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The bacterial two-hybrid system based on adenylate cyclase reconstitution in *Escherichia coli*

Aurélia Battesti [a], Emmanuelle Bouveret [b,*]

[a] NIH/NCI, 9000 Rockville Pike, Bethesda, MD 20892, USA
[b] CNRS, Aix-Marseille University, LISM, 31 Chemin Joseph Aiguier, 13009 Marseille, France

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ABSTRACT

The bacterial two-hybrid system based on the reconstitution of adenylate cyclase in *Escherichia coli* (BACTH) was described 14 years ago (Karimova, Pidoux, Ullmann, and Ladant, 1998, PNAS, 95:5752). For microbiologists, it is a practical and powerful alternative to the use of the widely spread yeast two-hybrid technology for testing protein–protein interactions. In this review, we aim at giving the reader clear and most importantly simple instructions that should break any reticence to try the technique. Yet, we also add recommendations in the use of the system, related to its specificities. Finally, we expose the advantages and disadvantages of the technique, and review its diverse applications in the literature, which should help in deciding if it is the appropriate method to choose for the case at hand.

1. Introduction

As an alternative to the widely used yeast two-hybrid method, several two-hybrid systems have been engineered in bacteria to study protein–protein interactions. One of them, developed by Karimova and collaborators in 1998, is based on the reconstitution of adenylate cyclase activity in *Escherichia coli* [1]. This bacterial two-hybrid system takes advantage of the specific features of the adenylate cyclase toxin from *Bordetella pertussis* to detect interactions between cytoplasmic proteins, as well as membrane proteins, from prokaryotes but also from eukaryotes. Previous reviews describing extensively this technique have already been published [2–5]. However, recently, the BACTH system has been much more frequently used in the molecular microbiology community and several improvements have been made on the technique. In this review, we would like to give basic and easy protocols, that we use routinely in our laboratories, to allow everyone to apply rapidly this technique to their research field. We will also describe the new tools available and summarize what was done in the last few years by using the BACTH system, discuss what is feasible, and underline the advantages and the successes of the technique to study protein–protein interactions.

2. Principle and development of the bacterial adenylate cyclase two hybrid system

The bacterial adenylate cyclase two hybrid (BACTH) system is based on the reconstitution of a regulatory cascade depending on cyclic adenosine 3′,5′-monophosphate (cAMP). cAMP is synthesized by a family of enzymes called adenylate cyclases. A peculiar member of this family is the adenylate cyclase of *B. pertussis*, the agent of the whooping cough. This enzyme is a toxin that becomes fully active in the host after binding to calmodulin, a protein found only in eukaryotic organisms. Following its activation, the *B. pertussis* adenylate cyclase toxin alters the host's functions by elevating the level of cAMP in this organism [6]. The *B. pertussis* adenylate cyclase has been extensively studied (for review see [7]). It is a large protein of 1706 amino acids with a catalytic activity that resides in its 400 first amino acids [8] (Fig. 1). This catalytic domain itself can be divided in two sub-domains: a 25 kDa fragment (residues 1–224) that contains the catalytic site and an 18 kDa fragment (residues 225–399) that contains the main calmodulin binding site [9].

Interestingly, when they are co-expressed in the presence of calmodulin, these two sub-domains interact and this interaction restores the synthesis of cAMP [10]. Such observation highlights the capacity of these two sub-domains to complement and recover the adenylate cyclase activity when they are brought close from each other. The BACTH system is based on this complementation. Indeed, the proteins of interest are fused to the T25 or the T18 sub-units. If the two proteins of interest interact in an otherwise

Abbreviations: BACTH, bacterial adenylate cyclase two-hybrid; cAMP, cyclic adenosine 3′,5′-monophosphate.

* Corresponding author. Fax: +33 04 91 71 21 24.
E-mail address: bouveret@imm.cnrs.fr (E. Bouveret).

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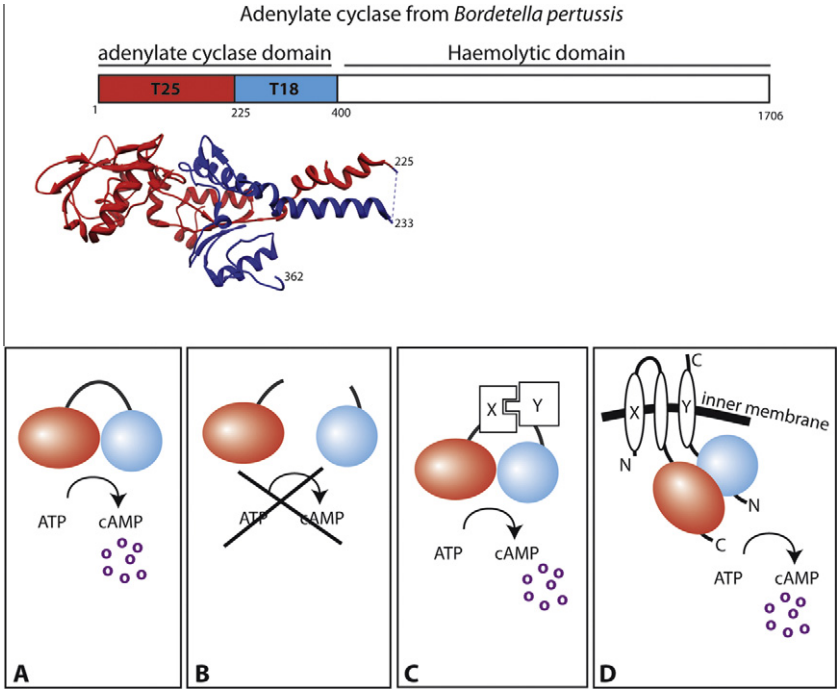

Fig. 1. Organization of *B. pertussis* adenylate cyclase and rational of the BACTH technique based on T18 and T25 domains reconstitution. The structure of the adenylate cyclase domain is shown with colour corresponding to the T18 and T25 domain limits [59]. A: When the adenylate cyclase domain alone is expressed in *E. coli cya*$^-$ cells, there is residual cAMP synthesis. B: When T18 and T25 domains are produced separately, no cAMP is produced. C: T18 and T25 domains put close together by the interaction of hybrid proteins X and Y restore adenylate cyclase activity. D: The technique functions for membrane proteins [17].

cya$^-$ strain, they fulfill the role of calmodulin in bringing together T18 and T25 domains and thus the adenylate cyclase activity is restored (Fig. 1). Newly synthesized cAMP interacts with the catabolite activator protein (CAP) and the cAMP/CAP complex binds to promoters and regulates transcription of several genes. Among these genes, the *lactose* and *maltose* catabolic operons are positively regulated by cAMP/CAP, and their activation can be easily detected by several assays in *E. coli*.

