Protein- protein interaction mutants to study structure and assembly of cellular machineries: its application to the prokaryotic divisome

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1. Introduction

Together with functional RNA, proteins are primarily responsible for manifold biological activities of the cell. Proteins define the enzymatic chemistries and transport processes characteristic of metabolic pathways, regulate gene expression and many other molecular functions, are involved in signal transduction, and make up the actual molecular and cellular machinery that fuels life. They are highly diverse and embed hierarchically many layers of molecular organization [1]. To fulfill their biological activity in the cell, most proteins function in association with protein partners, or as a part of large molecular assemblies. An exhaustive knowledge of the full complement of protein interactions in a cell, therefore, provides insight into the structure, properties and functions of the cell and its components. Therefore, analysis on the interactome is expected to produce several types of useful informations.

Bacteria, once seen as an amorphous vessel harboring a homogeneous solution of proteins, are now know to have an intricate and dynamic subcellular protein architecture that facilitates DNA replication, chromosome segregation, cell growth and behaviors as diverse as symmetric and asymmetric division, motility, chemotaxis, morphological differentiation, assembly into multicellular communities.

One of the most fascinating, best studied and conspicuous example of interactome, in the prokaryotic world, is the cytokinetic machinery, called also divisome or septosome. This is a multiprotein structure that mediates cell division and dictates the site of its formation. Although it seems so simple, the bacterial divisome construction poses some of the most fundamental and fascinating questions in cell biology, as how it could orchestrate the formation of a septum at a specific location and time within the cell cycle.

In the recent decade several studies have been performed to indentify the involved elements and to understand some fundamental aspects of this process. However the dynamic nature of the divisome, the low abundance of its constituents in combination with its embedding in the cytoplasmic membrane, has prevented thus far the successful isolation or reconstitution of the divisome [2]. In *Escherichia coli*, and in other rod-shaped bacteria at least 15 proteins play an essential role in this process. These proteins, 10 of which are essential under standard laboratory conditions, localize at the septum level according to a largely linear hierarchy:

FtsZ>(FtsA,ZipA,ZapA)>FtsK>FtsQ>(FtsL,FtsB)>FtsW>FtsI>FtsN>AmiC>EnvC

These proteins belong to various functional groups that participate in invagination, constriction of the three envelope layers and separation of the two daughter cells. To initiate cell division, FtsZ, a GTP-binding tubulin-like protein, forms an intracellular ring (Z-ring) at the division site, equidistant between the two cell poles, by self-assembly of FtsZ monomers. The Z-ring, beside its dynamic role in cytokinesis (i.e., assembly at the beginning and disassembly at the end of the division process) serves as a cytoskeletal scaffold for the recruitment of proteins of the cell division machinery [3, 4]. When both FtsA and ZipA are in place, the remaining proteins are recruited. These include bitopic, FtsQ, FtsL, FtsB, FtsI, FtsN and polytopic, FtsK, FtsW, membrane proteins. The first group is characterized by a short cytoplasmic N-terminus, a single transmembrane segment and a large periplasmic domain [5, 6, 7]. In the second group, FtsK is a large protein with distinct roles in cell division and in chromosome segregation [8, 9] and FtsW is a member of the SEDS (shape, elongation, division and sporulation) family of proteins [10]. Lastly, AmiC and EnvC, two peptidoglycan hydrolases that localize to the septal ring and play an important role in the separation of daughter cells, have been added to the list [11].

Despite all these knowledge, the exact molecular mechanisms underlying the process of the divisome assembly and septum formation are not yet known and several findings indicate that the pathway may not be as linear as first anticipated. Genetic data [12, 7, 13] as well as two-hybrid screens [14, 15] and biochemical techniques [16] indicate that these proteins have a complex web of interaction [14, 17, 15, 18, 19, 20, 21, 22] and that at least some of them assemble independently of their normal association with the divisome [16, 23]. Moreover, several of the division components can be completely bypassed via suppressor mutations or over-expression of other divisome components [24, 25]. In *B. subtilis*, another well studied model organism, beside some minor differences in the set of proteins that participate to the division process, the assembly of the divisome seems to occur in two steps. First, FtsA, ZapA, SepF (and probably EzrA) localize to the Z-ring and are dependent on FtsZ for their recruitment to the division site. Second, the membrane bound division proteins FtsQ/DiviB, FtsB/DivIC, FtsI/PBP2B, and probably FtsW, appear to be recruited

in a concerted or cooperative mode, rather than in a linear sequence, as these division proteins are all interdependent for septal localization [26]. However, the above mentioned studies in *E. coli*, indicating that the assembly of the divisome occurs as a complex web of protein-protein interactions rather than a series of binary interactions, suggests that perhaps the assembly of the division complex in these two organisms is more similar than what previously thought [27].

