Use of a two-hybrid assay to study the assembly of a complex multicomponent protein machinery: bacterial septosome differentiation

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The ability of each of the nine Escherichia coli division proteins (FtsZ, FtsA, ZipA, FtsK, FtsQ, FtsL, FtsW, FtsI, FtsN) to interact with itself and with each of the remaining eight proteins was studied in 43 possible combinations of protein pairs by the two-hybrid system previously developed by the authors’ group. Once the presumed interactions between the division proteins were determined, a model showing their temporal sequence of assembly was developed. This model agrees with that developed by other authors, based on the co-localization sequence in the septum of the division proteins fused with GFP. In addition, this paper shows that the authors’ assay, which has already proved to be very versatile in the study of prokaryotic and eukaryotic protein interaction, is also a powerful instrument for an in vivo study of the interaction and assembly of proteins, as in the case of septum division formation.

INTRODUCTION

In living organisms most of the cell functions are the result of complex multicomponent protein machineries and network activities (Alberts, 1998). A molecular study of these complexes needs to identify the constituent proteins and to establish, for each of them, their respective ability to form dimers, multimers or heterocomplexes with the other components; the identification of the domains that interact, both with themselves and/or with the other domains, is also necessary. A further level of knowledge concerns the integration of all of these data to determine the way in which the various components assemble in a given machinery.

An example of complex machinery in prokaryotes is the septosome, a complex structure formed by many different proteins responsible for cytokinesis. In Escherichia coli, and in other rod-shaped bacteria, the septosome differentiates in the cell centre at a particular time of the bacterial cycle, resulting in the formation of two daughter cells of the same size.

FtsZ is a key protein in the targeting and initiation of cytokinesis. Conditional mutants of ftsZ in E. coli fail to divide, yielding long filamentous cells that replicate and segregate their chromosomes but show no sign of division septa or cellular constrictions (hence the term fts, standing for ‘filamentation temperature sensitive’). Formation of long FtsZ polymers, starting from single subunits extending into the cytoplasm, is the first step towards septosome differentiation. This polymer forms a ring bound to the inner membrane (Bi & Lutkenhaus, 1991) and represents a sort of scaffold on which the other components of the division machinery are assembled. At least nine more proteins are currently known to participate in septum formation: FtsA, FtsK, FtsW, FtsQ, FtsI, FtsL, FtsN and ZipA (for reviews, see Rothfield et al., 1999; Margolin, 2000; Donachie, 2001) and YgbQ (or FtsB) (Buddelmeijer, 2002).

Cytological experiments have established that all these proteins co-localize at the septum level. In addition, it has been shown that ts mutations in the ftsZ gene result in the loss of co-localization of the eight remaining proteins, indicating that FtsZ is the first protein involved in the cell division process. These experiments demonstrated that FtsA localization to the Z-ring depends on FtsZ, whereas Z-ring localization does not depend on FtsA (Ma et al., 1996, 1997; Addinal et al., 1996; Addinal & Lutkenhaus, 1996). Evidence of a direct interaction between FtsZ and FtsA comes from yeast two-hybrid experiments (Wang et al., 1997) and from co-localization experiments with GFP-tagged FtsA (Ma et al., 1996, 1997; Addinal & Lutkenhaus, 1996).

Various authors have proposed models for the assembly of the septosome, based on their migration sequence by co-localization in the septum of the division proteins fused with GFP. The latter proteins are recruited in the septum soon after Z-ring formation in a linear sequence (for a review, see Rothfield et al., 1999) with two possible scenarios: (a) FtsA, ZipA, FtsW, FtsK, FtsQ, FtsL, FtsI, FtsN (Margolin, 2000); and (b) FtsA, ZipA, FtsK, FtsQ, FtsL,
FtsW, FtsI, FtsN (Chen & Beckwith, 2001). These scenarios were presented without taking into account the molecular interactions between the various components of the septosome. It is not yet known whether the sequence of protein recruitment in the septosome is also representative of the order in which they assemble with each other.