3. Bacterial two-hybrid protocols

3.1. Overview of the technique (Fig. 2)

An *E. coli* strain deleted of the gene coding for the endogenous adenylate cyclase (*cya*$^-$ strain) is transformed by both plasmids containing the T25 and T18 hybrids (Fig. 2A). Although *B. pertussis* adenylate cyclase usually needs to bind calmodulin to be fully active, it has been shown that in *E. coli*, a cryptic adenylate cyclase activity is detected even in absence of calmodulin [8]. There are then several ways to detect a positive interaction within the BACTH system. Indicator LB plates containing X-Gal, or MacConkey plates containing lactose or maltose as the sole carbon source are used for simple qualitative colorimetric assays (Fig. 2B). A positive interaction is detected on LB/X-Gal plates by the appearance of a blue color, and on lactose/maltose MacConkey plates by the appearance of a red color. For quantitative assays, direct cAMP measurement can be performed [11]. However, usually, an indirect β-galactosidase assay is used, following the classical Miller protocol [12]. β-galactosidase assays in 96-well plates have also been developed to screen several conditions and interactions at the same time (see Section 3.4).

3.2. Materials

We list below the material that will be needed to perform the protocols that are described.

3.2.1. Vectors

Two different sets of compatible bacterial two-hybrid vectors have initially been engineered to construct recombinant proteins in frame with the T18 and T25 sub-units. The first set consisted in two vectors permitting to fuse the T18 domain at the C-terminal and the T25 domain at the N-terminal end of the protein, with resistance to ampicillin and chloramphenicol respectively [1] (Table 1). Both hybrids were under the control of the *lacUV5* promoter. The second set was complete, enabling the tagging with T18 and T25 domains, either at the N-terminal or the C-terminal of the protein, with ampicillin and kanamycin resistances [13] (Table 1). In this second set of plasmids, the hybrid constructions are under the control of the wild type *lactose* promoter. The choice of the tag localization depends on the characteristics of each protein. Various tests can be performed to evaluate the incidence of the fusion on the protein functionality (see Section 3.6). The second set of vectors is available from Euromedex as a BACTH system kit (ref. EUK001). Later, derived vectors containing a modified MCS or an additional flag tag have been designed (Table 1) [14,15]. In these plasmids, the choice of cloning sites is reduced, but the EcoRI and XhoI sites are compatible with the widely used yeast two-hybrid vectors pEG202, pJG4-5, and pMW104 for example [16]. These plasmids are available through the Addgene plasmid repository (http://www.addgene.org). For the constructions of the hybrids, one has to make sure to add the stop codon in the cloned ORFs when using N-terminal tags, and to be careful with the reading frame for the C-terminal tags, because all the restriction sites are not in the same reading frame. Strong positive controls are available, such as the pT18-zip and pKT25-zip plasmids containing a DNA sequence coding for a leucine zipper motif [1] (Table 1).

3.2.2. Strains

Two *E. coli* strains are commonly used for BACTH experiments: DHM1 [17] and BTH101. The genotype of BTH101 is *F', cya-99, araD139, galE15, galK16, rpsL1 (Str*^{R}*), hsdR2, mcrA1, mcrB1, relA1* (the *relA1* mutations is not indicated in the Euromedex manual,

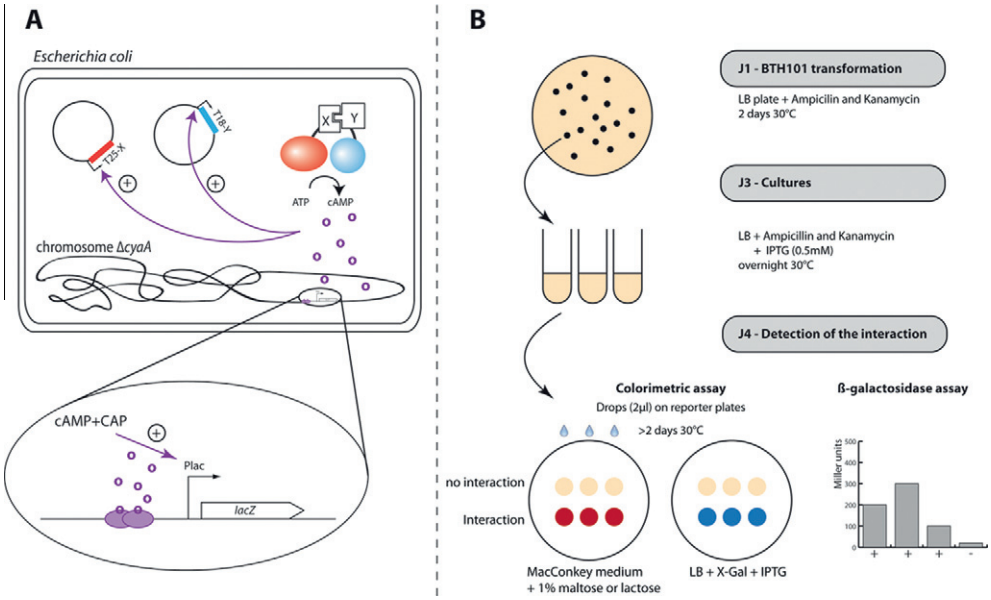

Fig. 2. BACTH protocol A: An *E. coli cya*$^{-}$ strain is transformed by the two compatible plasmids carrying the hybrids with T25 and T18 domains. The interaction of proteins X and Y brings back together domains T25 and T18 and restores cAMP synthesis. Diffusible cAMP then induces expression of lactose and maltose operons, and also increases by a positive feedback the expression of the hybrid genes. B: The different steps of the protocol we routinely use in our laboratories are outlined. See text for the detail.

Table 1
Vectors for the BACTH. In the description column, the promoter (*Plac* or *lacUV5*), the MCS, and its position relative to the T18 and T25 domains are indicated. Full sequences of most of these plasmids are included in the BACTH manual edited by Euromedex that can be freely downloaded on the Euromedex website. Sequences of pKT25linker, pUT18Clinker, pKT25-Flag, and pUT18C-Flag can be found at the following url: http://lism.cnrs-mrs.fr/Bouveret/Pages/plasmids.html. MCS: multiple cloning site.

