.

Figure Legends:

Figure 1. ACEMBL system components. (A) ACEMBL consists of Donor (pDC, pDK, pDS) and Acceptor (pACE) plasmids into each of which one or several genes, optionally as polycistrons, can be inserted by a variety of cloning routines. Ap, ampicillin; Cm, chloramphenicol; Kn, kanamycin; Sp, spectinomycin. R6Kψ, phage-derived conditional origin of replication; BR322, regular *E. coli* replicon. Transcriptional terminators are indicated as boxes filled in black. A multiplication modules is shown as boxes filled in blue. LoxP sequences are indicated as circles filled in red. T7 and Lac promoters are shown as triangles filled in black or grey, respectively. Promoters and terminators can be exchanged to different promoters (ara, trc, others) if needed. MIE stands for multi-integration element and indicates a polylinker that facilitates polycistron assembly *(15)*. **(B)** Cre-LoxP mediated fusion of one or several Donors with an Acceptor, each carrying one or several expression cassettes of interest, results in multigene expression constructs for protein complex production (diagram adapted from Ref. 16). The Cre reaction is an equilibrium reaction with the excision reaction being preferred over fusion. When equilibrium is reached, Donor and Acceptor plasmids co-exist with higher order multigene fusion constructs (AD, ADD, …). All DNA entities, educts and products, present in the Cre reaction are quasi bar-coded by their resistance marker combinations and cen be identified by transformation and subsequent challenge with antibiotics *(15)*.

Figure 2: The ACEMBL HTL expression construct. The hexa-histidine tags and the calmodulin-binding peptide (CBP) tag are marked in red and orange respectively (image adapted from Ref. 13). ara, arabinose promoter, trc, modified, tight lac promoter. LoxP sites are depicted as circles filled in grey, transcriptional terminators are shown as boxes filled in black.

Figure 3: Purification of the E. coli HTL. **(A)** Representative gel filtration/ion exchange elution profile of the HTL evidencing a single peak, demonstrating the copurification of all HTL subunits in a single complex. **(B)** SDS-PAGE analysis of peak fractions 16–24 (corresponding to elution volumes of the profile displayed in A marked by dotted lines). The asterisk represents a proteolytic break-down fragment of SecY.

