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1 **Protein-protein interaction: Tandem Affinity Purification in bacteria**

2 Julie P.M. Viala and Emmanuelle Bouveret

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4 Aix-Marseille University - CNRS - UMR7255 - Institut de Microbiologie de la Méditerranée,

5 Laboratoire d'Ingénierie des Systèmes Macromoléculaires, 31 Chemin Joseph Aiguier, 13009

6 Marseille, FRANCE

7

8 Correspondence: jviala@imm.cnrs.fr

9 Running head: TAP in bacteria

10

11 **Summary**

12 The discovery of protein-protein interaction networks can unveil protein complex(es) forming
13 cellular machinerie(s) or can reveal component proteins of a specific cellular pathway.
14 Deciphering protein-protein interaction networks therefore participates to a better
15 understanding of how the cell functions. Here, we describe the protocol to perform Tandem
16 Affinity Purification (TAP) in bacteria that enables the identification of the partners of a bait
17 protein under native conditions. This method consists in two sequential steps of affinity
18 purification, using two different tags. For that purpose, the bait protein is translationally fused
19 to the TAP-tag, which consists in a calmodulin binding peptide (CBP) and two IgG binding
20 domains of *Staphylococcus aureus* protein A (ProtA) that are separated by the Tobacco Etch
21 Virus (TEV) protease cleavage site. After the first round of purification based on the binding
22 of ProtA to IgG coated beads, TEV protease cleavage releases CBP-tagged bait-protein along
23 with its partners for a second round of purification on calmodulin affinity resin and leaves
24 behind protein contaminants bound to IgG. Creating the TAP-tag translational fusion at the
25 chromosomal locus allows detection of protein interactions occurring in physiological
26 conditions.

28 **Keywords**

29 Protein–protein interaction, protein complex, affinity purification, TAP, CBP, ProtA, TEV,
30 *E. coli*, *Salmonella*

31

32

33 **1. Introduction**

34 At the end of the nineties, mass spectrometry combined with genome sequencing rendered
35 possible the rapid and systematic identification of all the proteins present in a purified sample.
36 However, a protocol amenable to standardized and systematic purification of protein
37 complexes without any prior knowledge was missing. In 1999, the laboratory of B. Séraphin
38 in EMBL proposed such a generic procedure for the identification of protein complexes in
39 yeast [1]. This permitted the subsequent description of the full interactome of yeast [2, 3].
40 This method has since been used in a variety of organisms. We have first described its use in
41 bacteria [4], and it was soon after used to obtain the first interactome of *E. coli* [5].

42 One general principle of the TAP method is to use 2 successive steps of affinity
43 purification to lower as much as possible the amount of contaminants, together with an
44 elution preserving the interactions (without changing much the buffer chemical properties)
45 between these 2 steps. Specifically, the original TAP tag consists of 2 repeats of the IgG
46 binding domain of Protein A from *Staphylococcus aureus*, and a Calmodulin Binding Peptide,
47 separated by a TEV protease cleavage site (Fig. 1). However, it has to be noted that any
48 combination of affinity tags is potentially usable. Published examples are the GS-TAP
49 (Protein G and Strep tag), the SPA tag (CBP and 3Flag), the SF-TAP (Strep-tag II and Flag
50 tag), or the HB tag (6Histidine and Biotin) (see [6] for specific references). The second
51 general principle of the TAP procedure is to use physiological expression of the recombinant
52 tagged protein. This needs to be adapted to each organism of interest. For *E. coli* and closely
53 related bacteria, Lambda Red based recombination [7] combined with specific dedicated SPA
54 and TAP cassettes [8], makes it very easy to introduce the tag at the 3' extremity of the gene
55 on the chromosome to obtain the physiological production of a recombinant protein tagged at

its C-terminus (Fig. 2). If more convenient, however, TAP tag translational fusion can also be expressed from a plasmid (Fig. 3).