The two-hybrid system is an effective and quick tool for the in vivo study of protein–protein interaction which has been successfully employed in the study of many proteins both in prokaryotes and eukaryotes (for a review, see Hu et al., 2000). In this paper, we present an application of this method to the interaction between the nine division proteins and to 43 possible combinations of interaction between pairs of proteins. In addition, we have developed a model for their presumed sequence of interaction.

**METHODS**

**Media and chemicals.** LB broth for bacterial cultures and plating and SM for bacteria dilutions were as described by Miller (1972). The antibiotics used (Sigma) were ampicillin ($50 \mu\text{g mL}^{-1}$), chloramphenicol ($34 \mu\text{g mL}^{-1}$) and kanamycin ($30 \mu\text{g mL}^{-1}$). Synthetic oligonucleotides used in this work are listed in Table 1.

**Bacterial strains and plasmids.** Bacterial strains (all E. coli K-12 derivatives) and plasmids used in this work are listed in Table 2. Recombinant plasmids were constructed by cloning the genes for the division proteins in the SalI and BamHI restriction sites of pCP22 and pC134 vectors. The DNA of the gene of interest was obtained by PCR amplification using specific oligonucleotides carrying compatible restriction sites at their ends.

**General microbiological and recombinant DNA techniques.** Standard microbiological techniques were as described by Miller (1972). Standard procedures were used for small-scale plasmid preparations, endonuclease digestion, ligation, agarose gel electrophoresis, the elution of DNA fragments from agarose and bacterial transformation (Sambrook et al., 1989). PCR was carried out according to the recommendations of the manufacturer using the Taq DNA polymerase kit (Promega).

**β-Galactosidase assay and two-hybrid assay.** β-Galactosidase activity was assayed as described by Miller (1972). The two-hybrid assay was performed on bacterial cultures grown at 34 °C in B medium supplemented with $1 \times 10^{-4}$ M IPTG to OD$_{600}$ 0.5, as described previously (Di Lallo et al., 2001).

## RESULTS

The ability of each of the nine division proteins (FtsZ, FtsA, ZipA, FtsK, FtsQ, FtsL, FtsW, FtsI, FtsN) to interact with itself and with each of the other eight proteins was studied by the two-hybrid system previously developed by our group (Di Lallo et al., 2001). This system is constituted by a chimeric operator formed by the two hemi-sites of the P22 and 434 phage operators. These operators can be recognized and bound only by a hybrid repressor formed by two chimeric monomers: one can be bound by the N-terminal portion of phase 434 and the other by that of phase P22. The C-terminal domains of both are constituted by heterologous proteins (or protein domains) whose ability for interaction is under investigation. Only those proteins mediating an efficient dimerization of the two chimeric repressor monomers in vivo allow the formation of a functional repressor able to bind the P22–434 hybrid operator and shut off the transcription of the lacZ downstream reporter gene (Di Lallo et al., 2001); in this system the lacZ transcription is blocked by the heterodimeric repressor. This property