Name	Tag localization	Resistance	Origin of replication	Description	References
pT25	N-terminal	Cam	p15A	Derived from pACYC184 vector. lacUV5–T25–MCS(PstI–BamHI–KpnI)	[1]
pT18	C-terminal	Amp	ColE1	Derived from pBluescript IIKS. lacUV5–MCS(KpnI)–T18	[1]
pKT25	N-terminal	Kan	p15A	Derived from pSU40. Plac–T25–MCS(PstI–XbaI–BamHI–SmaI–KpnI–EcoRI)	[13]
pUT18C	N-terminal	Amp	ColE1	Derived from pUC19. Plac–T18–MCS(PstI–SalI–XbaI–BamHI–SmaI–KpnI–EcoRI)	[13]
pKNT25	C-terminal	Kan	p15A	Derived from pSU40. Plac–MCS(HindIII–SphI–PstI–XbaI–BamHI–SmaI–KpnI–SacI–EcoRI)–T25	[17]
pUT18	C-terminal	Amp	ColE1	Derived from pUC19. Plac–MCS(HindIII–SphI–PstI–SalI–XbaI–BamHI–SmaI–KpnI–SacI–EcoRI)–T18	[13]
pT25-zip	N-terminal	Cam	p15A	Derived from pT25. Sequence coding for the leucine zipper region of the GCN4 yeast protein. Positive control	[1]
pT18-zip	N-terminal	Amp	ColE1	Derived from pT18. Sequence coding for the leucine zipper region of the GCN4 yeast protein. Positive control	[1]
pKT25-zip	N-terminal	Kan	p15A	Derived from pKT25. Sequence coding for the leucine zipper region of the GCN4 yeast protein. Positive control	[13]
pUT18C-zip	N-terminal	Amp	ColE1	Derived from pUT18C. Sequence coding for the leucine zipper region of the GCN4 yeast protein. Positive control	[13]
pKT25linker(pEB354)	N-terminal	Kan	p15A	Derived from pKT25. Plac–T25–MCS(PstI–XbaI–EcoRI–ClaI–XhoI)	[15]
pUT18Clinker(pEB355)	N-terminal	Amp	ColE1	Derived from pUT18C. Plac–T18–MCS(PstI–XbaI–EcoRI–EcoRV–XhoI)	[15]
pKT25–Flag(pEB1029)	N-terminal	Kan	p15A	Derived from pKT25linker. Plac–T25–Flag–MCS(EcoRI–ClaI–XhoI)	[14]
pUT18C–Flag(pEB1030)	N-terminal	Amp	ColE1	Derived from pUT18Clinker. Plac–T18–Flag–MCS(EcoRI–EcoRV–XhoI)	[14]

but we have sequenced and verified it). The genotype of DHM1 is *F', cya-854, recA1, endA1, gyrA96 (Nal*^{R}*), thi1, hsdR17, spoT1, rfbD1, glnV44(AS).* Both strains are available in the BACTH system kit from Euromedex (ref. EUK001). BTH101 grows faster and shows stronger interaction signals than DHM1. However, some instability of plasmids can appear because it is Rec+, which is not the case for DHM1. The authors have only experience in the use of BTH101, but other labs do use DHM1.

3.2.3. Reagents and chemicals

Stock solutions:

- X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) stock at 20 mg/ml in dimethyl formamide (DMF) kept at −20 °C.
- IPTG (Isopropyl-β-D-thiogalactoside) stock at 0.1 M, filter sterilized and kept at −20 °C.
- $CaCl_2$ stock at 0.1 M kept at room temperature.
- Glycerol stock at 80% kept at room temperature.
- Antibiotic stock solutions: 25 mg/ml ampicillin and 10 mg/ml kanamycin kept at 4 °C.
- Maltose 20%, sterilized by filtration, kept at room temperature
- SDS (sodium dodecyl sulfate) 0.01%.
- Na_2CO_3 stock at 1 M.

Z buffer: 8 g of $Na_2HPO_4{\cdot}12H_2O$, 3.125 g of $NaH_2PO_4{\cdot}H_2O$, 0.375 g KCl, 0.123 g $MgSO_4{\cdot}7H_2O$ dissolved in 500 ml distilled water, adjusted to pH 7 if necessary. Immediately before use, 1.35 ml β-mercaptoethanol is added. The buffer is kept at 4 °C.

M63 medium: 13.6 g KH_2PO_4, 2 g $(NH_4)2SO_4$, 500 μg $FeSO_4$ $7H_2O$ and 245 mg $MgSO_4 \cdot 7H_2O$ in 1 liter of distilled water, adjusted to pH 7 with KOH.

LB (Luria–Bertani) broth: 10 g NaCl, 10 g tryptone, 5 g yeast extract in 1 liter of distilled water. Adjust pH to 7 with NaOH, and autoclave. Plates are prepared by adding 1.5% agar.

M63/Maltose plates: M63 medium supplemented with 0.2% maltose, kanamycin (30 μg/ml), ampicillin (50 μg/ml), Vitamin B1 (0.5 μg/ml), IPTG (0.5 mM) and X-Gal (40 μg/ml) and 1.5% agar.

MacConkey plates: For the MacConkey/maltose plates, suspend Difco™ MacConkey agar base powder (ref: 281810) in distilled water (following the indications on the bottle), and dissolve by boiling with frequent agitation. Autoclave at 121 °C for 15 min. The medium can also be used directly after 5 min boiling, without autoclaving. Add ampicillin (100 μg/ml), kanamycin (50 μg/ml), IPTG (0.5 mM), and 1% maltose. For the MacConkey/lactose plates, use the standard Difco™ MacConkey agar (ref: 212123) that already contains lactose, and omit IPTG.

LB–X-Gal plates: LB agar supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), X-Gal (40 μg/ml), and IPTG (0.5 mM).

ONPG (O-nitrophenyl-β-D-galactoside): 4 mg/ml of ONPG diluted in Z buffer, prepared just before use.

3.2.4. Specific material for the 96-well β-galactosidase assay

- 2.2 ml 96-well polypropylene block,
- standard 96-well microplates,
- multi-channel pipette,
- a spectrophotometer for 96-well microplates.

3.3. Basic protocol to test the interaction between a given pair of proteins

Here is the basic protocol that we use routinely in our laboratories (Fig. 2B). In addition to testing the pair of proteins of interest, positive controls are performed with T25-zip and T18-zip plasmids. Negative controls are performed with any pair of proteins that do not interact, for example the two empty T18 and T25 plasmids, or better, each protein of interest against the empty T18 or T25 plasmids.

Transformation. BTH101 (or DHM1) competent cells are prepared by treatment with $CaCl_2$. Cells are grown in LB at 37 °C until OD_{600nm} is comprised between 0.3 and 0.5. Cells are collected by centrifugation 10 min at 4000 rpm and the supernatant is discarded. The cell pellet is suspended in ½ volume of ice-cold $CaCl_2$ 50 mM and incubated 20 min on ice. Cells are again collected by centrifugation, and the supernatant is discarded. The cell pellet is suspended in 1/10 volume of ice–cold $CaCl_2$ 50 mM, 15% glycerol and incubated 20 min on ice. Aliquots of 500 μl can be stored at −80 °C or used immediately. For each assay, 0.5 μl (prepared with a standard miniprep kit) of the two plasmids carrying the T25 and T18 fusions are added to 100 μl of competent cells in an ice cold 1.5 ml tube and incubated on ice during 20 min. The cells are then heat shocked at 42 °C during 1.5 min followed immediately by 1 min on ice. Then, 1 ml LB is added and cells are placed at 37 °C for recovery for 1 h. Cells are collected by centrifugation at 8000 rpm for 3 min, and 1 ml of supernatant is discarded. The pellet is suspended in the remaining 100 μl of LB and cells are spread on LB plates containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. Plates are incubated at 30 °C for 48 h.