How these proteins find their place into the divisome has been investigated by making extensive use of mutants, fusions to fluorescent proteins and through the application of a topological recruitment assay [28]. Within the hierarchy, described above, a given protein requires all upstream proteins (to the left) to localize and is, in turn, required for the localization of proteins further downstream (to the right). One of the main problems is to understand the biological role of the involved players. In fact it is possible that, when many proteins form a complex, only few interactions are essential.

2. The two-hybrid methodologies for protein interactions studies

The identification and characterization of the interactions among the proteins that constitute the septal ring is important, as this information should help the comprehension of how these proteins work together during cytokinesis [29]. The first step is the identification of protein-protein interactions (PPI) among the division proteins. Many techniques, both *in vivo*, *in vitro* and *in silico*, have been developed to enable PPI studies. The two-hybrid assay (THA) is an *in vivo* methodology used in all biology sectors [30]. Among the many available THAs, the most widely used up until now remains the two-hybrid assay developed in *Saccharomyces cerevisiae* [31]. Since its development, the technique itself has been extensively improved and diversified [32, 33]. From the publication of two yeast interactome maps based on the yeast two-hybrid technology ten years ago, the quantity and variety of protein interaction data have increased rapidly. In particular, two-hybrid based interactome maps have been generated for model organisms such as *C. elegans*, Drosophila, bacteria and humans. Although data on the protein interactome are being accumulated rapidly, several surveys and analyses revealed that a significant proportion of the interaction data obtained by this high-throughput protein interaction assay is constituted by false positives and false negatives [34]. The reasons for these artifacts have in some cases been elucidated. False negatives may arise because the two partner proteins are not efficiently imported into the nucleus and/or are inefficiently folded. Alternatively, either partner might be titrated away by endogenous proteins. This problem is expected to be particularly severe when the proteins under study are yeast proteins. Similarly, yeast proteins might act as a bridge between the two proteins under study, thereby causing activation of the reporter gene (even if the two queried proteins do not interact directly). Furthermore, some proteins very often score as positive with several different baits, and the reason for this result is unclear.

These considerations prompted the development of a number of alternative approaches that could allow these limitations to be overcome, or at least complement the yeast two-hybrid method. Most of these are carried out in *E. coli* and are based on a variation of the two-hybrid approach [30, 35, 36, 37, 38, 39]. One of these, developed by Hu and colleagues in 1990 [30], exploits the characteristics of the λ repressor. This approach was used with several proteins, i.e. to the study of protein homodimerization in *Escherichia coli*, and it is therefore likely that (as the yeast two-hybrid method) it is relatively topology-insensitive. To extend this method to the study of protein heterodimerization, Di Lallo et al. (2003) [14] reported a method based on the construction of a chimeric operator formed by the two hemi-sites of the phage P22 and 434 operators. This chimeric operator can be recognized and bound only by a hybrid repressor formed by two chimeric monomers; one with the N-terminal portion of phage 434 and the other with that of phage P22. The C-terminal domains of both of them are composed of heterologous proteins (or protein domains) whose interaction ability is under investigation. Only those proteins that mediate efficient dimerization of the two chimeric repressor monomers *in vivo*, permit the formation of a functional repressor able to bind the P22-434 hybrid operator and shut off the synthesis of a downstream reporter gene [40, 14] (Figure 1). This method has proved to be a powerful instrument for an *in vivo* study of the interaction and assembly of proteins, as the case of septum division formation [14, 21, 20, 22]. Reconstitution of phage repressor function could reflect direct interactions and implies at the very least that the respective division proteins are in very close proximity, which is more than could be inferred from observations of colocalization by fluorescence microscopy [29].

