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1Chapter 24. Large complexes: Cloning strategy, production and purification

2Running Head: Membrane protein complex purification

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4attention abbreviations

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10

11i. Summary

12Membrane proteins can assemble and form complexes in the cell envelope. In Gram-negative
13bacteria, a number of multiprotein complexes including secretion systems, efflux pumps,
14molecular motors or pilus assembly machines comprise proteins from the inner and the outer
15membrane. Beside the structures of isolated soluble domains, only few atomic structures of these
16assembled molecular machines have been elucidated. To better understand the function and to
17solve the structure of protein complexes, it is thus necessary to design dedicated production and
18purification processes. Here, we present cloning procedures to overproduce membrane proteins
19into *E. coli* cells and describe the cloning and purification strategy for the Type VI secretion
20TssJLM membrane complex.

21

22ii. **Key words:** membrane protein complexes, *E. coli*, T7 overexpression, protein purification.

23

241. Introduction

25Protein overproduction results from cloning a gene of interest into a plasmid vector, downstream
26of a tightly regulated promoter, and from inducing its expression after plasmid transformation

27into a bacterial strain. For large protein complexes containing multiple subunits, the genes
28encoding the different subunits can be expressed under the control of an inducible promoter
29either from single plasmid containing a cluster of genes or from different compatible plasmids
30harboring single or multiple genes.

311.1 Cloning vectors

32Several inducible promoters have been described and are available to overexpress a gene of
33interest. These promoters are usually cloned into vectors that also contain the gene encoding the
34cognate regulatory protein and a transcriptional terminator to prevent non-productive
35transcription from downstream gene (**1-7**). The *tac* and *trc* promoters that contain the -35 and -10
36sequences from the *trp* and *lacUV5* promoters, respectively, have been optimized for high
37expression level (**1**). Other *E. coli* tightly regulated promoters such as the *tetA* promoter and its
38repressor TetR, the *araBAD* promoter and its cognate activator AraC (**3-6**). In addition,
39heterologous combinations of promoter/regulator binding sequences can be used (**2**). The last
40family of inducible promoters gathers sequences that are not recognized by the *E. coli* RNA
41polymerase but rather recognized by phage RNA polymerases such as the SP6, T3 and T7
42promoters. Vectors and strains that express the T7 RNA polymerase (T7RNAP) have been
43extensively developed. Three independent methods are used to regulate the T7 expression
44systems. First, the expression of the chromosomally- or plasmid-encoded T7 RNAP gene can be
45itself under the control of an inducible promoter (*see Note 1*). Second, the inhibition of the T7
46RNAP basal activity can be controlled by producing the T7 lysozyme under constitutive or
47regulated conditions (**8, 9**). Last, the transcription by the T7 RNAP can be repressed by the Lac
48repressor, adding the *lac* operator sequence (**10**), the *lacI* gene being cloned on the expression
49vector.

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

54- production of proteins in the cytoplasm, the periplasm or the membrane of *E. coli* (using the
55 addition of synthetic N-terminal signal-sequences such as that of the OmpA and PelB proteins).

56- compatible T7 expression vectors containing one or two T7 promoters to overproduce protein
57 complexes with up to eight subunits (see Duet Vectors from Novagen).

58- Protein tagging sequence (6 to 10 × His, *Strep*-Tag II...) or fusion partner (protein G,
59 glutathione-S-transferase: GST, calmodulin-binding peptide: CBP, Maltose binding protein:
60 MBP) to increase protein solubility or to simplify the purification using affinity chromatography
61 techniques. In addition, these tag affinity sequences can be removed using specific protease
62 (most used proteases: Tobacco Etch Virus Protease: TEV, enterokinase, Thrombin, Xa Factor,
63 PreScission...). For this purpose, the corresponding protease recognition sequence is inserted
64 either downstream (for N-terminal tagging) or upstream (for C-terminal tagging) of the tag
65 sequence. After protease digestion, purified protein complex is submitted to a new purification
66 step to remove the protease, the uncleaved protein and the tag peptide (for example affinity
67 chromatography to remove the His-tag peptide and the His-tagged TEV).

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

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

74T7RNAP and *E. coli* cell extracts, have been found efficient to produce milligram amounts of
75some membrane proteins (**11**).