Hybrid expression and interaction assay. After incubating the plates for 2 days at 30 °C, 3 ml of LB containing 100 μg/ml ampicillin, 50 μg/ml kanamycin and 0.5 mM IPTG are inoculated with several clones from the transformation plate (several clones are picked in order to reduce heterogeneity, see Section 3.6). Triplicates are performed for each pair of plasmids. Cultures are grown overnight at 30 °C with shaking. The next day, 2 μl of each culture are dropped on LB-X-Gal plates or on MacConkey/maltose or lactose plates (see Section 3.2.3). The plates are then incubated at 30 °C. MacConkey/Maltose or lactose plates can be incubated several days until a red coloration appears. Alternatively to the colorimetric assay on reporter plates, β-galactosidase assay is performed on the same overnight liquid cultures, following the classical Miller protocol [12] that we will not describe here. It has to be noted that the signal obtained by following this protocol with liquid precultures and drops on plates (Fig. 2B), gives higher signals than when the bacteria are directly plated on the reporter plates after transformation, or when the bacteria are reisolated on the reporter plates from the transformation plates.

3.4. β-Galactosidase assay in 96-well arrays (Fig. 3)

When using the BACTH technique, the number of assays to be performed grows exponentially even when not so many clones are tested. Indeed, for a given pair of proteins, one wants to test all the combinations possible between ORF fused to T18 or T25 sequences, and at the N-terminus or the C-terminus (this results in already eight assays just for two partners). Furthermore, due to some variability in the detection of interactions, it is necessary to perform replicates. Here, we describe the standard β-galactosidase assay in 96-well plates that we use routinely, doing 3, 4, or 6 replicates for each pair to be tested. A microplate spectrophotometer is required for this protocol. We use a TECAN machine, which permits the direct injection of the ONPG in the plate, and the measurement at 420 nm at different time points (see Fig. 3).

This β-galactosidase assay was adapted to 96-well array by modification of the Griffith and Wolf's method [18]. The bacteria are grown in a 2.2 ml 96-well polypropylene block, each well containing 0.6 ml of LB medium supplemented with 50 μg/ml kanamycin, 100 μg/ml ampicillin, and 0.5 mM IPTG. The block is positioned in an incubator with an inclination of about 45° in order to enhance cell agitation. After overnight growth at 30 °C, 50 μl of culture from each well are transferred with a multi-channel pipette into a flat-bottom microtiter plate already filled with 150 μl LB, in order to measure the OD_{600nm} in a microplate reader. In parallel, 200 μl of each culture are transferred into a glass tube containing 800 μl of Z buffer (see Section 3.2.3). One drop of SDS 0.01% and two drops of chloroform are added, and the glass tubes are mixed thoroughly during 10 s to permeabilize the cells. After letting chloroform settle down at the bottom of the tube, aliquots of 50 μl are transferred into a 96-well flat-bottom microplate containing 150 μl of Z buffer and pre-equilibrated at 28 °C in the microplate reader. Then, 40 μl of ONPG 0.4 % are dispensed and the enzymatic reaction is carried out at 28 °C for 15–20 min with measurement of OD_{420nm} every 2 min in the microplate reader. The relative β-galactosidase activity in each of the 96 samples is then calculated by simple Excel file manipulation. It corresponds to $((OD_{420nm}$ at time t2$-OD_{420nm}$ at time t1)/t2–t1 (min))/OD_{600nm}. The t2 and t1 time points are chosen to be located in the linear part of the kinetic.

One drawback of this approach is that the intensity of coloration of the ONP formed by the reaction is lower at the pH of the Z buffer as compared to that after classical addition of Na_2CO_3 [12]. This might lead to missing some weak interactions. Yet, the advantage is that the β-galactosidase activities can be accurately determined in parallel on multiple samples presenting a wide variation in enzymatic activities (e.g. negative and positive controls): the activities of samples expressing a high level of the β-galactosidase can be determined from the early time points of the kinetics, whereas those expressing a low level can be accurately determined after a prolonged incubation (as the variation in OD_{420nm} is larger at these times). Furthermore, there is no need to perform a blank

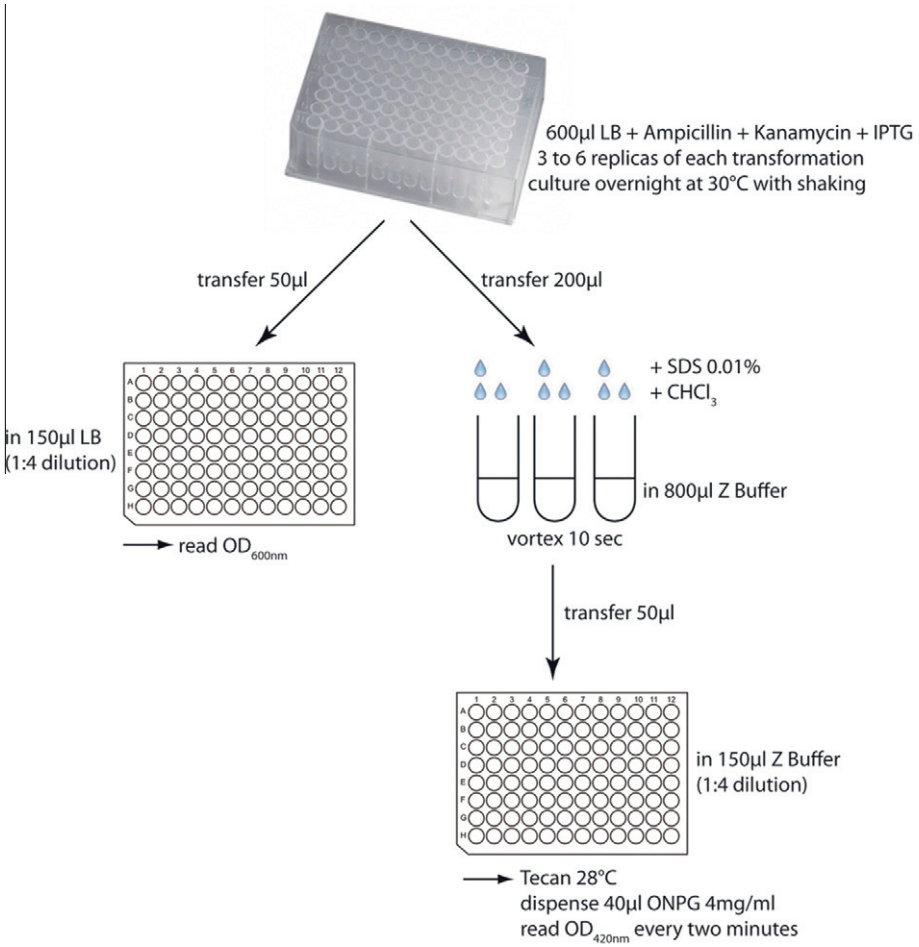

Fig. 3. β-galactosidase assay in 96-well plates. The different steps of the protocol we routinely use in our laboratories are outlined. See text for the detail.

reaction, neither to correct with the OD_{550nm}, because these terms disappear in the subtraction between the two time points.