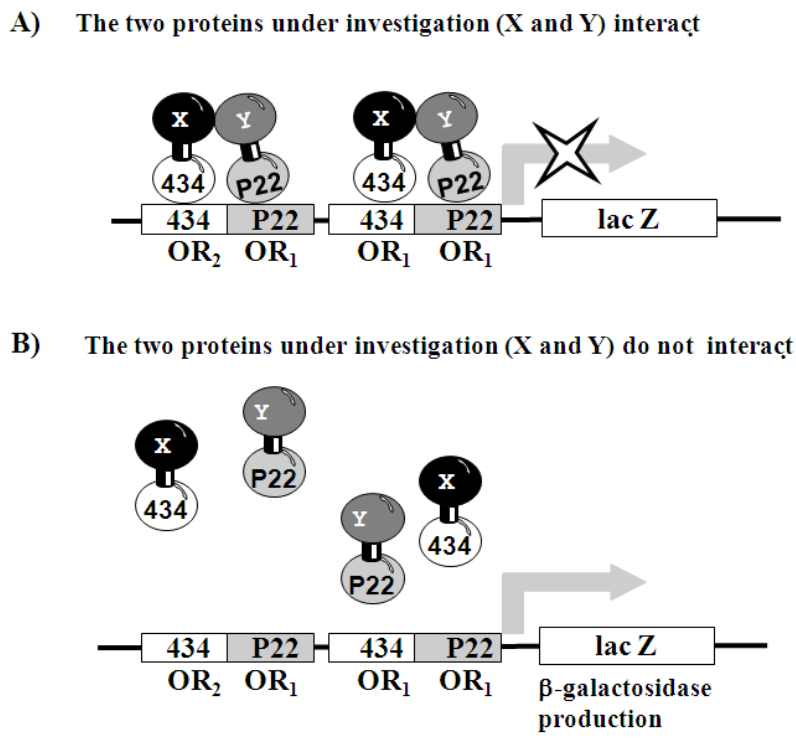

Fig. 1 The two phages two hybrid assay

Another bacterial two-hybrid system, used also to characterize the interactions between the *E. coli* proteins involved in the cell division machinery [15], was the bacterial adenylate cyclase two-hybrid system (BACTH) described in 1998 by Karimova and colleagues [36] (Figure 2). In this genetic test the proteins of interest are genetically fused to two complementary fragments of the catalytic domain of *Bordetella pertussis* adenylate cyclase. Interaction between the two proteins results in functional complementation between the two adenylate cyclase fragments leading to cAMP synthesis, which, in turn, can trigger the expression of several resident genes. The involvement of a signaling cascade offers the unique property that association between the hybrid proteins can be spatially separated from the transcriptional activation readout. This permits a versatile design of screening procedures either for ligands that bind to a given "bait," as in the classical yeast two-hybrid system, or for molecules or mutations that block a given interaction between two proteins of interest [36].

