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## **Protein-Protein Interactions: Oxidative Bacterial Two-Hybrid**

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### **Abstract**

Protein-protein interaction studies are essential to understand how proteins organize themselves into interaction networks and thus influence cellular processes. Protein binding specificity depends on the correct three-dimensional folding of the polypeptide sequences. One of the forces involved in the structuring and stability of proteins is the formation of disulfide bonds. These covalent bonds are formed post-transcriptionally by the oxidation of a pair of cysteine residues and can serve structural, catalytic, or signaling roles. Here, we describe an engineered *E. coli* adenylate cyclase mutant strain with an oxidative cytoplasm that promotes correct folding of proteins with disulfide bonds. This genetic background expands the set of host strains suitable for studying protein-protein interactions *in vivo* by the adenylate-cyclase two-hybrid approach.

Keywords: bacterial two-hybrid, oxidative cytoplasm, protein-protein interactions.

### **1. Introduction**

The formation of disulfide bonds participates in the folding and stability of many extra-cytoplasmic and secreted proteins in gram-positive and gram-negative bacteria. These bonds result from the oxidation of thiol groups between two cysteine residues in a polypeptide sequence. Disulfide bonds have multiple biological functions among proteins. They play structural roles or serve as redox sensors. Indeed, cysteines can be reversibly oxidized or reduced, which induces conformational switches of the protein in response to the redox state of the environment [1]. Structural disulfide bonds also tend to decrease the number of possible conformations for a given protein, a feature that is particularly important for periplasmic or secreted proteins in a harsh extracellular environment where they are subjected to multiple physico-chemical stresses. Thus, proteins functioning in these oxidative environments often rely on multiple disulfide bonds to increase their stability. This is the case for many

membrane proteins with signal transduction function [2], secreted toxins (cholera, diphtheria toxin), bacterial virulence factors like those of the T6SS [3] and pilins [4, 5].

For some proteins, disulfide bonds are essential to their functionality, as reduction of the cysteines can lead to protein denaturation. However, for other proteins, SS-bonds participate in protein stability but are dispensable. Correct pairing of cysteines during polypeptide folding can be critical for higher-order protein structuration and functionality. Indeed, mis-pairing of cysteines can result in misfolded and non-functional proteins. While disulfide bonds can form spontaneously in the presence of oxygen, the reaction is slow. In the bacterial periplasm, a dedicated range of thiol oxidoreductases, Dsb (for disulfide bond), catalyze the formation of SS-bonds (DsbA, DsbB) and the correction of mis-paired cysteines (DsbC) [1].

The Bacterial two-hybrid system (BACTH) in *Escherichia coli* is a popular technique for the study of protein-protein interactions *in vivo*. The method, originally developed by Karimova et al. is based on the reconstitution of a signaling cyclic adenosine monophosphate (cAMP) transduction cascade in the cytoplasm of an *E. coli* adenylate cyclase deficient strain [6]. Briefly, the two catalytic domains of *Bordetella pertussis* adenylate cyclase, named T18 and T25, are fused separately to proteins of interest. Interaction between the two tested proteins will bring the T18 and T25 domains in proximity in the cytoplasm, enabling the reconstitution of an active enzyme and leading to cAMP synthesis. This signal positively regulates the expression of the *lac* or *mal* operons, which can be assessed by growth on reporter medium (for review, see [7]).

In another chapter of this book, Ladant and coll. described the BACTH methodology in detail, based on the use of classical reporter strains such as BTH101 and DHM1. However, the cell cytoplasm is not permissive to the formation of disulfide-bonds due to the presence of numerous reductases and reducing agents such as glutathione. This feature limits the use of BACTH to proteins whose folding is not dependent on disulfide bonds. This limitation was first addressed by the development of modified plasmid backbones in a BACTH-TM system [8]. The insertion of transmembrane helices between the proteins of interest and the T18 and T25 fragments of the adenylate cyclase allows protein-protein interactions in the periplasm to be monitored by cAMP production in the cytoplasm.