76

77**1.2 Membrane protein complex overproduction and purification**

78Membrane protein complexes have been successfully overproduced and purified based on pBAD
79or T7 vectors. For example, the Tol proteins from the Tol cell envelope molecular motor were
80overproduced and specifically radiolabeled with ³⁵S-Methionine after cloning into a pT7-driven
81vector using restriction sites (RS) and ligation techniques (RSI) (**12, 13, see Note 3 and Note 4**).
82Sub-complexes from the Ton system (**14-17**), the PomA-PomB flagellar rotor (**18**), the AcrAB-
83TolC efflux pump (**19**), the Type IV and Type VI secretion systems (T4SS and T6SS,
84respectively; **20, 21**) and the β -barrel assembly machinery (**22**) have been also successfully
85purified using similar approaches.

86 In addition to the cloning strategy and the production to levels compatible with
87purification, studying these multiprotein machines also requires to extract and solubilize the
88protein complex without disrupting the contacts between the subunits. Finally, since the
89overproduction condition may induce stoichiometry artifacts, the tagging of the minor subunit or
90the specific tagging of different subunits should be performed to purify homogenous protein
91complexes (see below and section 3.2).

92 Here we describe the cloning strategy as well as the production, extraction, and
93purification of the T6SS TssJLM membrane complex (**21**).

94

95**2. Materials**

96**2.1 Cloning of the TssJLM complex**

971. Desalted oligonucleotides of up to 35 bases, 5' ends unphosphorylated
982. Restriction enzymes (NdeI, XhoI, DpnI)
993. T4 DNA ligase
1004. Thermoblock (16°C-42°C)
1015. *E. coli* DH5 α competent cells
1026. PCR thermocycler
1037. HPLC purified Mega-primer pairs (\geq 50 bases), 5' ends unphosphorylated
1048. vector: pRSF-Duet1
1059. High fidelity Taq DNA polymerase (Pfu turbo, Agilent)
10610. dNTPs, 10 mM stock solution in water
10711. Agarose gel electrophoresis system
- 1082.2 Production and purification of the TssJLM complex**
1091. IPTG stock solution: 0.1 M in water
1102. BL21(DE3): *E. coli* strain B F⁻ *ompT gal dcm lon hsdS_B(r_B⁻m_B⁻)* λ (DE3 [*lacI lacUV5-T7 gene*
- 1111 *ind1 sam7 nin5*]) [*malB*⁺] (λ^S)
1123. Lysozyme stock solution: 10 mg/mL in water
1134. DNase stock solution: 10 mg/mL in water
1145. TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) extemporaneously added
1156. EDTA stock solution: 0.5M in water
1167. MgCl₂ stock solution: 1 M in water
1178. Lysis buffer: 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA

1189. Solubilization buffer: 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.5% (w/v) n-
119dodecyl-β-D-maltopyranoside (DDM), 0.75% (w/v) decyl maltose neopentyl glycol (DM-NPG),
1200.5% (w/v) digitonin (Sigma-Aldrich)
12110. EDTA-free protease inhibitor tablets
122110. Affinity buffer: 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.05% (w/v) DM-NPG
12312. Imidazole-HCl, pH 8.0: stock solution 4M
12413. Incubation Shakers (16°-37°C)
12514. Centrifugation (from 5,000 to 100,000 × g)
12615. Glass potter
12716. Emulsiflex-C5 (Avestin)
12817. 5-mL StrepTrap HP and 5-ml HisTrap HP columns (GE Healthcare)
12918. Desthiobiotin
130

1313. Methods

1323.1: Cloning the TssJLM membrane complex

133To overproduce the T6SS TssJLM membrane complex, the *tssJ*, *tssL* and *tssM* genes have been
134assembled in an artificial operon with individual optimized ribosome-binding sites (*see Figure*
135**1**). In addition, based on previous data regarding permissive positions, each subunit has been
136tagged with a specific tag.

137 The pRSF-TssJ^{Strep}-TssL^{FLAG-His6}TssM plasmid was constructed by both RSI (*see Note*
138**4**) and RSI-free (*see Note 2, (23)*) cloning methods (**Figure 1**).