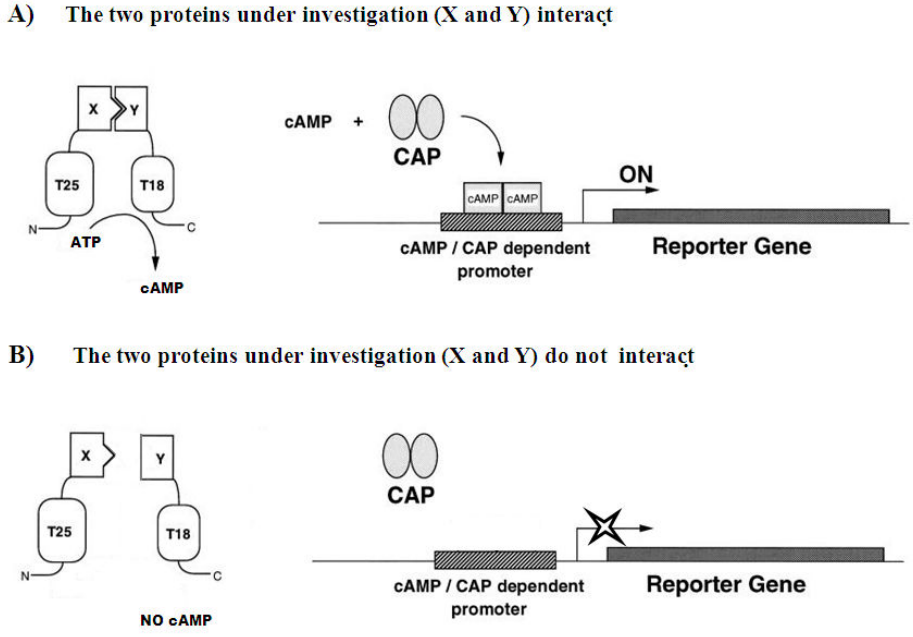

Fig. 2 The adenylate cyclase two-hybrid system

3. The interactome conservation

The function of most of the division proteins and the mechanisms by which cytoskeletal proteins participate in cell division, cell wall enlargement, DNA movement, and targeting of proteins to the cell poles are still to be discovered. Presently the only known property common of all the division proteins is their co-localization at the division site.

Modern cell biology methods for protein localization, in combination with DNA sequence comparisons and structural biology, allowed the identification of ubiquitous bacterial cytoskeletal elements present, for example, in model bacteria such as *Escherichia coli* or *Bacillus subtilis*. Although the amount of protein-protein interaction data has grown significantly, the only whole bacterial division protein interaction webs currently available are that of *E. coli* [14, 15, 21] and *S. pneumoniae* [20]. In this last organism, at least 12 proteins are known, or believed, to be involved in the process and, among them, nine, FtsZ, FtsA, ZapA, FtsK, DivIB/FtsQ, DivIC/FtsB, FtsL, FtsW e FtsI/ PBP2X, are in common with both other Gram-positive and Gram-negative bacteria. Using the bacterial two-phages two-hybrid assay (TP-THA) [40, 14] Maggi et al. (2008) [20] described a close net constituted by at least 37 homo and/or heterodimeric protein associations in which each protein generally interacts with more partners, up to 10 for FtsL and FtsZ, whereas the mean number of partners is two or three. PBP1A of *S. pneumoniae* is the only case described in which the protein interacts with a single partner, i.e. FtsL [20].

The comparison between the streptococcal division interaction web with that of *E. coli* showed that a significant number of the interaction were conserved [20] and allows the identification of protein-protein interaction representatives of a minimal common divisome in Bacteria. Peculiarities of each system could, therefore, be ascribed to additional division proteins typical of the single species.

The study of several different types of bacteria, including these having different shapes and growth patterns and those that growth under different conditions and pathogenic organisms will certainly broaden our the comprehension of structure and function of Eubacterial cytokinetic apparatus that could represent a starting point for the developing of an *in vitro* system for division protein assembly.

4. The biological role of the observed interactions

To clarify the mechanisms that regulate the assembly of the divisome's proteins in different bacteria is an obligatory step toward understanding the cytokinesis process, and also one of the most intricate due to the different factors involved. This elucidation requires not only the identification of all the proteins participating to the process, but also knowledge of their biological activity, the way by which they interact to form the sophisticated divisome machinery and their structure. The role of some division proteins, as a matter of fact may be to stabilize the structure in which they take part. In addition, it is possible that some interactions are only due to the protein's proximity to the complex without either determining the divisome formation or having a role in it.

The phylogenetic PPI conservation suggests their relevance for the bacterial vitality. To support this hypothesis, recently Barbati el al. (2010) [41] proposed to characterize the essential interactions necessary for the septum formation by isolating division protein interaction mutants and analyzing their biological relevance, searching for sequential and structural patterns that could be at the basis of the phylogenetic conservation of the divisome interactomes.

The behavior of interaction-defective mutants can provide an answer to this question, but the difficulty in predicting the phenotype of these mutants makes this search very arduous. For this reason Barbati et al. (2010) [41] developed an assay that enables PPI mutant selection regardless of their phenotype. The strategy for interaction mutant selection is based on the two phages two-hybrid assay adapted to interaction mutant selection by the use of an antibiotic resistance as a reporter gene. In this case, the interaction between the two proteins resulted in antibiotic sensitivity, whereas the loss of interaction conferred resistance to the bacterial strain. Therefore, turning on reporter gene expression highlights the loss of interaction due to a mutation in one of the genes for the two protein partners, and leads to direct selection of the mutants regardless of the mutant phenotype.