We present here the TAP protocol that has been successfully used in our institute to purify protein complexes of *E. coli*, *Salmonella*, and *Bacillus subtilis* [4, 9, 10, and unpublished results]. A detailed protocol for the SPA purification has been published before [11]. In order to isolate a protein complex by TAP, a strain producing a recombinant bait protein tagged with the TAP tag has to be constructed first (Fig. 1, step 1). Then, a soluble extract is prepared from a sufficient volume of bacteria (about 500 ml). The complex is enriched by a first step of affinity chromatography on IgG beads (Fig. 1, step 5). After washes, TEV protease is added, which cleaves the specific site located between the CBP and ProtA domains, resulting in the elution of the specifically bound material (Fig. 1, step 6). This material is purified a second time by affinity of the CBP tag with Calmodulin beads (Fig. 1, step 7). After washes, the purified complex is eluted by adding EGTA that chelates the calcium required for the CBP/Calmodulin interaction (Fig. 1, step 8). The totality of the purified material is analyzed on SDS-PAGE. Bands detected by Coomassie blue or Silver staining are cut from the gel and analyzed by mass spectrometry.

This consists in the basic TAP procedure. It has to be noted that this procedure can be amenable to adaptation or improvements depending on the specific needs. For example, the extensive washes and the duration of the procedure only allows for the recovery of relatively stable complexes. For the detection of more transient or unstable interactions, a cross-linking procedure can be applied before purification [12]. This might be helpful also for the purification of membrane complexes, where modifications have to be made in the protocol for solubilization of the membranes [3]. Finally, it is possible to play with the 2 tags to gain information on the organization of the complexes. Indeed, in some cases, one bait protein might participate to the formation of several types of complexes. To purify one specific type

of complex it is therefore possible to put the two tags on 2 distinct proteins that are both members of the desired type of complex (split tag method [9, 13]). Alternatively, it is possible to perform the subtraction method that consists in eliminating the unwanted complex(es) during the first purification step, by leaving it, for example, on IgG beads thanks to a partner protein of the bait that belongs to the unwanted complex and bears a non cleavable protA tag. The desired complex, made of untagged partner proteins, will elute with the bait after TEV protease cleavage [13, 14].

To our knowledge, the TAP procedure has not been used so much for the characterization of secretion systems in bacteria, certainly due to the difficulty of working with integral envelope components. However, it has proved to be powerful to identify the target of effectors of *Legionella* T4SS or *Pseudomonas* T6SS in eukaryotic host cells [15, 16]. In addition, as mentioned above, it is amenable to several improvements that might permit to identify unsuspected partners of the secretion machineries in the bacterium.

2. Materials

2.1 Media, stock solutions and reagents

1. 2YT: 16 g Yeast Extract, 10 g Tryptone, 10 g NaCl, make up to 1 L with distilled water. Autoclave and store at room temperature.
2. LB: 5 g Yeast Extract, 10 g Bactotryptone, 10 g NaCl, make up to 1 L with distilled water. Autoclave and store at room temperature.
3. PBS: 8 g NaCl, 0.2 g KCl, 0.2 g KH₂PO₄, 2.9 g Na₂HPO₄, make up to 1 L with distilled water. Autoclave and store at room temperature.