1391. PCR amplify the *tssJ* gene (encoding the TssJ lipoprotein) using High fidelity Taq DNA
140polymerase and primers containing the NdeI (5' primer) and the XhoI RS (3' primer) extensions.

141 The *Strep*-Tag II DNA sequence followed by a stop codon was introduced in the 3' primer (**Note**
142**5**). Check the correct amplification by agarose gel electrophoresis (AGE).

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

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

150 5. Transform *E. coli* DH5 α competent cells with about 30% of the ligation mix, plate on LB agar
151 containing the appropriate antibiotic (in case of pRSF-Duet, use kanamycin 50 μ g/mL) and
152 incubate overnight at 37°C.

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

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

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

163gene extension (35bp) including the stop codon followed by the pRSF-Duet sequence extension
164(35bp). Check correct amplification by AGE.

1659. Mix the *flag-tssL*, *his-tssM* PCR products and the pRSF–TssJ^{Strep} plasmid and PCR amplify
166with High fidelity DNA polymerase in a single reaction as described for the RSI-free technique
167(see **Note 2**).

16810. Digest the mixture with *DpnI*. Transform into *E. coli* DH5 α competent cells and plate on LB
169agar containing the appropriate antibiotic and incubate overnight at 37°C.

17011. Select positive clones containing the pRSF–TssJ^{Strep}-FLAG-TssL-^{6His}TssM plasmid by colony
171PCR screening using the same primers. Extract the plasmid DNA and check the accuracy of the
172cloned sequence by DNA sequencing.

173

174**3.2: TssJLM membrane complex, extraction and purification**

1751. Transform the expression vector (pRSF-TssJ^{Strep}-FLAG-TssL-^{6His}TssM) into the *E. coli*
176BL21(DE3) expression strain.

1772. Grow cells at 37 °C in 8 L of lysogeny broth (LB) to an optical density at 600 nm (A_{600}) ~ 0.7.
178Induce the expression of the *tssJLM* genes with 1.0 mM IPTG for 16 h at 16 °C (see **Note 7**).

1793. Pellet cells by centrifugation at 7,000 \times g for 20 min. Resuspend cell pellets in 300 mL of ice-
180cold Lysis buffer supplemented with 1 mM TCEP, 100 μ g/mL of DNase I, 100 μ g/mL of
181lysozyme and with one tablet of EDTA-free protease inhibitor. Add MgCl₂ to the final
182concentration of 10 mM.

1834. Break the cell suspension with an Emulsiflex-C5 by 4 passages at 15,000 psi (100 MPa).

184Pellet unbroken cells by centrifugation at 7,000 \times g (see **Note 8**).

1855. Pellet membranes by ultracentrifugation at 98,000 \times g for 45 min.

1866. Resuspend membranes in 120 mL of Solubilization buffer supplemented with 1 mM TCEP at 18722°C and homogenize membranes mechanically with a potter (duration about 45 min) (*see Note 1889*).

1897. Clarify the membrane suspension by centrifugation at $98,000 \times g$ for 20 min.

1908. Load the supernatant onto a 5-mL StrepTrap HP column and then wash with Affinity buffer at 1914 °C.

1929. Elute the TssJLM core complex in Affinity buffer supplemented with 2.5 mM desthiobiotin 193into a 5-mL HisTrap HP column.

19410. Wash the HisTrap HP column in Affinity buffer supplemented with 20 mM imidazole and 195proceed to the elution of the TssJLM core complex in the same buffer supplemented with 500 196mM imidazole.

19711. Pool the peak fractions and load onto a Superose 6 10/300 column equilibrated in 50 mM 198Tris-HCl, pH 8.0, 50 mM NaCl, 0.025% (w/v) DM-NPG (*see Note 10*). The TssJLM complex 199elute as a single monodisperse peak close to the void volume of the column.

200

2014. Notes

2021. *E. coli* T7RNAP expression systems result from (i) plasmid (pGP1-2) expression, the 203T7RNAP under the control of the Lambda P_L promoter is regulated by the plasmid encoded 204temperature-sensitive C1-857 repressor (**24**); (ii) chromosomal expression: the T7RNAP gene is 205under the control of the *lacUV5* (**25**) or the *ara* promoter (**26**); (iii) besides tight control of the 206chromosomal T7RNAP expression upon AraC control (**26**), infection by phages (M13 mGP1-2 207or Lambda CE6) encoding the T7RNAP (**27**, **28**) has proven to be efficient for the expression of 208toxic gene products.