Here, we present an evolution of the method that is now suitable to study both SS-bond folded and SS-bond independent proteins directly in the cell cytoplasm without the need of cloning in new vectors. The *E. coli* Oxi-BTH strain (Oxidative Bacterial Two-hybrid) is an adenylate-cyclase deficient strain engineered from the commercial SHuffle T7 Express strain (New England Biolabs) [9]. It is deleted for glutaredoxin reductase (*gor*) and thioredoxin reductase (*trxB*) genes and expresses a cytoplasmic version of the disulfide bond isomerase DsbC, promoting correct disulfide bond formation in the cell cytoplasm and proper oxidative folding of proteins containing multiple cysteines (Fig. 1A). The strain was validated in an interaction study between phage adhesion proteins and their corresponding bacterial receptor [9]. Indeed, the Oxi-BTH strain was able to detect binding between the two tested partners, whereas the BTH101 strain did not, demonstrating that SS-bond folding of the phage

adhesion proteins is essential for the interaction. However, the Oxi-BTH strain being *lacZ*, detection of the interactions was only possible through monitoring expression of the maltose operon, on MacConkey plates or Minimal Medium medium supplemented with maltose. In order to be able to follow the interactions on X-Gal plates, and also to permit quantification by beta-galactosidase assays, we reintroduced the *lacZ* gene in the Oxi-BTH genome to obtain the Oxi-Blue strain, now suitable to the use of the common X-gal reporter medium (Fig. 1B, Pellegrini et al., unpublished).

In conclusion, the Oxi-strains (Oxi-BTH and Oxi-Blue) are powerful tools to extend the scope of the two-hybrid approach to proteins susceptible to fold via SS-bonds, such as envelope proteins or secreted proteins. As the method relies on the use of the standard pUT18 and pKT25 vectors, the proteins of interest can be tested in parallel in both the regular BTH101 or DHM1 strain (reduction of the SS-bonds) and the Oxi- strains (SS-bond formation).

## 2. Materials

### 2.1 Equipment

1. Standard material for DNA cloning
2. Water bath or dry bath heater (37°C and 42°C)
3. Benchtop centrifuge
4. Wood applicators, such as Merck, ref#Z406430
5. 96 deep-well plates or culture tubes, sterile such as 96-well 2.2 mL deepwell plate (e.g., ABGENE, ref.#24704).
6. Microporous tape sheet, such as Porous film AeraSeal™ Clearline®
7. Sterile square Petri Dishes 120 x 15 mm
8. Multi-channel pipette 2 to 10 µL (*see Note 1*)
9. Incubator (30°C and 37°C)

### 2.2 Bacterial Media and solutions

1. Ampicillin stock solution (25 mg/mL), filter-sterilized. Store the solution at 4°C.
2. Kanamycin stock solution (25 mg/mL), filter-sterilized. Store the solution at 4°C.
3. IPTG (isopropyl 1-thio-β-D-galactopyranoside) stock solution, 100 mM in distilled water, filter-sterilized and stored at 4°C.
4. 2YT medium. Dissolve 16 g of Tryptone, 10 g of yeast extract and 5 g of NaCl in distilled water to reach a final volume of 1 L. Sterilize by autoclaving for 15 min at 121°C.
5. Lysogeny Broth (LB) medium and LB-agar plates. Dissolve 10 g NaCl, 10 g tryptone and 5 g yeast extract in 1 L distilled water. Sterilize the medium by autoclaving. Add 15 g of agar /L before autoclaving to make LB-agar plates. Supplement with ampicillin at 100 µg/mL and kanamycin at 50 µg/mL just before pouring the plates.

6. Maltose stock solution, 20%. Dissolve glucose-free maltose in distilled water (20 g in 100 mL) and filter-sterilize.

### 2.3 BACTH reporter media

There are three possible reporter media that are commonly used in the BACTH method. The M63/maltose and MacConkey/maltose media rely on the consumption of maltose by the bacteria and thus are adapted to the study of interactions in the Oxi-BTH and the Oxi-Blue strain backgrounds. The X-Gal/IPTG medium requires the use of a *lacZ*<sup>+</sup> strain and is therefore suitable for the Oxi-Blue strain only (*see Note 2*).

