Large complexes: Cloning strategy, production and purification

Eric Durand, Roland Lloubès

Chapter 24. Large complexes: Cloning strategy, production and purification

Running Head: Membrane protein complex purification

Eric Durand and Roland Lloubes

attention abbreviations

Laboratoire d'Ingénierie des Systèmes Macromoléculaires UMR7255, Institut de Microbiologie de la Méditerranée, CNRS/Aix-Marseille Université, 31 chemin Joseph Aiguier, 13402 Marseille cedex 20, France

E-mail: roland.lloubes@imm.cnrs.fr

i. Summary

Membrane proteins can assemble and form complexes in the cell envelope. In Gram-negative bacteria, a number of multiprotein complexes including secretion systems, efflux pumps, molecular motors or pilus assembly machines comprise proteins from the inner and the outer membrane. Beside the structures of isolated soluble domains, only few atomic structures of these assembled molecular machines have been elucidated. To better understand the function and to solve the structure of protein complexes, it is thus necessary to design dedicated production and purification processes. Here, we present cloning procedures to overproduce membrane proteins into *E. coli* cells and describe the cloning and purification strategy for the Type VI secretion TssJLM membrane complex.

ii. Key words: membrane protein complexes, *E. coli*, T7 overexpression, protein purification.

1. Introduction

Protein overproduction results from cloning a gene of interest into a plasmid vector, downstream of a tightly regulated promoter, and from inducing its expression after plasmid transformation

into a bacterial strain. For large protein complexes containing multiple subunits, the genes encoding the different subunits can be expressed under the control of an inducible promoter either from single plasmid containing a cluster of genes or from different compatible plasmids harboring single or multiple genes.

1.1 Cloning vectors

Several inducible promoters have been described and are available to overexpress a gene of interest. These promoters are usually cloned into vectors that also contain the gene encoding the cognate regulatory protein and a transcriptional terminator to prevent non-productive transcription from downstream gene (***1-7***). The *tac* and *trc* promoters that contain the -35 and -10 sequences from the *trp* and *lacUV5* promoters, respectively, have been optimized for high expression level ***(1).*** Other *E. coli* tightly regulated promoters such as the *tetA* promoter and its repressor TetR, the *araBAD* promoter and its cognate activator AraC (***3-6***). In addition, heterologous combinations of promoter/regulator binding sequences can be used ***(2)***. The last family of inducible promoters gathers sequences that are not recognized by the *E. coli* RNA polymerase but rather recognized by phage RNA polymerases such as the SP6, T3 and T7 promoters. Vectors and strains that express the T7 RNA polymerase (T7RNAp) have been extensively developed. Three independent methods are used to regulate the T7 expression systems. First, the expression of the chromosomally- or plasmid-encoded T7 RNAp gene can be itself under the control of an inducible promoter (*see* **Note 1**). Second, the inhibition of the T7 RNAp basal activity can be controlled by producing the T7 lysozyme under constitutive or regulated conditions ***(8, 9)***. Last, the transcription by the T7 RNAp can be repressed by the Lac repressor, adding the *lac* operator sequence ***(10)***, the *lacI* gene being cloned on the expression vector.

A large choice of cloning vectors with different characteristics is now available and can be selected based on the toxicity, stability and folding rate of the protein or protein complex to overproduce:

- production of proteins in the cytoplasm, the periplasm or the membrane of *E. coli* (using the addition of synthetic N-terminal signal-sequences such as that of the OmpA and PelB proteins).
- compatible T7 expression vectors containing one or two T7 promoters to overproduce protein complexes with up to eight subunits (see Duet Vectors from Novagen).
- Protein tagging sequence (6 to 10 × His, *Strep*-Tag II...) or fusion partner (protein G, glutathione-S-transferase: GST, calmodulin-binding peptide: CBP, Maltose binding protein: MBP) to increase protein solubility or to simplify the purification using affinity chromatography techniques. In addition, these tag affinity sequences can be removed using specific protease (most used proteases: Tobacco Etch Virus Protease: TEV, enterokinase, Thrombin, Xa Factor, PreScission..). For this purpose, the corresponding protease recognition sequence is inserted either downstream (for N-terminal tagging) or upstream (for C-terminal tagging) of the tag sequence. After protease digestion, purified protein complex is submitted to a new purification step to remove the protease, the uncleaved protein and the tag peptide (for example affinity chromatography to remove the His-tag peptide and the His-tagged TEV).