To conclude, any type of settings that can be implemented in the lab for high throughput β-galactosidase assay should be used. For example, a similar protocol for β-galactosidase assay in 96-well plates applied to BACTH has been published recently [19]. Finally, in parallel, 2 µl of the overnight cultures from the 96-well polypropylene block may also be directly replicated on indicator plates using a multi-channel pipette. Indeed, the indicator plates often display higher sensitivity than the β-galactosidase assay.

3.5. Library screening

3.5.1. Principle

The BACTH system is a simple and rapid tool to look for new partners of a protein of interest. For this purpose, T18 and T25 two-hybrid libraries have already been constructed with genomic DNA from *E. coli* [20,21] but also from *Mycobacterium tuberculosis* [22]. The screen is based on the ability of the cells to grow on minimal medium containing 0.2% lactose or maltose as the sole carbon source. Indeed, a cell devoid of adenylate cyclase cannot activate the catabolic *lactose* and *maltose* operons and by consequence is not able to use them as carbon source. On the contrary, bacteria containing a positive interaction are able to grow on this medium. This gives a positive selection procedure for the isolation of clones containing interacting proteins. In the section below, we will call "bait" the protein of interest and "preys" the candidate proteins selected by screening the BACTH library.

3.5.2. Protocol

In the following, we will consider that the bait protein is fused with the T18 domain and, therefore, that the screen is performed against a genomic library prepared in a pT25 vector.

Transformation and selection. BTH101 strain is transformed using the $CaCl_2$/heat shock method (see Section 3.3) by the plasmid expressing the T18-bait hybrid protein. Then, cells are prepared for DNA transformation by electroporation. Briefly, a 100 ml culture is grown until an OD_{600nm} around one. Then, cells are centrifuged 10 min at 4000 rpm at 4 °C. The resulting pellet is suspended in 50 ml of cold water and cells are centrifuged again. This step is repeated 2 times. Finally cells are suspended in 0.3 ml of cold water + 10% glycerol and incubated on ice for 20 min. Cells can be stored at −80 °C or be used immediately. Sixty microliters of cells are electroporated with 50–100 ng of the T25 library, 1 ml of LB is added and cells are incubated 90 min at 30 °C with shaking for recovery. After incubation, cells are centrifuged 3 min at 8000 rpm and the pellet is washed with 1 ml M63 medium (see Section 3.2.3). This step is repeated 4 times. After the last wash, OD_{600nm} of the sample is measured and about 10^6 cells are spread on selection plates and incubated at 30 °C. The selective plates contain M63 medium, 0.2% maltose, kanamycin (30 µg/ml), ampicillin (50 µg/ml), IPTG (0.5 mM) and X-Gal (40 µg/ml). Thus, a positive interaction not only allows the growth of the colony but also the appearance of a blue color. It can take 5–10 days for colonies to appear on the plates (Fig. 4A).

Analysis of the selected clones. Colonies growing on the selection plates are re-streaked on the same medium or on MacConkey/Maltose or lactose plates (Fig. 4B). If the signal is still positive,

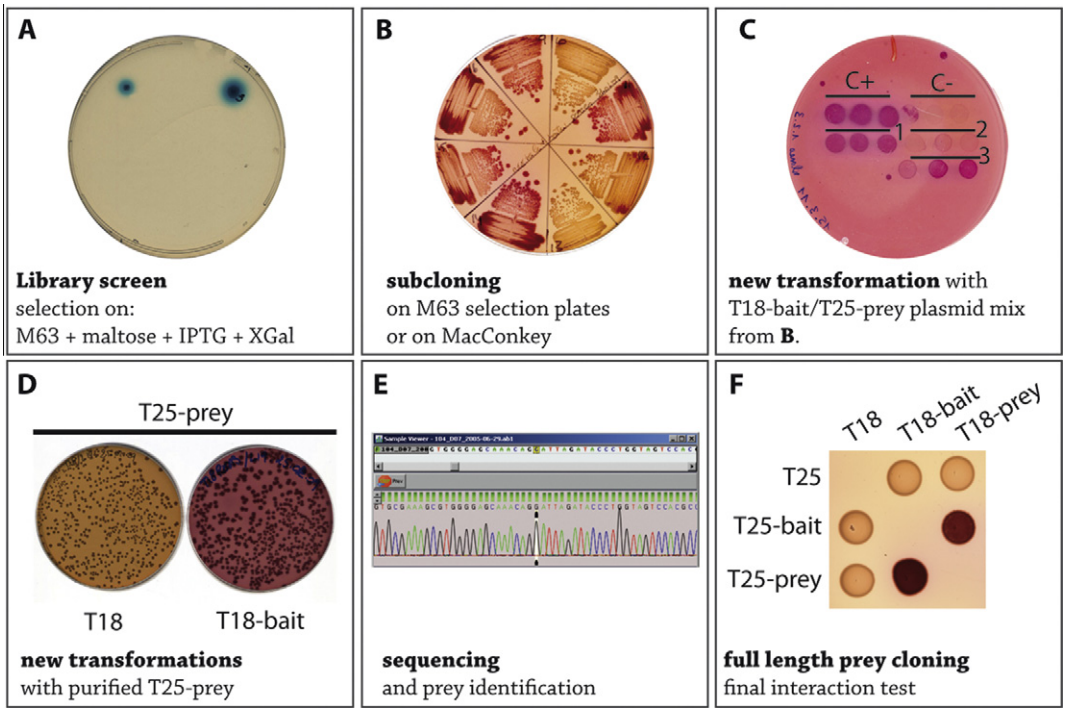

Fig. 4. Genomic library screening by BACTH. Each step of the procedure is illustrated. A: After 5 to 10 days at 30 °C, blue positive clones appear on the selective medium. B: Bacteria are re-isolated on selective medium. C: A classical interaction test is performed with the mix of plasmids obtained from bacteria at step B. D: After purification of the prey plasmids, interaction is tested against a control vector or against the bait. E: Positive clones obtained at step D are sequenced and the prey is identified. F: The full-length prey sequence is cloned in pT18 and pT25 and the interaction with the bait is again tested.

the DNA mix of the two T18 and T25 plasmids expressing the bait and the prey proteins is extracted, and the interaction is tested again in new BTH101 cells, following the standard protocol (see Section 3.3) (Fig. 4C). Then, to recover only the T25 plasmid coding for the selected prey protein, *E. coli* cells are transformed by the mix of two plasmids, and cells are incubated on plates containing only kanamycin. The T25-prey plasmid is then purified. To validate the prey, the T18-bait and the T25-prey plasmids are again used to transform BTH101 and tested for interaction (see Section 3.3) (Fig. 4D). BTH101 is also transformed by the T25-prey and the control empty T18 plasmid, to ensure that the prey is not a "sticky" or "autoactivating" protein by itself. If the interaction is positive and specific of the presence of the bait, the prey insert is sequenced and identified by BLAST search (Fig. 4E). The interaction must then be confirmed.