1. M63 medium supplemented with maltose plate :

Prepare a 5X M63 stock medium (dissolve 10 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 68 g KH<sub>2</sub>PO<sub>4</sub>, 2.5 mg FeSO<sub>4</sub>·7H<sub>2</sub>O in 1 L distilled water, adjust to pH=7.0 and sterilize by autoclaving. To prepare the reporter medium, dissolve 15 g agar in 800 mL distilled water and sterilize by autoclaving. Add 200 mL of the 5X M63 solution, 1ml of MgSO<sub>4</sub>·7H<sub>2</sub>O 1M, 10 ml of 20% maltose, 2 ml of 0.05% vitamin B1 (thiamin), antibiotics (50 µg/ml ampicillin; 25 µg/ml kanamycin), and IPTG 0.5 mM to induce full production of the protein fusions of interest. Mal<sup>+</sup> cells will form white colonies on the reporter medium after 2 to 7 days of incubation at 30°C (*see Note 3 and Note 4*)

2. MacConkey maltose plate :

Dissolve 40 g MacConkey agar base in 1 L H<sub>2</sub>O, mixed well until complete dissolution and autoclave (*see Note 5 and 6*). Just before pouring the plates, add 50 mL 20 % Maltose solution (1% final concentration), ampicillin 100 µg/mL, kanamycin 50 µg/mL. IPTG 0.5 mM can be included to insure full expression of the constructs. Mal<sup>+</sup> cells will form red colonies on the MacConkey medium after 24 to 72 h of incubation at 30°C (*see Note 4*).

3. LB X-gal plate :

LB agar medium is supplemented with 87.5 mg of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) dissolved in 500 µL of DMF (N, N-diméthylformamide)), ampicillin 100 µg/mL, kanamycin 50 µg/mL and IPTG 0.5 mM just before pouring the plates (*see Note 7*). Functional complementation of the adenylate cyclase can be detected by the observation of blue colonies after 24 to 72 hrs at 30°C (*see Note 4*).

### 2.4 Two-hybrid reporter strains and plasmids

1. *E. coli* reporter strain engineered to provide an oxidative cytoplasm and deleted of the adenylate cyclase gene *cya*. We recommend the Oxi-BTH strain [9] or the Oxi-Blue strain (Pellegri et al. unpublished) (*see Note2*).
2. BACTH vectors producing the proteins of interest fused to the *B. pertussis* T18 adenylate cyclase domain (such as pUT18 or pUT18C) (*see Note 8*).

3. BACTH vectors producing the proteins of interest fused to the *B. pertussis* T25 adenylate cyclase domain (such as pKT25 or pKNT25) (*see Note 8*).
4. Control vectors to test negative and positive signal of interaction between disulfide-bond folded proteins (*see Note 9*).

### 3. Methods

1. Prepare competent cells by standard protocol using CaCl<sub>2</sub> [10] (*see Note 10*).
2. Construct the BACTH plasmids to produce the hybrid proteins (*see Note 8 and Note 9*).
3. Introduce the couple of plasmids into the competent cells of choice by cotransformation: add 1 μL of pKT25 vector and 0.8 μL of pUT18 vector (i.e. 5 to 10 ng of each plasmid) in 40 μL competent reporter cells Oxi-BTH or Oxi-Blue cell. Mix contents by gently flicking the tubes and incubate on ice for 30 minutes. Transfer tubes to a dry bath heater that has been preheated to 42°C. Leave the tubes in the rack for exactly 45 seconds. Rapidly transfer the tubes to an ice bath. Allow the cells to chill for 2 min. Add 600 μL 2YT to each tube. Incubate cultures for 60 min in a dry bath heater set at 37°C with agitation to allow expression of the antibiotic resistance genes encoded by the plasmids. Spread the transformed competent cells onto LB-agar plate supplemented with ampicillin and kanamycin using sterile glass beads. Use the appropriate volume of cell suspension so as to obtain isolated clones on the plates (*see Note 4*).
4. Invert the plates and incubate at 37°C for 24-30 hours, or at 30°C for 48-72 hours (*see Note 11*).
5. Prepare the sterile 96-deepwell plate : for each co-transformation to test, fill a well with 600 μL of LB medium supplemented with ampicillin 100 μg/mL, kanamycin 50 μg/mL, and IPTG 0.5 mM.
6. For each cotransformant to test, collect 4 to 6 colonies with a wooden stick and disperse the cells in one growth medium-filled well (*see Note 1*).
7. Cover the deepwell plate with a porous adhesive film.
8. Incubate the deepwell plate at 30°C with shaking at 180 rpm for 24 to 30 hrs.
9. Spot 3.5 to 4 μL of each culture with a multichannel pipette on the reporter medium square plate (M63 minimal medium plate, MacConkey plate or LB X-gal plate for Oxi-Blue) (*see Note 1*).
10. Incubate at 30°C for 24 to 48 h (LB/X-Gal and MacConkey medium) or longer (M63/maltose medium) and monitor the interaction signal (*see Note 4 and Note 12*).