Currently, cloning into these vectors is facilitated by PCR techniques based on gene and plasmid amplifications, leading to restriction site/ligation free cloning methods. These techniques have proven to be efficient to perform gene expression strategies in short delay (*see* section 3.1 and **Note 2**).

If none of the overproduction strategies are sufficient to produce and purify protein complexes, *in vitro* alternatives such as cell-free transcription-translation systems, based on

T7RNAp and *E. coli* cell extracts, have been found efficient to produce milligram amounts of some membrane proteins ***(11)***.

1.2 Membrane protein complex overproduction and purification

Membrane protein complexes have been successfully overproduced and purified based on pBAD or T7 vectors. For example, the Tol proteins from the Tol cell envelope molecular motor were overproduced and specifically radiolabeled with ^{35}S-Methionine after cloning into a pT7-driven vector using restriction sites (RS) and ligation techniques (RSl) (***12, 13***, *see* **Note 3 and Note 4**). Sub-complexes from the Ton system (***14-17***), the PomA-PomB flagellar rotor (***18***), the AcrAB-TolC efflux pump (***19***), the Type IV and Type VI secretion systems (T4SS and T6SS, respectively; ***20, 21***) and the β-barrel assembly machinery (***22***) have been also successfully purified using similar approaches.

In addition to the cloning strategy and the production to levels compatible with purification, studying these multiprotein machines also requires to extract and solubilize the protein complex without disrupting the contacts between the subunits. Finally, since the overproduction condition may induce stoichiometry artifacts, the tagging of the minor subunit or the specific tagging of different subunits should be performed to purify homogenous protein complexes (see below and section 3.2).

Here we describe the cloning strategy as well as the production, extraction, and purification of the T6SS TssJLM membrane complex (***21***).

2. Materials

2.1 Cloning of the TssJLM complex

1. Desalted oligonucleotides of up to 35 bases, 5' ends unphosphorylated

2. Restriction enzymes (NdeI, XhoI, DpnI)

3. T4 DNA ligase

4. Thermoblock (16°C-42°C)

5. *E. coli* DH5α competent cells

6. PCR thermocycler

7. HPLC purified Mega-primer pairs (≥ 50 bases), 5' ends unphosphorylated

8. vector: pRSF-Duet1

9. High fidelity Taq DNA polymerase (Pfu turbo, Agilent)

10. dNTPs, 10 mM stock solution in water

11. Agarose gel electrophoresis system

2.2 Production and purification of the TssJLM complex

1. IPTG stock solution: 0.1 M in water

2. BL21(DE3): *E. coli* strain B F^- *ompT gal dcm lon* $hsdS_B(r_B^-m_B^-)$ λ(DE3 [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*]) [$malB^+$] (λ^S)

3. Lysozyme stock solution: 10 mg/mL in water

4. DNase stock solution: 10 mg/mL in water

5. TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) extemporaneously added

6. EDTA stock solution: 0.5M in water

7. $MgCl_2$ stock solution: 1 M in water

8. Lysis buffer: 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA

9. Solubilization buffer: 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.5% (w/v) n-dodecyl-β-D-maltopyranoside (DDM), 0.75% (w/v) decyl maltose neopentyl glycol (DM-NPG), 0.5% (w/v) digitonin (Sigma-Aldrich)

10. EDTA-free protease inhibitor tablets

10. Affinity buffer: 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.05% (w/v) DM-NPG

12. Imidazole-HCl, pH 8.0: stock solution 4M

13. Incubation Shakers (16°-37°C)

14. Centrifugation (from 5,000 to 100,000 × g)

15. Glass potter

16. Emulsiflex-C5 (Avestin)

17. 5-mL StrepTrap HP and 5-ml HisTrap HP columns (GE Healthcare)

18. Desthiobiotin

3. Methods

3.1: Cloning the TssJLM membrane complex

To overproduce the T6SS TssJLM membrane complex, the *tssJ, tssL* and *tssM* genes have been assembled in an artificial operon with individual optimized ribosome-binding sites (*see* **Figure 1**). In addition, based on previous data regarding permissive positions, each subunit has been tagged with a specific tag.

The pRSF–TssJ^{Strep}-TssL^{FLAG}-^{His6}TssM plasmid was constructed by both RSl (*see* **Note 4**) and RSl-free (see **Note 2**, ***(23)***) cloning methods (**Figure 1**).

1. PCR amplify the *tssJ* gene (encoding the TssJ lipoprotein) using High fidelity Taq DNA polymerase and primers containing the NdeI (5' primer) and the XhoI RS (3' primer) extensions.