- 103 4. 1 M Tris pH 8 stock solution: Dissolve 121 g of Tris base in 800 ml of distilled water,
104 make up to 1 L with distilled water once the pH has been adjusted to 8 with HCl. Autoclave
105 and store at room temperature.
- 106 5. 5 M NaCl stock solution: Dissolve 292 g NaCl in 1 L of distilled water. Autoclave and
107 store at room temperature.
- 108 6. 10 % Nonidet P-40 (NP-40 or Igepal): Mix 10 ml NP-40 to 90 ml of distilled water,
109 0.2 μ m filter and store at room temperature (note 1).
- 110 7. 0.5 M EDTA ($\text{C}_{10}\text{H}_{14}\text{N}_2\text{Na}_2\text{O}_8 \cdot 2\text{H}_2\text{O}$): Dissolve 18.6 g of EDTA to 80 ml of distilled
111 water, make up to 100 ml with distilled water once the pH has been adjusted to 8 with
112 10 N NaOH (note 2). Autoclave and store at room temperature.
- 113 8. 1 M DTT: Dissolve 1.54 g in 10 ml distilled water. Store at -20°C .
- 114 9. 1 M magnesium acetate ($\text{Mg}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$): Dissolve 10.7 g magnesium acetate
115 in 50 ml distilled water, 0.2 μ m filter and store at room temperature.
- 116 10. 1 M imidazole: Dissolve 3.4 g imidazole to 50 ml distilled water, 0.2 μ m filter and
117 store at -20°C .
- 118 11. 1 M CaCl_2 : Dissolve 11.1 g to 100 ml distilled water. Autoclave and store at room
119 temperature.
- 120 12. 1 M EGTA: Dissolve 19 g to 40 ml distilled water, make up to 50 ml with distilled
121 water once the pH has been adjusted to 8 with 10 N NaOH (note 2), 0.2 μ m filter and store at
122 4°C .
- 123 13. 0.1 M PMSF: Dissolve 87.1 mg PMSF to 5 ml isopropanol. Prepare 1 ml aliquots and
124 store at -20°C (note 3)

- 125 14. 16 mg/ml sodium deoxycholate: Dissolve 160 mg sodium deoxycholate to 10 ml water
126 and 0.2 μ m filter. Store at room temperature.
- 127 15. β -mercaptoethanol (stock is at 14.3 M)
- 128 16. Liquid Trichloroacetic acid (stock is 100 %)
- 129 17. Acetone
- 130 18. Ethanol
- 131 19. Peroxidase Anti-Peroxidase antibody (PAP) (Sigma, # P1291)
- 132 20. AcTEVTM protease (Invitrogen, # 12575015)
- 133 21. IgG sepharose 6 fast flow (GE Healthcare, # 17-0969-01)
- 134 22. Calmodulin affinity resin (Agilent, # 214303)
- 135 23. Disposable chromatography columns (Bio-Rad, # 7311550)

136

137 2.2 Buffers

- 138 1. Protein A binding buffer : 10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% NP-40

139 Approximately 50 ml will be required per sample per experiment.

140 Prepare 500 ml containing 5 ml of 1M Tris-HCl pH 8, 15 ml of 5 M NaCl, 5 ml of
141 10% NP-40 and 475 ml of distilled water. Store at 4°C.

- 142 2. TEV cleavage buffer : 10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% NP-40,
143 0.5 mM EDTA, 1 mM DTT

144 Approximately 1.5 ml will be required per sample per experiment.

145 Prepare 100 ml containing 1 ml of 1M Tris-HCl pH 8, 3 ml of 5M NaCl, 1 ml of 10% NP-40,
146 100 μ l of 0.5 M EDTA, 100 μ l of 1 M DTT (note 4) and 95 ml of distilled water. Store at 4°C.

147 3. Calmodulin binding buffer : 10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% NP-40,
148 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl_2 , 10 mM β -mercaptoethanol

149 Approximately 40 ml will be required per sample per experiment.

150 Prepare 500 ml containing 5 ml of 1M Tris-HCl pH 8, 15 ml of 5M NaCl, 5 ml of 10% NP-40,
151 500 μ l of 1 M magnesium acetate, 500 μ l of 1 M imidazole, 1 ml of 1 M CaCl_2 , 348.5 μ l of
152 14.3 M β -mercaptoethanol (note 5) and 473 ml of distilled water. Store at 4°C.