Figure 4: HTL-RNC supercomplex. (A) Interaction of the HTL^{DDM} with ribosome nascent chain complexes (RNC) analysed by sedimentation experiments and SDS-PAGE. Molecular weight marker is shown on the left. Commassie stained protein bands corresponding to HTL components are marked on the right. **(B)** Atomic model of the ribosome-SecYEG complex fitted into a preliminary cryo-EM structure of the RNC^{FtsQ}-HTL^{DDM} complex. The density of the HTL is larger than SecYEG alone, and an additional strong connection to the ribosome was identified (indicated by the red arrow).
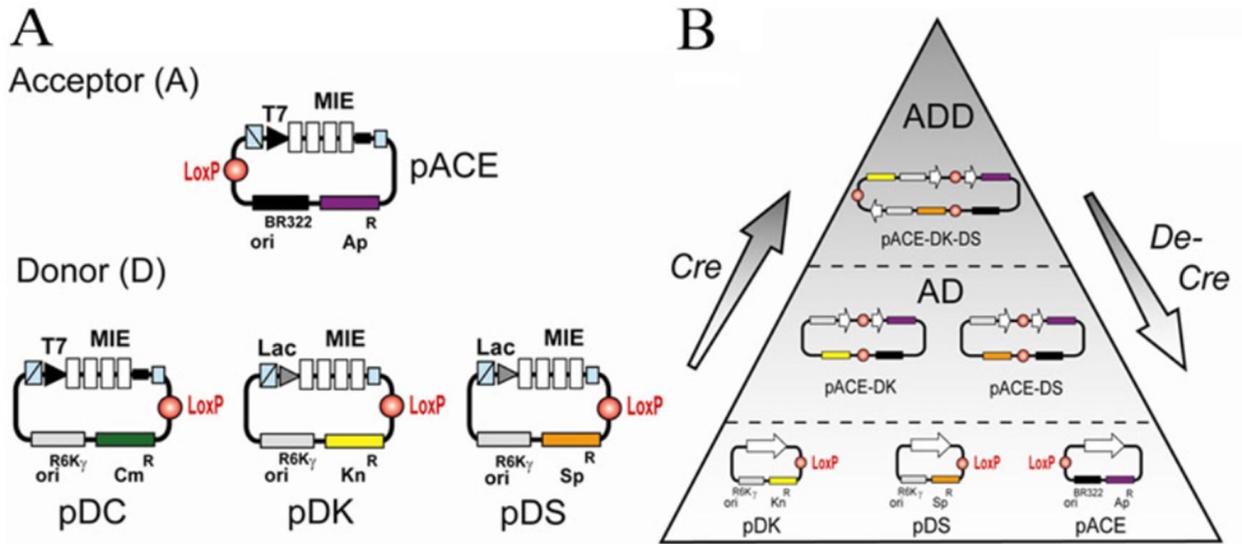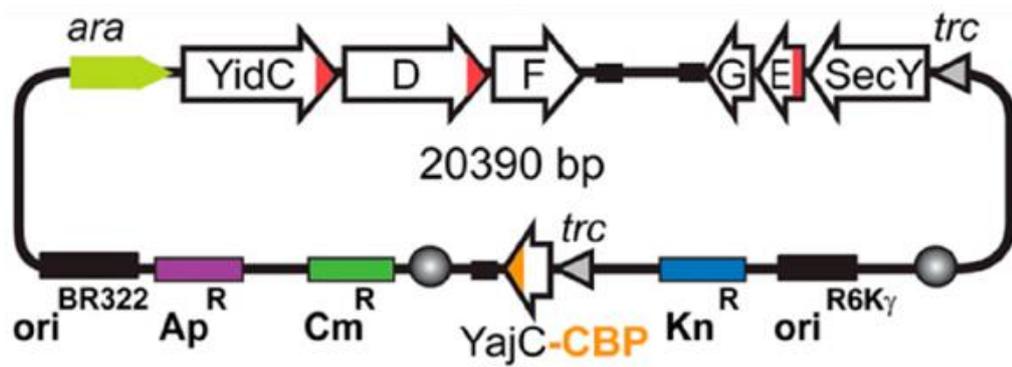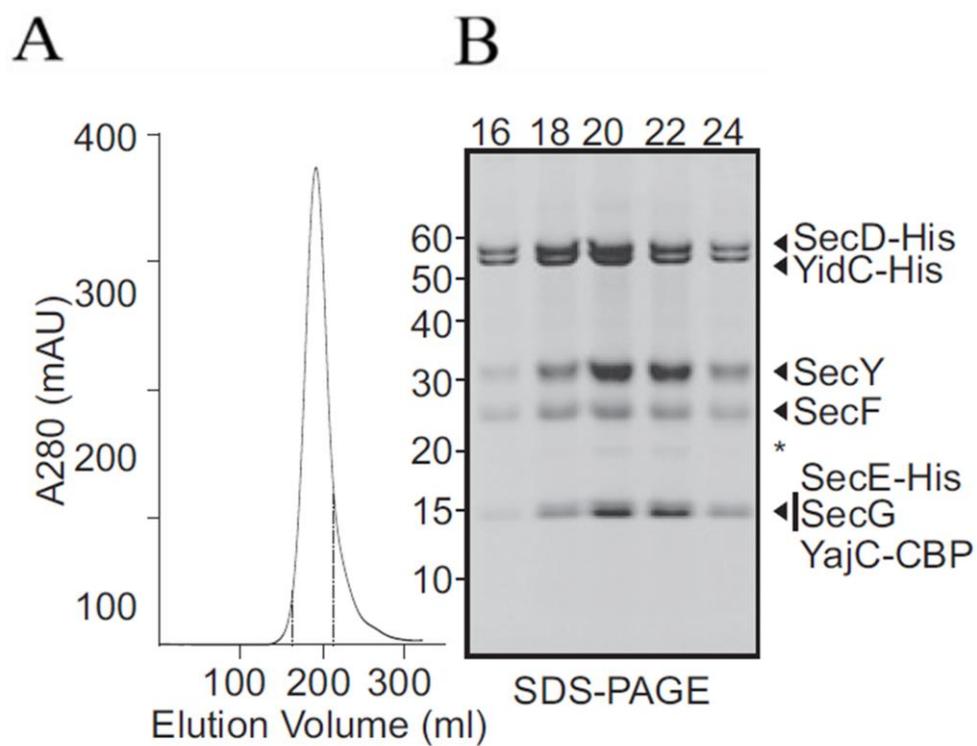
Figures:

Figure 1.

Figure 2.

Figure 3.

Figure 4.