The *Strep*-Tag II DNA sequence followed by a stop codon was introduced in the 3' primer (**Note 5**). Check the correct amplification by agarose gel electrophoresis (AGE).

2. Digest the PCR product and the pRSF-Duet vector with NdeI and XhoI restriction enzymes (sites present into the pRSF-Duet MCS2).3. Mix the digested PCR amplified fragment with about 50 ng of the digested pRSF-Duet vector, both digested with NdeI and XhoI, with a molar excess ratio of insert/vector of (2/1 to 5/1, inversely proportional to the DNA length of the insert).

4. Add 1 U of T4 DNA ligase to the DNA mixture plus its specific buffer in a total volume of 15 µl and incubate at 16-20°C for at least 2 hours.

5. Transform *E. coli* DH5α competent cells with about 30% of the ligation mix, plate on LB agar containing the appropriate antibiotic (in case of pRSF-Duet, use kanamycin 50 µg/mL) and incubate overnight at 37°C.

6. Select positive clones containing the pRSF–TssJ^{Strep} plasmid by colony PCR screening using the same primers. Extract the plasmid DNA and check the accuracy of the cloned sequence by DNA sequencing.

7. PCR amplify the *tssL* gene using (i) a 5' primer containing a 5' extension (22bp) corresponding to the *Strep*-TagII and the stop codon followed by the RBS sequence (*see* **Note 6**) and the 5'-FLAG-*tssL* extension (63bp), and (ii) a 3' primer containing the 3'-*tssL* gene extension (35bp) including the stop codon followed by RBS, the ATG and the 5'-His6-*tssM* extension (14bp). Check correct amplification by AGE.

8. PCR amplify the *tssM* gene using (i) the 5' primer complementary to the 3' end of *tssL* gene, RBS, His tag and 5' sequence of *tssM* gene (20bp) and (ii) the 3' primer containing the 3'-*tssM*

gene extension (35bp) including the stop codon followed by the pRSF-Duet sequence extension (35bp). Check correct amplification by AGE.

9. Mix the *flag-tssL*, *his-tssM* PCR products and the pRSF–TssJ^{Strep} plasmid and PCR amplify with High fidelity DNA polymerase in a single reaction as described for the RSl-free technique (*see* **Note 2**).

10. Digest the mixture with *DpnI*. Transform into *E. coli* DH5α competent cells and plate on LB agar containing the appropriate antibiotic and incubate overnight at 37°C.

11. Select positive clones containing the pRSF–TssJ^{Strep}-^{FLAG}TssL-^{6His}TssM plasmid by colony PCR screening using the same primers. Extract the plasmid DNA and check the accuracy of the cloned sequence by DNA sequencing.

3.2: TssJLM membrane complex, extraction and purification

1. Transform the expression vector (pRSF-TssJ^{Strep}-^{FLAG}TssL-^{6His}TssM) into the *E. coli* BL21(DE3) expression strain.

2. Grow cells at 37 °C in 8 L of lysogeny broth (LB) to an optical density at 600 nm (A_{600}) ~ 0.7. Induce the expression of the *tssJLM* genes with 1.0 mM IPTG for 16 h at 16 °C (*see* **Note 7**).

3. Pellet cells by centrifugation at 7,000 × g for 20 min. Resuspend cell pellets in 300 mL of ice-cold Lysis buffer supplemented with 1 mM TCEP, 100 µg/mL of DNase I, 100 µg/mL of lysozyme and with one tablet of EDTA-free protease inhibitor. Add $MgCl_2$ to the final concentration of 10 mM.

4. Break the cell suspension with an Emulsiflex-C5 by 4 passages at 15,000 psi (100 MPa). Pellet unbroken cells by centrifugation at 7,000 × g (*see* **Note 8**).

5. Pellet membranes by ultracentrifugation at 98,000 × g for 45 min.

6. Resuspend membranes in 120 mL of Solubilization buffer supplemented with 1 mM TCEP at 22°C and homogenize membranes mechanically with a potter (duration about 45 min) (*see* **Note 9**).

7. Clarify the membrane suspension by centrifugation at 98,000 × g for 20 min.

8. Load the supernatant onto a 5-mL StrepTrap HP column and then wash with Affinity buffer at 4 °C.

9. Elute the TssJLM core complex in Affinity buffer supplemented with 2.5 mM desthiobiotin into a 5-mL HisTrap HP column.

