**Protein pulldown assays to monitor the composition of the bacterial RNA degradosome**

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**Running head**: pulldown assay of RNA degradosome

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**Abstract**

The method of co-immunoprecipitation (co-IP or pulldown) enables to identify proteins interacting in macromolecular assemblies, through the purification of a key protein by affinity chromatography using specific antibodies immobilized in a matrix. The advantages of the use of epitope-tagged proteins are that they usually do not disrupt the structure of the protein and are recognized by commercially available antibodies. Here we describe the utilization of an epitope-tagged version of *Caulobacter crescentus* RNase E in order to determine the composition of the RNA degradosome under different growth conditions. Several proteins that interact with the RNA degradosome were identified.

**1. Introduction**

In all kingdoms of life, deciphering the nature of complex networks of intra-cellular protein-protein interactions has been proven to be crucial for understanding the machinery that underpins cellular processes. The capacity of proteins to form macromolecular assemblies allows the creation and re-modelling of functional networks, and can boost the efficiency and processivity of enzymatic pathways by colocalizing players that perform specific functions.

The method of co-immunoprecipitation (co-IP or pulldown) is one of the most commonly used to determine the interactions among proteins *in vivo*. This technique relies on a stable interaction being formed between two (or more) proteins that remain bound even in the presence of a specific antibody against one of them.

Initial studies demonstrating the efficacy of co-IP of interacting proteins were predominantly carried out using monoclonal antibodies against one of the protein partners, which could then be captured by protein A from *Staphylococcus aureus* and immobilized on agarose beads (Chenais et al., 1977). The specificity and affinity of protein A for the Fc portion of immunoglobulins, especially IgGs, and its ability of being immobilized on a solid support made it a good choice for pulling down protein complexes. This strategy is still a popular method for identifying protein interactomes, however the major disadvantages of this technique is the ability to generate high quality antibodies against the target protein, and the assumption that antibody binding will not displace partner molecules.

However, these drawbacks have largely been overcome with the advent of commercially available monoclonal antibodies against specific epitopes that can be incorporated into target proteins by genetic manipulation. These epitopes are designed to be small, so that they do not disrupt the structure of the protein, and are usually appended to the N- or C-termini of the target protein. Several such epitopes are now commonly used as affinity tags, with their respective specific antibodies being commercially available. Amongst the most commonly used tags are the His-tag, Strep-tag and the FLAG-tag.

In many bacterial species, a multienzymatic complex termed the RNA degradosome is responsible for the turnover of the vast majority of cellular RNA. The RNA degradosome is assembled on the framework of the core enzyme, RNase E, which has two distinct domains, a catalytic N-terminal domain and a non-structured C-terminal region that is the site of interaction with other protein and RNA components. The composition of the RNA degradosomes varies among bacteria and in distinct physiological situations, allowing the cell to adjust its RNA metabolism accordingly, but generally consists of a DEAD-box RNA helicase, polynucleotide phosphorylase (PNPase) and a central metabolic enzyme (reviewed in Hardwick and Luisi, 2013; Bandyra and Luisi, 2018). The utilization of an attached epitope to the N-terminus of RNase E has provided an easy and reliable way to determine the composition of the RNA degradosome in several bacteria (Miczak et al., 1996, Aguirre et al., 2017, Hardwick et al, 2011).

Here we describe the use of genomically encoded, epitope tagged-RNase E in protein pulldown assays in order to determine the composition of the RNA degradosome in the bacterium *Caulobacter crescentus* at two distinct growth temperatures. The protein complexes recovered by this method were resolved by SDS-PAGE and the individual proteins were then identified by mass spectrometry.

**2. Materials**

All solutions were prepared using ultrapure water (MilliQ) and analytical grade reagents. The manufacturers of specific reagents are indicated.

**Bacteria whole extract preparation**

1. Lysis Buffer: 20 mM Tris-Cl, pH 7.5, 250 mM NaCl, 5 mM EDTA, complete EDTA free protease inhibitor cocktail (Roche).
2. PYE medium: 0.2 % peptone, 0.1% yeast extract, 0.02% MgSO4, 0.5 mM CaCl2 (Ely, 1990). Prepare a 1 M CaCl2 solution by dissolving 11.09 g CaCl2 in 100 ml water and sterilize by filtration. For a litre of PYE medium, dissolve 2 g of peptone, 1 g of yeast extract and 0.2 g of MgSO4 in 1 litre of water. Autoclave. Before use add 0.5 ml of the CaCl2 solution for a final concentration of 0.5 mM (see Note 1).