3.5.3. Good practice to validate an interaction

The identification of a partner by screening a library with BACTH, or by testing interactions between proteins of interest, is only the beginning of a series of experiments that need to be performed in order to validate the interaction and then establish its physiological relevance. In this section, we wish to describe the good practice approach that has been already extensively described in the case of the yeast two-hybrid technique, for which much more experience has been gathered [23]. The doctrine consists in confirming the interaction by another technique, accumulating evidence that the interaction is specific (no interaction with homologs for example), isolating mutations breaking and then restoring the interaction, and any other data showing its physiological relevance.

Confirmation of the interaction. Frequently, the plasmid isolated during the screening of the BACTH library does not contain the full coding sequence of the protein but just a fragment. The first step to validate the interaction is to clone the full-length prey protein in the BACTH vectors and check the interaction with the bait protein in the various available combinations of plasmids (Fig. 4F). If a positive signal is still detected, the interaction can then be demonstrated by another method as for example co-purification experiments [20,22]. For this purpose, we have designed a set of vectors for BACTH (Table 1) and for purification with 6His, CBP, or TAP N-terminal tags, which all have in frame restriction sites [14]. It is then easy to transfer the insert from the two hybrid vectors to the affinity purification vectors, in order to confirm the interaction by purification or pull-down experiments. The cloning sites of these modified BACTH vectors are also compatible with yeast two-hybrid vectors (see Section 3.2.1). Yeast two hybrid can also be a second technique to validate an interaction found with BACTH. The advantage to test an interaction in a heterologous organism is to determine if the interaction is direct between the two partners or involves other proteins. Detection of a positive interaction, in this case in yeast, would imply that the interaction between the two partners is direct.

Functional link. The establishment of a functional link is also necessary to prove the physiological significance of an interaction. If a homologous protein involved in another physiological pathway exists, it is a good control to test the specificity of the interaction [24]. Mutants affecting the interaction can also be studied: it could be mutants from both partners, known to be defective for their functions and tested for their ability to interact. The reverse is also true: mutants impaired in their interaction with the protein partner can be tested to check if they are affected in their functions.

In the same order of idea, mapping the protein domain involved in the interaction may also give useful information to understand the link between the two proteins. In this case, interactions are tested between one protein and several truncated forms of the protein partner [24].

Finally, a functional link can also be established by overproducing or deleting one of the protein and see if it has any effect on the function of the protein partner.

3.6. Technical recommendations

In this part of the review, we will go through the different steps of the BACTH technique to give some indications and tricks to improve the different assays.

Construction of the fusion plasmids. The two plasmids of the BACTH system express the fusion proteins from wild type *lactose* promoter. However, the LacI repressor is not encoded by the plasmids, which might lead to the accumulation of mutations during the cloning process if the fusions provoke growth disadvantage. Therefore, during the construction of the plasmids, it is better to use *E. coli* strains that have a high level expression of LacI (lacIq), such as XL1-Blue. Glucose may also be added to the growth medium to further repress expression. This might be very important when constructing genomic libraries.

Hybrid production. As for every technique based on the production of recombinant proteins, it is necessary to prove that the recombinant hybrid protein is correctly produced. Expression of the hybrid DNA is under the control of the *lac* promoter. During BACTH experiment, it is induced by IPTG addition. However, *lac* expression also requires the presence of the cAMP/CAP complex [25]. This implies that the cAMP produced when there is an interaction contributes to the promoter induction. On the contrary, when only one plasmid is present in the *cya*$^-$ strain, or when there is no interaction between the two proteins tested, the level of induction of the two-hybrid proteins is low because of the lack of cAMP. Thus, correct production of the hybrid protein should not be tested in the BTH101 or DHM1 strains but rather in a *cya*$^+$ strain. On the contrary, two interacting proteins can contribute to signal amplification through cAMP synthesis, and also to each other stabilization and, by consequence, to a better detection.

In most two-hybrid systems, recombinant protein production and stability are verified by Western blot using antibodies against the fused domains. However, such antibodies directed against T18 and T25 domains of adenylate cyclase were not clearly proposed in the first description of BACTH. To fill this gap, we first exploited the property of the T18 sub-unit of the *B. pertussis* adenylate cyclase to bind calmodulin [10]. The T18-fused recombinant proteins can therefore be detected by Western blot using biotinylated calmodulin, for example by using the CBP detection kit from Stratagene [14]. Additionally, calmodulin binding property allows the direct purification of T18-tagged proteins on calmodulin coated beads [26]. We have also developed two new vectors with a Flag epitope inserted at the N-terminus, between the T18 and T25 domains and the ORF of interest (Table 1). The addition of a Flag tag in the two-hybrid constructions does not affect the interaction between the two partner proteins [14]. Now, several antibodies have been developed against the adenylate cyclase of *B. pertussis*, and some of them can be used to detect hybrid proteins from the BACTH system. For example, the monoclonal 3D1 antibody from Santa Cruz recognizes the T18 sub-unit [19,26]. The use of b300 polyclonal antibody from Santa Cruz to detect the T25 domain has also been reported, however we do not have experience with it [27].

Hybrid functionality. Together with verifying correct production of the hybrid protein, proving that this hybrid protein is still functional can be a plus. However, the possibility to do so is specific of each research project. When mutants are available and a phenotype can be detected, one can test complementation by the hybrid vectors. For example T18- or T25-SpoT recombinant proteins were functional in *spoT* mutant strains [24] and T18-ACP was functional in an *acpP* thermosensitive mutant strain [28]. The correct localization of the hybrids can also be tested by fractionation experiments, especially for membrane proteins.

Tricks to improve the detection of an interaction. One critical step to detect an interaction can be the incubation time of the transformation plates. Although the required incubation time is certainly dependent of the strength of the interaction, we have noticed that incubation shorter than 48 h might not be sufficient to accumulate enough cAMP and detect a signal. Sometimes, cAMP accumulation in bacteria containing interacting hybrids can be directly inferred from the aspect of the colonies on the LB agar plates. Indeed, after prolonged growth, the colonies have irregular outlines and a rougher aspect than the parental *cya*$^-$ strain (A. Battesti, personal communication). Moreover, overnight cultures corresponding to a positive interaction usually reach a higher OD_{600nm} than the negative ones.