209**2.** The-RSI-free cloning strategy allows the insertion of a gene at a precise position on a target
210plasmid but does not require the presence of RS. The restriction free cloning method consists in
211two sequential PCR amplifications using only two primers. These primers (with ≥ 25 bp
212homology extension) are designed to hybridize on the 5' and 3' ends of the gene of interest and
213contain additional extension to hybridize on the expression vector at a selected position
214downstream of the regulate promoter sequence. First, the gene of interest is amplified by PCR,
215then the amplified mega-primer pairs containing the gene are annealed to the vector of interest. A
216new amplification with High fidelity polymerase produce a linear gene-vector amplification (**29**).
217The gene is thus plasmid included into a nicked and circular DNA molecule. The PCR reaction is
218treated by *DpnI* RE to digest the unwanted Dam methylated plasmids templates. Competent
219*E.coli* cells are further transformed with the annealed DNA complex directly. An alternative-
220method has been developed (**30**). It uses PCR amplified gene containing at least 15 bp extensions
221that are homolog to each end of the linearized vector (PCR amplified or RS digested). PCR DNA
222and vector treated with T4 polymerase, to create 5' overhangs, are annealed to form a
223recombinant plasmid.

224**3.** Addition of a T7 promoter by a RS-dependent cloning strategy. RS present in the upstream
225sequence of the gene of interest can be used to insert a synthetic T7 promoter DNA fragment
226which is formed by two overlapping oligonucleotides containing the T7 consensus promoter
227sequence of 23 bases corresponding to: 5'-taatacgaactcactataggaga-3'. The T7 promoter sequence
228is inserted upstream of the Ribosome Binding Site (RBS) of the gene of interest (it is also
229possible to insert the T7 promoter upstream of the natural promoter sequence). For this purpose,
230the synthetic DNA fragment contains additional 5' and 3' end extensions that are complementary
231of the sticky ends of the RS present in the plasmid (blunt ended RS are also possible to use but

232with lower ligation efficiency and random insertion). Two complementary desalted
233oligonucleotides (ON) of about 35 bases, 5' end unphosphorylated are hybridized following heat
234denaturation in ultrapure water and further cooling at room temperature. The annealed DNA
235fragment is further ligated into RS digested plasmid. It is possible to favor the selection of
236positive clones using Synthetic DNA that do not recreate the initial RS. Then, heat inactivated
237ligation mix is digested with the RE corresponding to the RS that has been destroyed upon DNA
238fragment ligation (Example: DNA fragment insertion into the EcoRI RS: after EcoRI digestion,
239the overhang EcoRI: 5'-AATTC... should be filled with the synthetic sequence: 5'-AATTX....,
240where the X nucleotide does not correspond to C nucleotide). It is noticeable that this fast
241technique does not require plasmid sequencing. It should be used for plasmids harboring
242resistance and regulatory genes in the opposite orientation from that of the T7 regulated gene(s)
243**(31)**.

244**4.** The general RSI-dependent strategy is often used for cloning genes of interest into the MCS of
245expression vectors. The genes can be either purified from RS digestion **(32)** or obtained from
246PCR amplification using primers that contain additional RS extensions. Then, the PCR amplified
247DNA is RE digested and inserted into the cognate RS of the MCS present in the vector **(16)**.

248**5.** Since lipoproteins undergo a post-translational modification at their N-termini **(33)**, the *Strep*-
249Tag II affinity tag sequence was introduced at the C-terminal of TssJ. It is important to note that
250the positions of affinity tags (Strep, FLAG and His Tags) have been rationally chosen to
251maintain functional proteins.

252**6.** To optimize the production of the TssL and TssM protein, the endogenous RBS-ATG 5'
253sequence in front of the genes were replaced (in the 5' primer sequence) by the consensual

254sequence “**AAGGAGATATACATATG**” (**34**) (RBS and start codon are presented, bold and
255italic letters respectively).