10. Wash the HisTrap HP column in Affinity buffer supplemented with 20 mM imidazole and proceed to the elution of the TssJLM core complex in the same buffer supplemented with 500 mM imidazole.

11. Pool the peak fractions and load onto a Superose 6 10/300 column equilibrated in 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.025% (w/v) DM-NPG (*see* **Note 10**). The TssJLM complex elute as a single monodisperse peak close to the void volume of the column.

4. Notes

1. *E. coli* T7RNAp expression systems result from (i) plasmid (pGP1-2) expression, the T7RNAp under the control of the Lambda P_L promoter is regulated by the plasmid encoded temperature-sensitive C1-857 repressor ***(24)***; (ii) chromosomal expression: the T7RNAp gene is under the control of the *lacUV5* ***(25)*** or the *ara* promoter ***(26)***; (iii) besides tight control of the chromosomal T7RNAp expression upon AraC control ***(26)***, infection by phages (M13 mGP1-2 or Lambda CE6) encoding the T7RNAp ***(27, 28)*** has proven to be efficient for the expression of toxic gene products.

2. The-RSl-free cloning strategy allows the insertion of a gene at a precise position on a target plasmid but does not require the presence of RS. The restriction free cloning method consists in two sequential PCR amplifications using only two primers. These primers (with ≥ 25 bp homology extension) are designed to hybridize on the 5' and 3' ends of the gene of interest and contain additional extension to hybridize on the expression vector at a selected position downstream of the regulate promoter sequence. First, the gene of interest is amplified by PCR, then the amplified mega-primer pairs containing the gene are annealed to the vector of interest. A new amplification with High fidelity polymerase produce a linear gene-vector amplification ***(29)***. The gene is thus plasmid included into a nicked and circular DNA molecule. The PCR reaction is treated by *DpnI* RE to digest the unwanted Dam methylated plasmids templates. Competent *E.coli* cells are further transformed with the annealed DNA complex directly. An alternative-method has been developed ***(30)***. It uses PCR amplified gene containing at least 15 bp extensions that are homolog to each end of the linearized vector (PCR amplified or RS digested). PCR DNA and vector treated with T4 polymerase, to create 5' overhangs, are annealed to form a recombinant plasmid.

3. Addition of a T7 promoter by a RS-dependent cloning strategy. RS present in the upstream sequence of the gene of interest can be used to insert a synthetic T7 promoter DNA fragment which is formed by two overlapping oligonucleotides containing the T7 consensus promoter sequence of 23 bases corresponding to: 5'-taatacgactcactatagggaga-3'. The T7 promoter sequence is inserted upstream of the Ribosome Binding Site (RBS) of the gene of interest (it is also possible to insert the T7 promoter upstream of the natural promoter sequence). For this purpose, the synthetic DNA fragment contains additional 5' and 3' end extensions that are complementary of the sticky ends of the RS present in the plasmid (blunt ended RS are also possible to use but

with lower ligation efficiency and random insertion). Two complementary desalted oligonucleotides (ON) of about 35 bases, 5' end unphosphorylated are hybridized following heat denaturation in ultrapure water and further cooling at room temperature. The annealed DNA fragment is further ligated into RS digested plasmid. It is possible to favor the selection of positive clones using Synthetic DNA that do not recreate the initial RS. Then, heat inactivated ligation mix is digested with the RE corresponding to the RS that has been destroyed upon DNA fragment ligation (Example: DNA fragment insertion into the EcoRI RS: after EcoRI digestion, the overhang EcoRI: 5'-AATTC... should be filled with the synthetic sequence: 5'-AATTX...., where the X nucleotide does not correspond to C nucleotide). It is noticeable that this fast technique does not require plasmid sequencing. It should be used for plasmids harboring resistance and regulatory genes in the opposite orientation from that of the T7 regulated gene(s) ***(31)***.

4. The general RSl-dependent strategy is often used for cloning genes of interest into the MCS of expression vectors. The genes can be either purified from RS digestion ***(32)*** or obtained from PCR amplification using primers that contain additional RS extensions. Then, the PCR amplified DNA is RE digested and inserted into the cognate RS of the MCS present in the vector ***(16)***.

5. Since lipoproteins undergo a post-translational modification at their N-termini ***(33)***, the *Strep*-Tag II affinity tag sequence was introduced at the C-terminal of TssJ. It is important to note that the positions of affinity tags (Strep, FLAG and His Tags) have been rationally chosen to maintain functional proteins.