### 4. Notes

1. We recommend the use of multichannel pipettes (2 to 10 μL) for the regular spotting of the cultures on the reporter plates, especially when testing large number of protein pairs, which is often the case in BATCH assays. To facilitate the spotting, we recommend planning the culture in the deepwell plate as ready for the spotting. Beware not to pipet the bottom of the well, as dead cells tend to sediment.

2. Strain genotypes:

Oxi-BTH: Shuffle T7 Express (*fhuA2 lacZ::T7 gene1* [lon] *ompT ahpC gal latt::pNEB3-r1-cDsbC* (Spec<sup>R</sup>, lacI<sup>q</sup>) *ΔtrxB sulA11 R(mcr-73::miniTn10--Tet<sup>S</sup>)2* [dcm] *R(zgb-210::Tn10 --Tet<sup>S</sup>) endA1 Δgor Δ(mcrC-mrr)114::IS10*), *Δcya*

Oxi-Blue: Oxi-BTH *ΔphoA lacZ<sup>+</sup>*

DHM1: F-, *cya-854, recA1, endA1, gyrA96* (Nal<sup>r</sup>), *thi1, hsdR17, spoT1, rfbD1*, glnV44(AS).

BTH101: F-, *cya-99, araD139, galE15, galK16, rpsL1* (Str<sup>r</sup>), *hsdR2, mcrA1, mcrB1*.

3. X-Gal can be added to the M63/Maltose reporter medium to facilitate the observation of the colonies.
4. The Oxi-BTH and Oxi-Blue strains have a reduced growth rate compared to the BTH101 and DHM1 strains. We recommend increasing the time of recovery to 1 hr after transformation of the Oxi-strains. Growth on the reporter plate media and development of the signal will also be longer than for the regular BACTH strains, from 24 to 72 hours (LB/X-Gal and MacConkey/Maltose media) and 2 to 7 days (M63/Maltose medium).
5. MacConkey medium can be simply boiled in a microwave for 5 min to avoid autoclaving. This method is suitable for immediate utilization of the reporter medium. It is best not to store the poured plates for later use, as sterilization is less effective than when autoclaving.
6. Not all the MacConkey agar media have equivalent quality. Difco # 216830 is recommended.
7. X-Gal solution in DMF must be prepared extemporaneously.
8. The T18 and T25 domains can be fused to either the C-terminus or the N-terminus of the proteins of interest. However, fusions can generate steric hindrance incompatible with an interaction between the partners. Similarly, the position of the T18 or T25 domain may be incompatible with the reconstitution of adenylate cyclase activity if the reporter domains are too far apart in the complex between the partners. It may therefore be interesting to test both N-terminal and C-terminal fusions of the proteins of interest to overcome these potential limitations.
9. Several options of proteins devoid of disulfide bonds and suitable as controls are available (pUT18-zip, pKT25-ZIP, pUT18-Pal and pKT25-TolB [6, 9]). For positive control of disulfide-bond dependent interaction in the Oxi-strains, we recommend to use the filamentous phage minor coat protein pIII and its bacterial receptor TolA (either pIII-N1<sup>CTX</sup>/ TolA3<sup>V. cholerae</sup> or pIII-N1<sup>M13</sup>/ TolA3<sup>E. coli</sup>), [9] and Fig.1B.
10. Lac<sup>+</sup> or Mal<sup>+</sup> revertants may spontaneously occur. Before preparing the competent cells, the *cya<sup>-</sup>* reporter strains from the frozen stock should be reisolated on the reporter medium of choice (MacConkey/maltose or LB/X-Gal/IPTG plates) and grown overnight at 37 °C to insure the selection of a white colony to start the culture.
11. To run more sustainable experiments with less disposable plastic plates and to obtain isolated clones, it is possible to spread two (or more) co-transformations on a single LB-agar plate supplemented with ampicillin and kanamycin. After the recovery step, centrifuge the

transformed cells for 5 min at 8,000 rpm, discard the supernatant so that only about 50  $\mu$ L remains and resuspend the pellet in this volume by pipetting. Divide the plate in half and carefully drop the suspension at one end of the half plate. With a wooden stick, streak the cells from the drop to isolate colonies on the plate. The other half of the plate can be used to spread another transformation.