153 4. Calmodulin elution buffer : 10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% NP-40,
154 1 mM magnesium acetate, 1 mM imidazole, 2 mM EGTA, 10 mM β -mercaptoethanol

155 Approximately 1 ml will be required per sample per experiment.

156 Prepare 100 ml containing 1 ml of 1M Tris-HCl pH 8, 3 ml of 5M NaCl, 1 ml of 10% NP-40,
157 100 μ l of 1 M magnesium acetate, 100 μ l of 1 M imidazole, 200 μ l of 1 M EGTA, 69.7 μ l of
158 14.3 M β -mercaptoethanol (note 5) and 94.5 ml of distilled water. Store at 4°C.

159 5. TCA Washing Buffer: Mix 70 ml acetone, 20 ml ethanol, 5 ml 1 M Tris pH 8 and 5 ml
160 distilled water. Store at 4°C.

161

162 2.3 Equipment

163 1. Centrifuge tubes and rotor compatible with spinning volumes of 250 ml, 10 ml and
164 50 ml, at approximately 5 000 rpm, 15 000 rpm and 6 000 rpm, respectively.

2. Sonicator, French press or Cell disruptor
3. Disposable chromatography columns of 10 ml
4. Wheel

3. Methods

3.1 Preamble

Construction of the TAP-tagged protein translational fusion

A translational fusion between the protein of interest and TAP tag, either at the chromosomal locus or on an appropriate plasmid has to be constructed. Translational fusion at the chromosomal locus will allow a physiological expression, while constructing the translational fusion on a plasmid may be more amenable.

A C-terminal TAP tag translational fusion can be introduced at the chromosomal locus using the λ red recombination system [7]. To prepare the appropriate PCR product, use pJL72 plasmid as template (this latter harbors a cassette made of the TAP tag and the kanamycine resistance gene, Fig. 2A) [8], design a forward primer that contains, in the 5'-end, the 45 nucleotides that are immediately upstream the stop codon of the gene of interest, followed by the sequence 5'-TCCATGGAAAAGAGAAG-3' (this sequence will hybrid to the CBP tag, Fig. 2B) and design a reverse primer that contains at its 5'-end the reverse complement 45 nucleotides that are immediately downstream the stop codon of the gene of interest, followed by the sequence 5'-CATATGAATATCCTCCTTAG-3' (Fig. 2A).

Alternatively, the sequence corresponding to the open reading frame of the gene of interest can be cloned in the plasmid pEB587 [17] (Fig. 3A), which allows a N-terminal TAP tag translational fusion (Fig. 3B) under the control of the PBAD arabinose inducible promoter.

Verifying the expression of the TAP-tag translational fusion by western blot

Prepare a cytoplasmic or a crude protein extract (note 6), load 10 μ g of protein extract (or proteins corresponding to a bacterial sample of 0.3 OD₆₀₀ unit) on a SDS-PAGE and proceed to transfer and western blot to verify production of the hybrid protein (note 7).

A one step western blot can be performed using PAP antibody (note 8) and using an appropriate substrate to detect horseradish peroxidase activity. In our experience, the detection of the tagged protein in crude extracts using this PAP antibody is mandatory for a successful TAP purification.

3.2 Protein cytoplasmic extract

1. Day 1 - Inoculate 10 ml of 2YT media with a bacterial colony and grow overnight at 37°C with shaking (note 6).
2. Day 2 - Dilute culture 1:100 in 500 ml LB and grow 5h30 at 37°C with shaking until OD₆₀₀ $\approx$ 2- 3.
3. Pellet bacteria by centrifugation 20 min 5 000 rpm at 4°C.
4. Wash once with cold PBS, transfer to 50ml centrifuge tubes, centrifuge again 10 min 6 000 rpm at 4°C, discard supernatant and freeze bacterial pellet with liquid nitrogen.

206 Keep frozen bacterial pellet at -80°C until you are ready to prepare cytosolic protein extract
207 and proceed to Tandem Affinity Purification.

208 5. Day 3 - Resuspend frozen bacterial pellet with 10 ml of Protein A binding buffer
209 containing 0.5 mM PMSF (note 3).

210 6. Use sonication, French Press or Cell disruptor to break bacterial cells (note 9).