256 **7.** Growth and induction at 37°C for 3 hours led to very low biomass and protein yield. The
257culture was conducted at 37°C until $A_{600} \sim 0.7-0.9$ before shifting the incubator at 16°C prior to
258induction by IPTG. The final OD after the 16H induction was around 1.4-1.6. It is important not
259to induce around 0.4 OD since the cell would stop growing shortly after.

260**8.** It is important to note that whereas many cell disrupting protocol have been tried, only lysis
261with Emulsiflex produced a stable and homogenous sample of the TssJLM membrane core
262complex.

263**9.** This specific combination of detergents (same recipe than that used for the purification of the
264T4SS (**20**)) gave the higher extraction yield of the TssJLM membrane complex. Other isolated
265detergents (Triton-X-100, n-Octyl- β -D-Glucoside, DDM or DM-NPG) gave poor extraction
266yields. All detergent buffers were prepared extemporally the day of the purification. Digitonin
267tends to precipitate in high salt buffers and without other detergents. Adding DDM and DM-
268NPG prevent digitonin precipitation.

269**10.** DM-NPG was chosen because the solubilized TssJLM complex is stable and since this
270detergent is successful for high-resolution structural biology studies giving very clear and
271reproducible EM background after negative staining. Using low salt concentration (50 mM
272maximum) was key to prevent aggregation of the TssJLM complex. The affinity purification
273steps were of paramount importance to isolate a stoichiometric complex. Indeed, a far excess of
274the TssJ lipoprotein was eliminated during the second His column.

275

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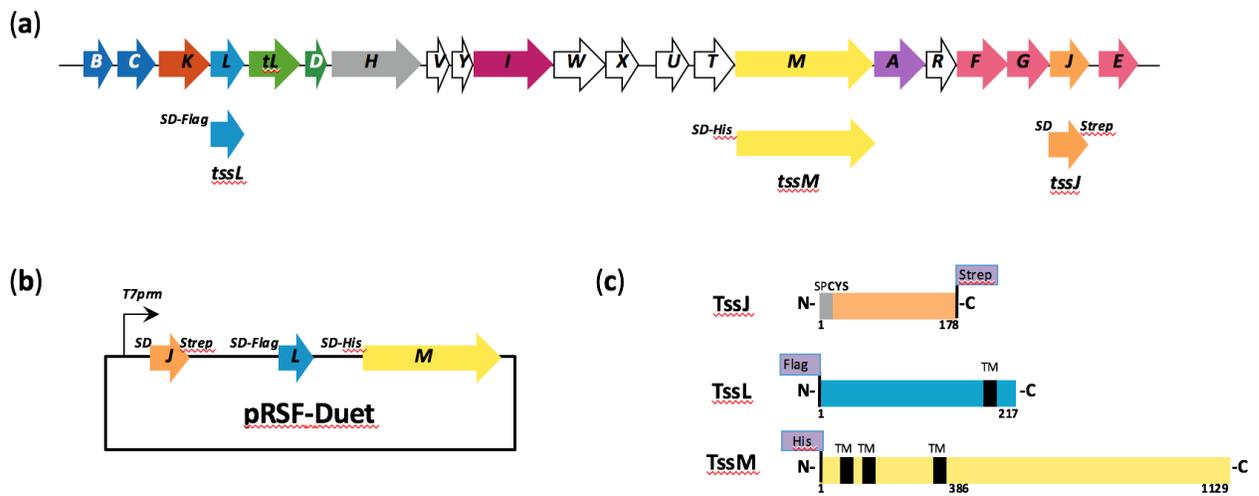
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373 **Figure 1: Cloning of the synthetic *tssJLM* operon encoding the T6SS membrane core**

374 **complex.** (a) The *tssL*, *tssM* and *tssJ* genes were PCR amplified from the entero-aggregative *sci-*
 375 *I* T6SS operon. DNA sequences were added encoding an optimized RBS at the 5' end of each
 376 gene and sequences encoding a C-terminal Strep-II tag for TssJ and N-terminal 6×His and Flag
 377 tags for TssM and TssL, respectively. (b) The *tssJLM* artificial operon was cloned in the pRSF-
 378 Duet vector. (c) Schemes representing the protein constructs, the sub-domain boundaries and
 379 some indicated characteristics (TM, transmembrane segments; SP, signal peptide; CYS, acylated
 380 cysteine).

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