6. To optimize the production of the TssL and TssM protein, the endogenous RBS-ATG 5’ sequence in front of the genes were replaced (in the 5’ primer sequence) by the consensual

sequence "**AAGGAG**ATATACAT*ATG*" (***34***) (RBS and start codon are presented, bold and italic letters respectively).

7. Growth and induction at 37°C for 3 hours led to very low biomass and protein yield. The culture was conducted at 37°C until A_{600} ~ 0.7-0.9 before shifting the incubator at 16°C prior to induction by IPTG. The final OD after the 16H induction was around 1.4-1.6. It is important not to induce around 0.4 OD since the cell would stop growing shortly after.

8. It is important to note that whereas many cell disrupting protocol have been tried, only lysis with Emulsiflex produced a stable and homogenous sample of the TssJLM membrane core complex.

9. This specific combination of detergents (same recipe than that used for the purification of the T4SS ***(20)***) gave the higher extraction yield of the TssJLM membrane complex. Other isolated detergents (Triton-X-100, n-Octyl-β-D-Glucoside, DDM or DM-NPG) gave poor extraction yields. All detergent buffers were prepared extemporally the day of the purification. Digitonin tends to precipitate in high salt buffers and without other detergents. Adding DDM and DM-NPG prevent digitonin precipitation.

10. DM-NPG was chosen because the solulilized TssJLM complex is stable and since this detergent is successful for high-resolution structural biology studies giving very clear and reproducible EM background after negative staining. Using low salt concentration (50 mM maximum) was key to prevent aggregation of the TssJLM complex. The affinity purification steps were of paramount importance to isolate a stoichiometric complex. Indeed, a far excess of the TssJ lipoprotein was eliminated during the second His column.

5. References

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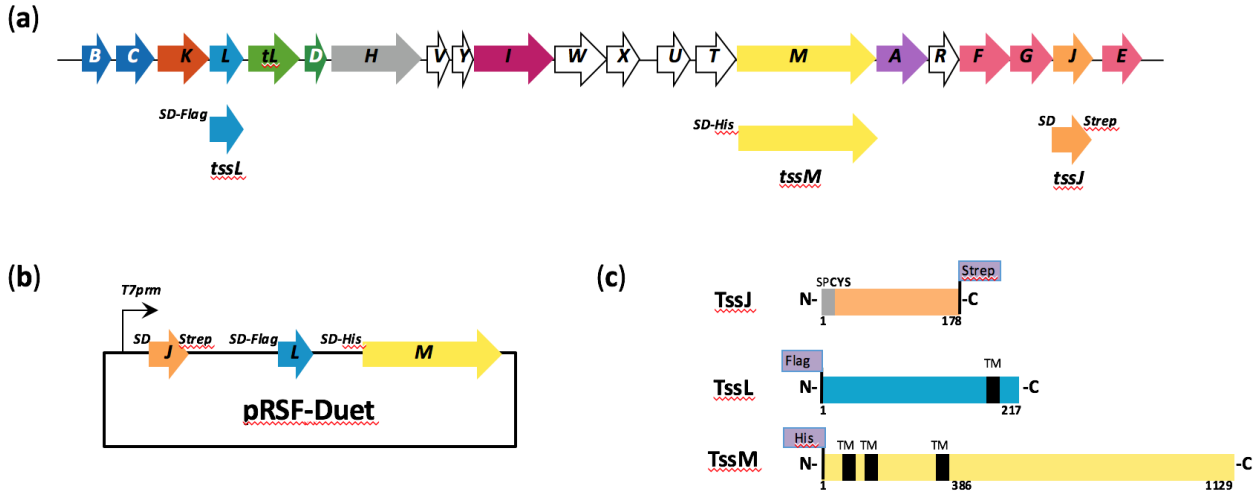

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373Figure 1: **Cloning of the synthetic *tssJLM* operon encoding the T6SS membrane core**

374**complex.** (**a**) The *tssL*, *tssM* and *tssJ* genes were PCR amplified from the entero-aggregative *sci-*

375*1* T6SS operon. DNA sequences were added encoding an optimized RBS at the 5' end of each

376gene and sequences encoding a C-terminal Strep-II tag for TssJ and N-terminal 6×His and Flag

377tags for TssM and TssL, respectively. (**b**) The *tssJLM* artificial operon was cloned in the pRSF-

378Duet vector. (**c**) Schemes representing the protein constructs, the sub-domain boundaries and

379some indicated characteristics (TM, transmembrane segments; SP, signal peptide; CYS, acylated

380cysteine).

381