### Table 1. Oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5\’–3\’)</th>
<th>Gene</th>
<th>Position</th>
<th>Site</th>
</tr>
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<tbody>
<tr>
<td>ftsI –1F</td>
<td>GCGTCGACCATGAAAGAGCGCCGGAAGAC</td>
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<td>SalI</td>
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<td>ftsI 1767R</td>
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<td>ftsI</td>
<td>1767</td>
<td>BamHI</td>
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<tr>
<td>ftsK –1F</td>
<td>GCGTCGACCTTGAGCCAGGAATACATTG</td>
<td>ftsK (N-term)</td>
<td>−1</td>
<td>SalI</td>
</tr>
<tr>
<td>ftsK 2172R</td>
<td>CGGGATCCAGACAGCAACGCTATGGG</td>
<td>ftsK (N-term)</td>
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<td>BamHI</td>
</tr>
<tr>
<td>ftsK 1971F</td>
<td>GCGTCGACAGCAGCAACGCTATGGG</td>
<td>ftsK (C-term)</td>
<td>1971</td>
<td>SalI</td>
</tr>
<tr>
<td>ftsK 3990R</td>
<td>GAGATCCTTAGCAACGGCGGTTGGG</td>
<td>ftsK (C-term)</td>
<td>3990</td>
<td>BglII</td>
</tr>
<tr>
<td>ftsL –1F</td>
<td>GCGTCGACCATGATCGCAAG</td>
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<td>SalI</td>
</tr>
<tr>
<td>ftsL 365R</td>
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<td>365</td>
<td>BamHI</td>
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<tr>
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<td>−1</td>
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</tr>
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<td>1059</td>
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</tr>
<tr>
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<td>SalI</td>
</tr>
<tr>
<td>ftsQ 831R</td>
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<td>ftsQ</td>
<td>831</td>
<td>BglII</td>
</tr>
<tr>
<td>ftsW –1F</td>
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<td>ftsW</td>
<td>−1</td>
<td>XhoI</td>
</tr>
<tr>
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<td>1245</td>
<td>BamHI</td>
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<tr>
<td>zipA –1F</td>
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<td>−1</td>
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</tr>
<tr>
<td>zipA 85F</td>
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<tr>
<td>zipA 987R</td>
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<td>987</td>
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<tr>
<td>zapA –1F</td>
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<td>SalI</td>
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<tr>
<td>zapA 330R</td>
<td>CGGGATCTCTACATCAGATTTTGTGCTG</td>
<td>zapA</td>
<td>330</td>
<td>BamHI</td>
</tr>
</tbody>
</table>

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is exploited in the study of heterodimer formations in division proteins. We constructed two series of nine recombinant plasmids: the coding sequence of each of the nine division proteins was fused in-frame with the N-terminal domain of phage 434 in the first series, and with that of P22 in the second series. We therefore obtained plasmids coding for 18 chimeric repressors.

Pairs of these plasmids (one of which harboured the 434 hybrid repressor, and the other the P22 hybrid repressor) were transformed into the *E. coli* strain R721, carrying the 434–P22 chimeric operator. We obtained 45 bacterial strains, with which 43 possible combinations of division protein couples were studied.

### Interactions of 43 combination pairs among the nine division proteins

These combinations refer to only one of the two genetic configurations; i.e. where two different proteins (X and Y) were examined, the data reported in the table refer to cI<sub>434</sub>X–cP<sub>22</sub>Y only. However, we found that in most cases, the interactions were observed in both genetic configurations: the cI<sub>434</sub>X–cP<sub>22</sub>Y pair reduced β-galactose as well as the cI<sub>434</sub>Y–cP<sub>22</sub>X pair (data not shown).

Before analysing the results of this study, we need to evaluate the sensitivity of the assay. The assay sensitivity has already been analysed in previous work (Di Lallo et al., 2001). We have observed, for example, a residual β-galactosidase synthesis of 5% (standard deviation ±0.25) for the pairs of reconstructed repressors (434-negative and P22-negative). This value represents the lower limit of the assay. In addition, the study of various prokaryotic and eukaryotic proteins which are positive with the *Saccharomyces cerevisiae* two-hybrid assay, or have been shown to interact by other methods, showed that the residual β-galactosidase synthesis varies from 13% to 43%, depending on the pair of proteins analysed (data not shown). This range could be explained either by the interaction strength, which differs from one pair of proteins to another, or by the different experimental conditions necessary to optimize the interactions, e.g. the protein concentration may be different. Theoretically, in the case of non-interaction, the β-galactosidase synthesis should be similar to that obtained in the parental strain without plasmids. However, we cannot exclude that an
interaction, although transient, between the N-terminal domain of the fusion proteins and the operator site could interfere with the assay by slightly repressing the β-galactosidase synthesis.