The different indicator plates used to perform qualitative assay have different sensitivities. We observed that the MacConkey/lactose plates give slightly higher signals than the MacConkey/maltose plates, and both MacConkey plates are much more sensitive than the LB-XGal plates because they can be incubated longer at 30 °C with the negative control remaining white. Besides, the MacConkey reporter plates will often allow the detection of a weak interaction that cannot be detected by β-galactosidase assay. Furthermore, even if we recommend our standard conditions (LB, IPTG, Ampi/Kana) for β-galactosidase assay, one has to keep in mind that an interaction can be detected or not depending on the medium used. In this regard, some authors reported the use of richer medium [29], and some have evidenced a clear effect of the medium on their specific interaction [30].

Due probably in part to the expression regulation by cAMP but also to the difference in copy number between the two plasmids, the BACTH system presents certain heterogeneity. Indeed, if cells are directly plated on indicator plates after recovery, a mix between white and red colonies can be observed. We have found that starting cultures with several colonies help to cope with this issue.

Finally, the detection of an interaction can sometimes be improved by the deletion of already known interactants (A. Battesti, personal communication). This can be tested for example if an interaction initially detected *via* a BACTH library screen cannot be reproduced. Indeed, as the medium used to do the screen is a harsh condition for the cell, some secondary mutations can appear in the genome allowing the interaction between two proteins. In some circumstances the expression of a third protein can inversely have a positive effect if this protein is needed to form a more stable complex or stabilize an interaction [17,31] (see Section 5.2).

4. Advantages/disadvantages

When considering the potential drawbacks of a technique, it often happens that its disadvantages might turn out to be advantages and vice versa. This is especially true for the BACTH, as shown by the following examples.

4.1. Expression system

In order to correctly analyze the results of a BACTH experiment, one has to take into account the principle guiding recombinant protein expression. The two hybrid proteins are expressed from two compatible plasmids that have distinct replication origins: the pT25 plasmids have a p15A origin (low copy number), while the pT18 plasmids have a ColE1 origin (high copy number). However, the same promoter in the two plasmids, i.e. the wild type promoter of the *lactose* operon, drives the expression of the hybrids. This results in different relative amounts of the two hybrid proteins that are produced. Because the stoichiometry between two

partner proteins can be critical for their interaction, the interaction has to be tested in the two possible combinations of T18 and T25 fusions (it is not rare to detect an interaction in only one case). In order to resolve this problem, authors have designed new plasmids with the same copy number [32]. The issue may also be resolved by putting the two hybrid genes on the same plasmid.

The use of the wild type P*lac* promoter itself may come as a surprise. Because of the positive feedback loop driven by the cAMP (see Section 3.6), it is only if an interaction occurs that the cAMP level will rise and allow full expression of the hybrid proteins. This results in a threshold effect, which may be the strength of the technique, by keeping the rate of false interactions due to sticky proteins or misfolding very low. Indeed, in order to avoid this auto-activation, BACTH plasmids containing a mutant P*lac* promoter not inducible by cAMP (*lacUV5*) have been constructed, but it increased the rate of detection of false positive interactions (D. Ladant, personal communication). On the other hand, this auto-amplification might be the reason for the heterogeneity in interaction that is observed for some interactions in the β-galactosidase assays (see Section 3.5). This is therefore a feature of the technique that is both advantageous and disadvantageous.

4.2. Recombinant protein production and false positive

As it is the case for any two-hybrid technique, independently of the principle of interaction detection, production of recombinant proteins may cause problems. The fusion to T18 or T25 domains may cause the protein to be badly folded, unstable, or simply prevent the interaction with its partners. Furthermore, proteins may have intrinsic tendency to interact with any protein, what is often called "sticky proteins".

Currently, no cases of auto-activators in the BACTH, meaning proteins that alone are able to bind to T18 or T25 domains have been reported in the literature. In our experience, we did not find such auto-activators either, but they might exist, as calmodulin-like proteins have been described in bacteria [33]. It would be interesting to list all the sticky proteins and also the potential auto-activators that will certainly appear from library screening studies. However, there have not been so many screens published till now, and unfortunately authors usually do not list the false positives.

4.3. BACTH can be used with membrane proteins

One clear advantage of the BACTH technique is the possibility to study membrane protein interactions. Indeed, because the output of the interaction is a diffusible cAMP signaling molecule, the interaction can take place anywhere in the cell, hence at the membrane. Providing that the hybrid proteins are correctly inserted in the membrane, with the T18 and T25 domains facing the cytoplasm, the interaction can be detected (Fig. 1D). This has been first described for multiple interactions between Fts proteins of *E. coli* [17], and then for several other membrane proteins [21,34–36]. The interaction can be reduced down to interaction between transmembrane segments alone fused to T18 or T25 domains. Consequently, BACTH has been the technique of choice to study small transmembrane peptides involved in the regulation of membrane proteins [37–39]. However, we observed that for membrane proteins, unspecific weak signal of interaction is more often observed than for soluble proteins, maybe due to overcrowding of the membrane. In this case, it is therefore very important to perform specificity controls with unrelated proteins. For example, MalF and MalG hybrids that interact strongly with each other can be good controls [17].

4.4. Reporter detection

The direct consequence of an interaction in the BACTH is reconstitution of adenylate cyclase activity, and ideally that is this activity that should be measured to quantify the level of interaction. Even if cAMP levels can be measured [11], usually the output of the BACTH assay is the level of expression of the *lactose* and *maltose* operons. Therefore, colorimetric assay on reporter plates or quantification of β-galactosidase activity is only an indirect measure of the interaction, involving a complex signaling cascade. Indeed, *lactose* and *maltose* operons are subjected to complex regulation and are affected by a variety of signals in addition to cAMP [25]. Because the assay depends on wild type promoters and reporter genes, there is the possibility of indirect effects on the expression level caused by the recombinant proteins. For example, we have been blocked in our studies on enzymes involved in stringent response, because (p)ppGpp affects *lactose* and *maltose* operon transcription (Battesti and Bouveret, unpublished).

One advantage of the use of wild type *lactose* and *maltose* operon expression as the output for BACTH is the possibility to perform positive selection of interacting proteins on minimal media containing only lactose or maltose. This is especially powerful for library screening (see Section 3.5). Inversely, this setup theoretically permits a reverse system, in which it is this time the absence of interaction that is positively selected, by incubating cells in presence of lambda phage. Indeed, the lambda phage receptor LamB is encoded within the *maltose* operon, and its absence would render the cells resistant to lambda phage. This may prove to be useful to identify residues involved in an interaction, however we are not aware of any studies that would have used this system.

4.5. The host is E. coli

The BACTH relies on the expression of recombinant proteins in *E. coli* cells. Therefore, for use with eukaryotic proteins, this might cause a problem if proteins have to be post-translationally modified. Yet, it can be viewed as an advantage to get rid of indirect interactions [40] and it might be the only solution to study membrane protein interactions. On the contrary, for studies of bacterial proteins, it is not possible to exclude that the detected interaction is indirect, especially for bacteria closely related to *E. coli*. Yet, for researchers working on *E. coli*, this technique is very powerful. Indeed, it permits the dissection of complexes, by playing with the genetic background of the strain used for the BACTH [41], or by modifying the growth conditions [30].