This system was applied to the search of two sets of interaction mutants in *E. coli* division proteins FtsI and FtsQ [41, 42]. The obtained results shown that this method for protein-protein interaction mutant selection is hard-hitting, since more than 90% of the clones selected for antibiotic resistance, carrying the mutagenized gene, showed loss of the particular protein-protein interaction due, in most cases, to a single mutation. Moreover, the presence of a single mutation in each mutant is important for performing fine dissection of the interaction domain(s) of a particular protein. Therefore, the selection of FtsI and FtsQ interaction mutants could constitute a substantial contribution to structural studies of these proteins, enabling genetic map construction of the interaction sites.

At the beginning, the behavior of *ftsQ* point mutants, where the respective protein is impaired in its ability to interact with its *E. coli* division partners, was analyzed [42]. FtsQ is a highly conserved component of the divisome that plays a central role in the assembly of early and late cell division proteins. The biological activity of this protein is still largely unknown, but its ability to interact with many components of the divisome was described by both two hybrid assays and co-immunoprecipitation experiments. The study of *ftsQ* point mutants yielded two important results. *i*) The identity of the FtsQ residues involved in interactions with the other divisome proteins. These results strengthen the previous genetic data describing the FtsQ functional domains [21] and identify, in agreement with structural data [43], two

domains involved in FtsI, FtsN and FtsQ interactions. *ii*) The biological relevance of the mutated FtsQ residues in protein-protein interactions, as deduced by the effects of aminoacylic substitutions evaluated through the study of the ability to complement bacterial strains depleted for FtsQ, to localize at the division site and to recruit the downstream division proteins.

It is important to note that this strategy is not restricted to the cell division proteins, but its application could be generalized to the study of other complex systems where numerous proteins interact.

5. The division proteins interactions as potential targets for new antibacterial drugs

The paucity of effective drugs for the treatment of bacterial infections prompted the scientific community to think about novel strategies for discovering new classes of antibacterial agents, since, actually, most of the new drugs are merely variant of older overused antibiotics. Among the new putative targets, bacterial cell division is one essential process that is not yet targeted by clinically approved antibacterials and, moreover, most of the divisome components are characterized by essentiality and prokaryotic specificity. Different high-throughput assays have been developed to search for inhibitors of components of the bacterial cytoskeleton. In these last 10 years, various natural or synthetic inhibitors of the FtsZ protein or the FtsZ-ZipA complex or FtsA were identified. Although it is not known whether or not these compounds could have a medical application, these data confirm the hypothesis that these proteins represent an excellent antibacterial target [44].

Cell-based assays for the detection of cell division inhibitors as well as FtsZ GTPase assays led to the identification of several compounds that inhibit the polymerization of FtsZ, blocking bacterial cell division. Such inhibitors might not only be valuable tools for basic research, but might also lead to novel therapeutic agents against pathogenic bacteria. For example, the polyphenol dichamanetin, the 2–alkoxycarbonylaminopyridine SRI-3072, and the benzophenanthridine alkaloid sanguinarine inhibit the GTPase activity of FtsZ and exhibit antimicrobial activity [44]. Other divisome components, and, amongst them, FtsQ, would be taken into account as potential targets for new antibiotics. As far as FtsQ is concerned, some considerations are in favor of its potential application as antibiotic target: (*i*) as the other simple or multi-spanning membrane proteins, FtsQ is located external to the cytoplasm. This location allows, to the inhibitors, a more easily accession to this protein than intracellular proteins and removes the problematic issue of resistance development due to drug-efflux pumps *(ii)* FtsQ as well as its partners proteins FtsL and FtsB with which form a sub-complex, is phylogenetically conserved; *(iii)* FtsQ interacts with many other molecular partners and some residues involved in these interactions could be therefore account as targets for synthetic oligopeptides to be used as competitors for these interactions. Furthermore, unlike FtsZ and FtsA, there are no identified human homologues to this protein [44], thereby increasing its potential as antibacterial target drugs.

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