What is the range of the residual β-galactosidase synthesis that determines whether or not two proteins interact with each other? On the basis of a series of results obtained with this assay, we will assume that the residual level of β-galactosidase synthesis between 13% and 50% represents positive interaction whereas values beyond this range are considered negative interactions. Although this is an empirical determination, we submitted the data reported in Table 3 to the statistical program SPSS for cluster analysis. The program showed two clearly distinct clusters: cluster I from 13–17% (FtsZ–FtsZ, FtsZ–FtsA, ZipA29–328–FtsZ, true positives in accordance with results of other authors), to 45% (ZipA–ZipA interaction); and cluster II, from 80% (ZipA–FtsA) to 90–100% (FtsK–FtsA, FtsK–FtsN, etc.). Cluster I can be subdivided into three groups, according to the interaction strength hypothesis, whereas cluster II is homogeneous.

On this basis, we shall thus assume that the interacting groups revealed by the assay (Table 3) are the following: FtsZ–(FtsA, ZipA, FtsK); FtsW–(FtsL, FtsN, FtsI); FtsL–(FtsK, FtsQ, FtsW); FtsI–(FtsA, FtsQ, FtsN, FtsI); FtsQ–(FtsK, FtsI, FtsN, FtsL). The results suggest a large number of interactions amongst these proteins. Many of the interactions described here have so far failed to reveal themselves in yeast-based assays, and many represent newly identified molecular interactions (see Discussion). These data, which

**Table 3. Homo- and heterodimerization of the nine division proteins with the 434/P22 two-hybrid assay**

<table>
<thead>
<tr>
<th></th>
<th>pC1434</th>
<th>pC1434</th>
</tr>
</thead>
<tbody>
<tr>
<td>FtsZ</td>
<td>416</td>
<td>17 %</td>
</tr>
<tr>
<td>FtsA</td>
<td>617</td>
<td>322</td>
</tr>
<tr>
<td>FtsQ</td>
<td>2056</td>
<td>84 %</td>
</tr>
<tr>
<td>FtsL</td>
<td>2150</td>
<td>88 %</td>
</tr>
<tr>
<td>FtsN</td>
<td>2515</td>
<td>100 %</td>
</tr>
<tr>
<td>FtsI</td>
<td>2547</td>
<td>100 %</td>
</tr>
<tr>
<td>FtsW</td>
<td>2250</td>
<td>100 %</td>
</tr>
<tr>
<td>FtsK1–724</td>
<td>833</td>
<td>34 %</td>
</tr>
<tr>
<td>ZipA</td>
<td>1162</td>
<td>46 %</td>
</tr>
<tr>
<td>ZipA29–328</td>
<td>373</td>
<td>15 %</td>
</tr>
</tbody>
</table>

FtsZ–FtsZ 17 %, FtsZ–FtsA 25 %, FtsQ–FtsL 84 %, FtsL–FtsN 100 %, FtsI–FtsW 100 %, FtsK–FtsQ 34 %, ZipA–FtsZ 46 %, ZipA29–328–FtsZ 15 %, true positives in accordance with results of other authors, to 45 % (ZipA–ZipA interaction); and cluster II, from 80 % (ZipA–FtsA) to 90–100 % (FtsK–FtsA, FtsK–FtsN, etc.). Cluster I can be subdivided into three groups, according to the interaction strength hypothesis, whereas cluster II is homogeneous.
still need to be confirmed by other methodologies, could be a starting point in understanding the protein–protein interactions at the septal ring.

**DISCUSSION**

The data reported in Table 3 suggest that there are three groups of interaction proteins. The first is represented by the cytoplasmic proteins FtsZ, FtsA, ZipA and FtsK. This group is characterized by the fact that FtsA, ZipA and FtsK interact with FtsZ but not each other, although two of these (FtsA and ZipA) are able to homodimerize.