**FLAG-tag protein pulldowns**

1. Binding buffer: 20 mM Tris-Cl, pH 7.5, 200 mM NaCl.
2. Prepare ahead the anti-FLAG® agarose (Sigma Anti-FLAG® M2 Affinity Gel, A2220) by centrifuging 200 l of the agarose slurry at 1000 x g (see Notes 2 and 3) for 1 min and resuspending gently in 200 l of cold binding buffer. Repeat this step once and resuspend the final agarose pellet in 100 l of cold Elution Buffer. Store at 4°C.
3. Elution buffer: Prepare a stock of 3 x FLAG® peptide (Sigma F4799) by solubilizing the lyophilized powder into 20 mM Tris-Cl, pH 7.5, 100 mM NaCl for a final concentration of 5 mg/ml. Store at -20°C. For use, dilute the FLAG peptide to 0.25 mg/ml in Elution buffer.

**SDS polyacrylamide gel**

1. Pre-cast gels: NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.5 mm, 10-well (Invitrogen™) gradient gels 4-12% (NP0321BOX), stored at 4°C.
2. 20x MES Running buffer: 1M MES, 1M Tris base, 2 % wt/v SDS, 20 mM EDTA (pH 7.3). Store at RT. For use, dilute 50 ml of the 20 x solution in 950 ml water.
3. Sample loading buffer: Trupage LDS sample buffer 4x (Sigma-Aldrich). Resuspend 20 μL of protein elution with 5 μL of LDS sample buffer supplemented with 50 mM DTT. Heat at 90°C for 5 minutes prior to loading on the SDS-PAGE gel.
4. Gel Staining: Coomassie Blue G (Sigma B0770). Prepare a solution by weighing 1 g of Coomassie Blue G and resuspend in 90 ml of water for a final concentration of 1%. For 1 litre Gel Stain, mix 890 ml water with 10 ml of the Coomassie Blue 1% solution, 100 ml ethanol and 2.94 ml HCl (concentrated, 10 M). Store at RT.

**3. Methods**

**Bacteria whole cell extract preparation**

1. Grow *Caulobacter crescentus* strains in 1 litre PYE medium at 30°C with aeration until an Optical Density (OD600) of around 0.5. The strains must be engineered to produce a FLAG-tagged RNase E (FLAG-RNE) (see Note 4). When low temperature is desired, grow the cultures up to OD600 of 0.2 at 30°C, and then lower the temperature to 15°C and incubate for 2h (the final OD600 is ~ 0.4) (see Note 5). Centrifuge the bacterial cultures at 4,200 rpm in a Beckman IS4.2 rotor for 20 min and gently resuspend the cell pellets in 30 ml of cold binding buffer. Freeze at -80°C until use.
2. Lyse the cells by two passages through a high-pressure homogenizer at 500 bar (Emulsiflex, Avestin) and centrifuge the lysates at 30000 x g at 4C for 30 min in a Sigma rotor 12150-H (see Note 6).

**Protein pulldown**

1. Transfer the cell extract supernatants (30 ml) into conical Falcon tubes and incubate with 50 μl of anti-FLAG affinity gel previously equilibrated in binding Buffer. Agitate the tubes gently in a swinging plate in a cold room (4°C) for 2h.
2. Leave the tubes standing upright for 30 min at 4°C for sedimentation of the anti-FLAG affinity gel at the bottom of the conical tubes.
3. Collect the anti-FLAG affinity gel with a Pasteur pipette and transfer into a mini-chromatography column (Thermo-Scientific). Wash three times with 500 μl of lysis buffer, centrifuging the tubes at 1000 x g between washes.
4. Elute the proteins by adding to the top of the dry gel 30 μl of the FLAG peptide previously diluted to 0.25 mg/ml in Elution buffer and incubate for 10 minutes at 4°C (see Note 7).
5. Centrifuge the tubes at 1000 x g in a collection tube to obtain the eluted proteins.

**SDS-PAGE**

1. Assemble the pre-cast gels in an electrophoresis tank and add the running buffer to the tank (see Note 8).
2. Add 5 l of the Sample buffer to each sample and heat to 95°C for 10 min (see Note 9).
3. Load the molecular weight marker and the samples (25 l) in the gel, together with a negative control lane with a pulldown from a strain carrying the RNase E without the FLAG tag. Run the electrophoresis at 150 V for 75 min.
4. Turn off the power supply and disassemble the gel separating the plastic plates with the aid of a spatula. Remove the stacking gel and put the gel in water. Heat for 1 min in the microwave.
5. Remove the water, add the Gel Staining solution and heat for 1-2 min in microwave. Leave overnight at RT.
6. Remove the Gel Staining solution and heat in water for 1-2 min for several rounds until the background is clear. The results of pulldown analyses of proteins from *C. crescentus* associating with FLAG-tagged RNase E in different conditions can be seen in Figure 1.
7. Cut the desired protein bands and prepared for trypsinization and mass spectrometry.