211 7. Centrifuge 30 min 15 000 rpm at 4°C and save supernatant, which is the cytoplasmic
212 protein extract.

213

214 3.3 Tandem Affinity Purification

215 From here, carry out all procedures with gloves to avoid contamination of your sample(s)
216 with keratine.

217 1. Put 200 μ l of IgG sepharose beads in a disposable chromatography column and wash
218 by gravity with 5 ml of Protein A binding buffer.

219 2. *Binding of the Protein A tag to IgG sepharose beads.* After washing the beads, close
220 the bottom of the chromatography column and, using a pipette, transfer 9 ml of the
221 cytoplasmic protein extract. Close the top of the column and put on a wheel for 2 hours at 4°C.

222 3. Remove first the top plug of the column and then the bottom one. Leave the unbound
223 material flow by gravity and discard.

224 4. Wash the IgG beads still in the column by gravity, with 3 times 10 ml of Protein A
225 binding buffer.

226 5. *TEV protease cleavage.* Close the bottom of the column, fill it with 1 ml of TEV
227 cleavage buffer and 100 units of AcTEVTM protease. Close the top of the column and put on a
228 wheel at room temperature for one hour.

229 6. Remove the top and bottom plugs and recover elution by gravity. Add an additional
230 200 μ l of TEV cleavage buffer in the column in order to recover as much material as possible
231 from the sides of column.

232 7. Add 3 ml of calmodulin binding buffer and 3 μ l of 1M CaCl₂ (note 10) to the elution
233 fraction.

234 8. *Binding by the CBP tag part on calmodulin affinity resin.* In a new disposable
235 chromatography column, put 200 μ l of calmodulin affinity resin, and wash it with 5 ml of
236 Calmodulin binding buffer. Then, close the bottom of column.

237 9. Add the 4.2 ml elution fraction (obtained at steps 6 and 7). Close the top of column
238 and put on wheel for 1 hour at 4°C.

239 10. Remove first the top plug of the column and then the bottom one. Leave the unbound
240 material to flow by gravity and discard.

241 11. Wash calmodulin affinity resin by gravity, with 3 times 10ml of calmodulin binding
242 buffer

243 12. *Elution.* Elute with 5 times 200 μ l of Calmodulin elution Buffer.

244 13. Pool fraction 2, 3 and 4 and proceed to TCA precipitation of elution fractions (1),
245 (pooled 2, 3 and 4), and (5).

246

247 3.4 TCA precipitation

248 1. To each of the eluted protein samples, add 1/100th of 16 mg/ml sodium deoxycholate.

249 Vortex and leave on ice 30 min.

250 2. Add TCA to 10% final. Vortex and leave on ice 30 min.

251 3. Centrifuge 15 min 15 000 rpm at 4°C.

252 4. Wash twice with TCA wash buffer.

253 5. Leave the pellet to dry on bench and resuspend in 20 μ l protein loading buffer 1x.

254

255 3.5 Analysis by SDS-PAGE and mass spectrometry

256 1. Load the totality of the samples on SDS-PAGE (note 11) and stain with Coomassie
257 Blue.

258 2. Unstained and then rinse with distilled water.

259 3. Cuts bands to identify partner proteins by mass spectrometry (note 12).

260

261 **4. Notes**

262 **Note 1:** Gently agitate the solution for complete dissolution of NP-40 if necessary.

263 **Note 2:** EDTA and EGTA may not be soluble until pH had been adjusted to 8 with
264 10 N NaOH.

265 **Note 3**: PMSF crystallizes at -20°C, therefore heat PMSF aliquot to 37°C to redissolve PMSF
266 before use. We use PMSF as generic protease inhibitor but protease inhibitor cocktail can be
267 used alternatively.

268 **Note 4**: Add DTT to the volume of buffer you will need when starting the experiment. DTT
269 is necessary for TEV activity.

270 **Note 5**: Add β-mercaptoethanol to the volume of buffer you will need when starting the
271 experiment.