FtsZ and FtsA are able to interact both with themselves and with each other, according to the observations reported in the literature on the *S. cerevisiae* two-hybrid system (Wang et al., 1997). Membrane protein ZipA also belongs to this group since it is able to form heterodimers with FtsZ (ZipA–FtsZ) as also reported by Haney et al. (2001). Furthermore, the two-hybrid assay reveals a ZipA homodimerization not described by other authors. Interestingly, these ZipA interactions are observed despite the fact that ZipA is a membrane-anchored protein. ZipA is, in fact, formed by five domains: amino acids (aa) 1–6 are a charged periplasmic domain; aa 7–28 are a transmembrane segment; aa 86–185 are called the P/Q domain; and aa 186–328 are a globular domain. The globular domain of ZipA has been shown to bind the conserved C-terminal peptide of FtsZ (Hale & de Boer, 1997; Hale et al., 2000; Haney et al., 2001; Liu et al., 1999; Moy et al., 2000; Ohashi et al., 2002). The interactions described in Table 3 can be explained by the hypothesis that a portion of the fusion may not be correctly inserted in the membrane as well as by the fact that the chimeric protein cI–ZipA could show affinity for both the cytoplasmic membrane and the operator site. The observed interactions could therefore be the resultant of these two effects. According to this interpretation, we observed that the residual β-galactosidase activity obtained with a pair of ZipA proteins lacking the periplasmic domain and transmembrane segment (aa 1–28) becomes 15% (±6.1%) instead of 46% (±5.4%) obtained with the whole protein.

In the second group we find three division proteins. FtsL, FtsN and the polytopic membrane protein FtsW are able to interact among themselves; FtsL and FtsN are also able to homodimerize; in addition, FtsW also interacts with FtsL. The third group is made up by FtsL and FtsQ proteins, which could have a binding function between the two previous groups. As a matter of fact, FtsL heterodimerizes with FtsK (belonging to the first group), FtsW (belonging to the second group) and FtsQ (the other ‘connection’ protein). The FtsQ–FtsN interaction is supported by genetic data consisting in the fact that the overexpression of FtsN partially suppresses FtsQ1(ts) (Dai et al., 1993). The interaction between FtsQ and FtsK, and between FtsL and FtsI, has not been so far described. The reason why these interactions have not been identified could be that the double hybrid *S. cerevisiae*, much exploited for these investigations, may not be an appropriate tool, as already hypothesized by Guzman et al. (1997). In fact, these authors suggest adopting a prokaryotic double hybrid in order to detect the interactions between division proteins. In any case, the present work aims at identifying the subdomains of the FtsQ protein specifically involved in the interaction with the various partners identified during this study.

As for the pair FtsA–FtsL, results were obtained only with the combination cI434FtsA–cIP22FtsL (i.e. with FtsA cloned in a low-copy-number plasmid) and with an IPTG induction of 90 min. The bacterial strain carrying the two plasmids does not grow under other experimental conditions, suggesting that a precise ratio of these proteins is necessary for cell physiology.

A new protein, ZapA, has recently been discovered in *Bacillus subtilis* which localizes to the Z-ring and stabilizes it, probably by promoting the bundling of FtsZ protofilaments (Gueiros-Filho & Losick, 2002; for a review, see Margolin, 2003). We studied the interaction between the orthologous *E. coli* ZapA protein, inferred from the *B. subtilis* zapA sequence, and the division gene products. We observed that ZapA strongly interacts with both FtsZ and FtsA (19% and 25% of residual β-galactosidase activity, respectively) whereas no interaction was detected with ZipA. These results suggest that this protein may act early in *E. coli*, perhaps stabilizing the newly formed Z-ring, as hypothesized by Gueiros-Filho & Losick (2002) in *B. subtilis*. Further investigations will be necessary to elucidate the possible role of this protein in *E. coli* cell division.

**A model for septosome assembly**

It was possible to develop a model of the temporal sequence of assembly according to the following premise. If protein A is the first to be recruited in the division site and interacts with protein B but not with C, while B and C interact with each other, we may conclude that the protein assembly sequence is A/B/C. Taking this observation into account, the interaction groups described in Table 3 can be subdivided into early interactions, in which proteins FtsZ, ZipA, FtsA, FtsK participate, and late interactions, in which FtsW, FtsN, FtsL participate. It seems that, out of these two groups, FtsL and FtsQ proteins could have a binding role.