12. Following the initial recommendations on the BACTH method, Oxi-BTH and Oxi-Blue transformant cells spotted on the reporter media should be incubated at 30°C to obtain efficient complementation activity. However, due to the slow growth rate of the Oxi-Strains, it may be worth testing the incubation at 37°C.

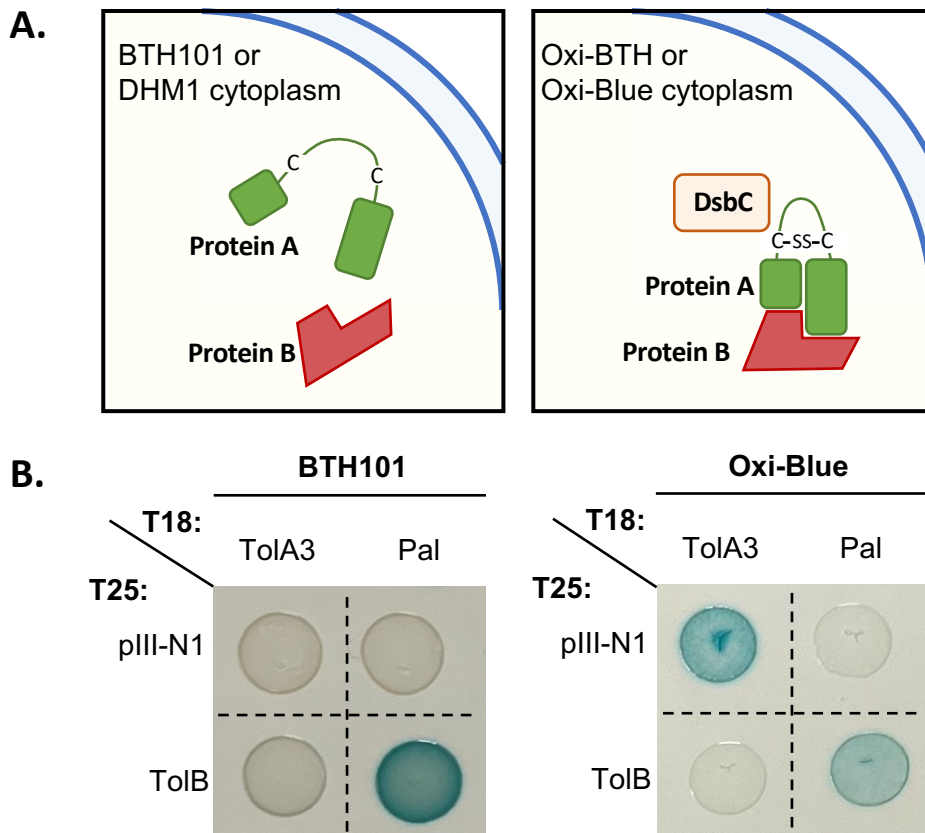
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**Fig.1 Bacterial Adenylate Cyclase Two-Hybrid assay in oxidative reporter strains. A.** Schematic representation of an interaction dependent on the correct folding of a protein by a disulfide bond. The interaction is not possible in the reducing cytoplasm of the BTH101 or DHM1 strains. In contrast, the Oxi-BTH and Oxi-Blue strains are engineered to have an oxidizing cytoplasm and to produce a cytoplasmic form of the disulfide bond isomerase DsbC. This environment is favorable to correct folding of proteins with thiol-bonds. **B.** Example of an interaction assay performed between the CTX filamentous phage minor coat protein pIII-N1 and its receptor, the TolA3 domain of *Vibrio cholerae*. The proteins of interest are fused to the T18 and the T25 domains of *B. pertussis* adenylate cyclase, as described in [9], and co-produced in either the BTH101 or the Oxi-Blue reporter strains spotted on LB/X-Gal/IPTG medium. The blue color indicates an interaction between the partners. As pIII-N1 folds via four disulfide bonds, the interaction with its partner can only be visualized in the Oxi-Blue strain. The interaction between TolB and Pal serves as a positive control in both strains, as it is not dependent on the redox state of the proteins.