5. What can be done with BACTH?

In order for the reader to know if the technique will be helpful for her or his research, we will try here to summarize the types of proteins and interactions that have been studied successfully so far by BACTH. We will also give examples of the questions that can be asked by playing with the method.

5.1. Subjects covered (types of organisms and proteins)

Quite expectedly, most publications referring to BACTH deal with bacterial protein interactions. Indeed, studies have been performed not only in *E. coli*, but also in any bacterial phylum. However, as demonstrated in the founder paper [1], this technique can also be successfully applied to eukaryote protein interactions. For example, interactions between Laminin proteins of mouse [42] or between proteins of the yeast Set1 complex have been evi-

denced by BACTH [40]. Interaction between viral proteins has also been reported [43], and this suggests that the technique may be used for studying interactions between virus and host proteins. Even if the examples are scarce (yet it is not certain that we are aware of all of them), they indicate that the technique can be used for eukaryote and viral protein studies.

In the last ten years, about hundred papers have been published making use of the BACTH. Interestingly, there are clearly recurrent processes that are studied in bacteria using this technique. It concerns proteins involved in cell division in *E. coli*, *Synechocystis*, *Bacillus subtilis* or other bacteria [17,21,44,45]. Two-component system proteins have also been repeatedly studied by this technique [46,47]. It has also been the technique of choice to study newly identified small proteins that form transmembrane segment [37–39]. Two explanations can be given for these preferred subjects: first, the BACTH appears as the perfect approach for rapid screening of membrane protein interactions (see Section 4.3). Second, when protein interactions have been reported once, they are often studied by the same approach by the other scientists in the same field of research. Because there are still not so many interaction studies with BACTH that are published, both explanations certainly apply here.

BACTH has been used successfully for screening genomic libraries of *E. coli* and *M. tuberculosis* [20–22]. Other studies should be published soon of screens performed in *E. coli* or in *P. aeruginosa* (A. Battesti, unpublished; L. Houot, 2012, in press). Some studies also systematically checked the protein–protein interactions between an important set of proteins of interest in a systematic way [17,36,44]. In this kind of approach, β-galactosidase assay in 96-well plate might prove to be very useful.

5.2. Technical developments and applications

When browsing the literature, several original use of the BACTH, besides simply testing a pair of proteins or screening a library, have been reported. These approaches are a promise for powerful developments and applications.

- Chemical screening to identify inhibitors

Recently, Paschos et al. screened a compound collection in order to find molecules that inhibit the dimerization of VirB8, assayed by BACTH [29]. About 30,000 molecules were screened, demonstrating here again the suitability of the BACTH for high throughput studies.

- Influence of the genetic background

For researchers working in *E. coli*, it is possible to test the effect of gene deletions in the BTH101 strain on the interactions that are studied. It is for example possible to abolish transient interaction by removing an intermediate involved in the interaction. Indeed, interactions between some enzymes of molybdenum cofactor synthesis were abolished when the first step of synthesis was mutated [41]. Similarly, the interactions between nitrate reductase A and the enzymes involved in molybdenum cofactor synthesis strictly require the presence of mature Moco (abolished in *moa* or *mob* mutants) [31]. Because the technique is amenable to high throughput studies, screening mutant library obtained by transposon mutagenesis or on the reverse, screening an expression library, should be possible. Both positive and negative effects could then be screened. For example, the production of the two proteins NarH and NarJ restores the interaction between NarG and the Mo proteins [31]. This is a typical case of three-hybrid: on a single plasmid, a hybrid protein is expressed together with a non hybrid. This could lead either to competition or to enhancement of the interaction in the case of ternary complexes. Examples of the different possibilities are nicely shown in the case of Fts division proteins in *E. coli* [17].

- Characterization of an interaction

Because of the simplicity of the method and different possibilities of screening for gain or loss of interaction, the method can be used for dissecting a given interaction that has been detected. Moreover, the characterization of the residues or domain involved can be part of validating the interaction (see Section 3.5.3). Following studies are examples of such careful dissection experiments. The domains and residues involved in the interaction between Crl and SigmaS of *E. coli* were identified in both proteins by truncation and mutagenesis experiments [48,49]. The same was done for characterizing the interaction between SpoT and Acyl Carrier Protein [24,26]. The dimerization of CbpA protein was dissected by truncation and alanine scanning mutagenesis, permitting the identification of a hydrophobic surface involved in the dimerization [30]. A screen for a gain of interaction between membrane proteins TrwE and TrwB have permitted the isolation of point mutations indicating an interaction between the transmembrane regions of these proteins [50]. Finally, the association of cell division proteins FtsL and FtsB through leucine zipper motifs has been dissected [19].

- Other applications based on T18/T25 reconstitution

The ability to detect the reconstitution of adenylate cyclase from T18 and T25 domains association is amenable to other applications than protein–protein interaction studies. This system is indeed well adapted to screen for the synthesis of a full-length polypeptide consisting of the fused T18 and T25 domains. For example, by inserting linkers between the T18 and T25 sequences, it is possible to screen genetically for events such as premature codons [51] or presence of specific protease cleavage sites [52,53].

6. Other bacterial two-hybrid systems

This review is dedicated to the BACTH technique. However, it is important to mention that other bacterial two-hybrid systems are available. The major alternatives to the BACTH in *E. coli* are techniques that are based on the activation of transcription, similar to the classical yeast two-hybrid [54,55]. One complete system called BacterioMatch®II is distributed by Stratagene. In this system, one of the proteins is fused to a component of the RNAP (either α or ω subunit), while the other is fused to specific DNA binding domain (Zif or λcI). In this case, engineered test reporters (genes coding for resistance to antibiotics or for β-galactosidase) contain the Zif or λcI binding box in their promoter sequence. These techniques conserve most of the advantages of working in *E. coli*, yet they do not allow studying membrane protein interactions. However, they are powerful to study interactions involved in gene regulation [56]. Furthermore, we want to point out the existence of dedicated plasmids for Gateway cloning [57], which should unable high throughput screening of interactions, in order to describe interactomes such as what is done with yeast two-hybrid. Even without using these Gateway plasmids, a high throughput study of *Mycobacterium tuberculosis* interactome has been performed using this technique [58].

7. Concluding remarks

We have described the current applications of the BACTH system. Its use has increased in the recent years in the community

of microbiologists, and we hope this review will be helpful for newcomers to the technique. It is rapid, easy, and very reliable, with few reported case of false positives or false negatives. However, due to some drawbacks that have been exposed here, there is still space for improvement, such as designing other reporters than β-galactosidase, like GFP or antibiotic resistance, and the construction of other plasmids for Gateway cloning for example.

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