272 **Note 6**: Also plan to prepare a protein extract of an untagged strain as negative control of the
273 experiment.

274 **Note 7**: Translational TAP tag fusion adds 20 kDa to the mass of the protein of interest;
275 3 kDa corresponds to the CBP tag and 15 kDa to the Protein A tag.

276 **Note 8**: Immunoglobulins will bind the protein A fragment of TAP tag.

277 **Note 9**: French Press or Cell disruptor might be more gentle to preserve protein complexes.

278 **Note 10**: Addition of extra CaCl₂ is required to quench EDTA that was previously necessary
279 for TEV protease activity.

280 **Note 11**: Usually a 12% SDS-PAGE allows visualization of low and high molecular weigh
281 proteins.

282 **Note 12**: Use one blade per band.

283

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331

Guidelines

1. Construction

2. Check Expression of the TAP-tagged bait protein by Western blot with PAP antibody

3. Production

4. Cytoplasmic protein extract

Add:

5. Protein A binding

partner bait contaminants

IgG beads

6. TEV cleavage

TEV protease

7. CBP binding

Calmodulin beads
 Ca^{2+}

8. Elution + TCA precipitation

EGTA

9. SDS-PAGE and staining

Cut band and analyze by Mass spectrometry

Fig. 1. Guidelines for the overall Tandem Affinity Purification procedure

Fig 2. Scheme of the procedure for creation of a C-terminal TAP-tag translational fusion at the chromosomal locus (**A**) and the corresponding nucleotidic and protein TAP-tag sequences (**B**). CBP sequence is in clear purple; upper cases at the beginning of the nucleotidic CBP sequence corresponds to the primer sequence mentioned in 3.1, TEV protease cleavage site is in yellow and Protein A sequence is in dark purple with the stop codon in red.

A

B

```

atggcaggcCTTGCGCAACACGATGAAGCCGTGGACAACAAATTCAACAAAGAACAACAA
M A G L A Q H D E A V D N K F N K E Q Q
AACGCGTTCTATGAGATCTTACATTTACCTAACTTAAACGAAGAACAACGAAACGCCTTC
N A F Y E I L H L P N L N E E Q R N A F
ATCCAAAGTTTAAAGATGACCCAAGCCAAAGCGCTAACCTTTTAGCAGAAGCTAAAAAG
I Q S L K D D P S Q S A N L L A E A K K
CTAAATGATGCTCAGGCGCCGAAAGTAGACAACAAATTCAACAAAGAACAACAAACGCG
L N D A Q A P K V D N K F N K E Q Q N A
TTCTATGAGATCTTACATTTACCTAACTTAAACGAAGAACAACGAAACGCCTTCATCCAA
F Y E I L H L P N L N E E Q R N A F I Q
AGTTTAAAGATGACCCAAGCCAAAGCGCTAACCTTTTAGCAGAAGCTAAAAAGCTAAAT
S L K D D P S Q S A N L L A E A K K L N
GGTGCTCAGGCGCCGAAAGTAGACGCGAATtgtgatatacctacaactgctcttgaaaat
G A Q A P K V D A N C D I P T T A S E N
ttatatattttcaaggtgaactaaagagaagatggaaaaagaatttcataagccgtctcagca
L Y F Q G E L K R R W K K N F I A V S A
gccaacgcctttaagaaaatctcatcctccggggcacttgtcgagtcgacTCTAGActcg
A N R F K K I S S S G A L V E S T L D S
agttcgacCTGCAGGcatgcAAGCTTg
S S T C R H A S L

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Fig 3. Map of plasmid pEB587 used to create a N-terminal TAP-tag translational fusion under the control of the P_{BAD} arabinose inducible promoter (A) and the corresponding nucleotidic and protein TAP-tag sequences (B). Protein A sequence is in dark purple, TEV protease cleavage site is in yellow, CBP sequence is in clear purple, the multicloning site is underlined and restriction enzyme sites are indicated.