**4. Notes**

1. The MgSO4 and CaCl2 ions may precipitate when the medium is autoclaved, so it is best to add the CaCl2 after autoclavation.
2. The centrifugation of the affinity resin must be very gentle or the beads will be damaged. Do not use speeds over 1000 x g when equilibrating, washing or eluting the proteins from the resin.
3. The affinity resin suspension is viscous. For ease of pippeting use a wide bore tip, or remove the end of a standard pipette tip with scissors.
4. The addition of the codons for the epitope in bacterial proteins is done with the use of vectors carrying the modified version of the gene, either by in-trans expression from the vector or by substituting the chromosomal copy of the gene. The methods of choice have to be those adequate for each bacterial species. In any case, it is best to have only the gene for the tagged protein present in the cell, to avoid competition of the untagged protein for the resin.
5. Consider that the temperature drop is slow when growing large volumes such as 1 litre culture. In our hands, after moving a 1 litre culture from 30°C to 15°C, with agitation, it took approximately 2 h for the medium temperature to be homogeneously at 15°C.
6. The method for cell disruption must be chosen with care. The disruption must be mild so that the protein complexes do not dissociate during sample preparation. We did not get good results with sonication due to the heating of the samples and inconsistent cell breakage. Milder treatments such as lysozyme followed by freeze-thawing cycles proved to be more efficient, although it takes very long for the cells to thaw and proteolysis is more difficult to control. The best method was the use of a high-pressure homogenizer, which was fast, reproducible, and preserved the RNA degradosome complexes.
7. The buffer composition should be adjusted according to the stability of the desired complexes. Stable protein complexes can withstand high ionic strength buffers, but weaker interactions may require an optimization of the salt concentration and pH.
8. Remember to remove the tape at the bottom of the gels and to make sure there are no bubbles at the bottom that may prevent the electrical current to pass through the gel.
9. It is useful to briefly centrifuge the samples after boiling to remove insoluble debris and particles of agarose that cause distortions in the sample migration.

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**Table 1**: Some commonly used affinity tags

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| --- | --- | --- | --- | --- |
| Tag | Sequence | Affinity | Advantagesa | Disadvantagesa |
| FLAG | DYKDDDDK | Monoclonal Antibody | Small; immobilized antibody matrix commercially available; N- or C-terminal |  |
| His-tag | (H)6-14 | Ni2+ ions | Small; Ni2+ chelate matrixes commercially available | Non-specific interactions with His-rich proteins |
| HA-tag | YPYDVPDYA (Hemagglutinin peptide from Influenza virus) | Monoclonal antibody | Small; immobilized antibody matrix commercially available; N- or C-terminal |  |
| c-myc-tag | EQKLISEEDL | Monoclonal antibody | Small; immobilized antibody matrix commercially available; N- or C-terminal |  |
| SBP-tag | MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP (Straptavidin binding peptide) | Streptavidin | Straptavidin-conjugated matrix commercially available; N- or C-terminal |  |
| MBP | Maltose binding protein | Amylose matrix | Increases solubility; matrix commercially available | Could prevent partner recognition by steric hindrance; non-specific binding |
| GST | Glutathione S-transferase | Glutathione affinity antibody | matrix commercially available | Could prevent partner recognition by steric hindrance; non-specific binding |
|  |  |  |  |  |

a Advantages and disadvantages to its use in pulldown assays of protein complexes.

**LEGENDS**

**Figure 1.**  Pulldown analyses of proteins associating with FLAG-RNAse E in different conditions. (A) Cultures of *C. crescentus* were grown at either 30°C (cold shock -) or 15°C (cold shock +) up to midlog phase and RNA degradosomes were isolated. The strains used are indicated as: FLAG-RNE, NA1000 (wt) containing a tagged FLAG-RNase E (FLAG-RNE); wt, NA1000 with no tagged proteins as negative control. RNase A was added to some of the extracts as indicate. The proteins associated with the RNA degradosome are indicated by arrowheads and identified on the right. (Reproduced from reference Aguirre et al., 2017 under a Creative Commons Attribution 4.0 International license).