These data, together with the data from the literature on Z-ring differentiation, suggest the following model of localization and assembly of division proteins. (a) FtsZ polymerization and Z-ring formation are the first steps towards septosome differentiation (Bi & Lutkenhaus, 1991); since both FtsA and ZipA interact with FtsZ but not with each other, they could interact with FtsZ early but independently from each other as described by Liu et al. (1999) and by Hale & de Boer (1999). This result agrees with previous data on FtsA and ZipA recruitment at the Z-ring level (Ma et al., 1996; Addinal et al., 1996; Addinal & Lutkenhaus, 1996; Ma et al., 1997). (b) Soon after, FtsK, the
membrane protein able to interact with the cytoplasmic FtsZ, is recruited since localization data show FtsK to be dependent upon FtsA and ZipA (Hale & de Boer, 2002). (c) At this point, two proteins follow: FtsL, by interacting with both FtsK and FtsW, and FtsQ, by interacting with FtsK and FtsL. (d) FtsW, in turn, interacts with FtsL and FtsN, which also interact with each other. In addition, FtsL interacts with FtsK but its localization to the septum depends on the presence of FtsW. FtsL also interacts with FtsQ and FtsA. Before interacting with the other proteins, FtsL and FtsN probably dimerize or polymerize, since the two-hybrid assay indicated their ability to homodimerize. Since FtsL, FtsW, FtsL and FtsN recruitment depends on FtsQ (Addinall et al., 1997; Ghigo et al., 1999; Weiss et al., 1999), it is plausible to believe that it localizes after FtsK and that, at a later stage, the other proteins could interact with FtsQ for septosome assembly.


**Potential importance and limitations of the findings**

It is often desirable to be able to assay the ability of a particular protein domain to associate with one or several alternative partners, or various proteins which, by interacting with each other, can assemble in a complex machinery. In this context, various two-hybrid assays have been developed, in both prokaryotes and eukaryotes, to study protein–protein interactions.

In this paper, we have shown that our assay, which has already proved to be very versatile in the study of prokaryotic and eukaryotic protein interactions, is also a powerful instrument for an in vivo study of the interaction and assembly of proteins, as in the case of septum division formation.

As far as the reliability of the assay is concerned, we addressed the problem regarding the ability to discriminate between direct interactions and false positives or negatives according to the reasoning here described. False positives could be due to bridging proteins. As septum proteins are likely to be present in a large complex, bridging proteins could contribute to at least some of the positive results of the assay. Although it would be rather difficult to rule out this hypothesis (which would require a comparison between these results and those obtained by other groups through other methodologies), as regards the present work, recent genetic data seem to minimize the relevance of false molecular interactions.

We selected FtsZ mutants impaired in homodimerization. One of these mutants, ZD4 (for which fusion with GFP shows that the protein is diffused in the cytoplasm and that it does not localize in the septum), is unable to homodimerize by our bacterial two-hybrid assay although it still interacts with FtsA (unpublished data). FtsA normally interacts with FtsZ and, if we follow the reasoning applied to false positives, then it should behave as a bridging protein; this possibility should have revealed itself through our assay as a false positive interaction for FtsZ. Instead, this was not observed even when the intracellular level of FtsA was increased. Formally, such an approach could potentially be extended in order to exclude possible false positives from the assay.

Negative results could be due to the fact that one or both the proteins to be tested are not present at all in the cell (due to a defect in expression or translation of the coding gene or to protein degradation) or are present in insufficient amount. However, if a protein pair does not show any interaction but each of the two proteins is able to interact with another protein, the negative result could be significant. Moreover, one cannot exclude that a particular fusion might fold so as to expose the surface for interaction with one partner but not another.

In conclusion, although the data here reported need confirmation through other methodologies, they represent an important step in obtaining an accurate dissection of E. coli septum differentiation.

**ACKNOWLEDGEMENTS**

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