Phage-Display and Correlated Mutations Identify an Essential Region of Subdomain 1C Involved in Homodimerization of Escherichia coli FtsA

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ABSTRACT FtsA plays an essential role in Escherichia coli cell division and is nearly ubiquitous in eubacteria. Several evidences postulated the ability of FtsA to interact with other septation proteins and with itself. To investigate these binding properties, we screened a phage-display library with FtsA. The isolated peptides defined a degenerate consensus sequence, which in turn displayed a striking similarity with residues 126–133 of FtsA itself. This result suggested that residues 126–133 were involved in homodimerization of FtsA. The hypothesis was supported by the analysis of correlated mutations, which identified a mutual relationship between a group of amino acids encompassing the ATP-binding site and a set of residues immediately downstream to amino acids 126–133. This information was used to assemble a model of a FtsA homodimer, whose accuracy was confirmed by probing multiple alternative docking solutions. Moreover, a prediction of residues responsible for protein-protein interaction validated the proposed model and confirmed once more the importance of residues 126–133 for homodimerization. To functionally characterize this region, we introduced a deletion in ftsA, where residues 126–133 were skipped. This mutant failed to complement conditional lethal alleles of ftsA, demonstrating that amino acids 126–133 play an essential role in E. coli. Proteins 2003;50:192–206. © 2002 Wiley-Liss, Inc.

Key words: bacterial cell division; FtsA; homodimerization; phage-display library; correlated mutation analysis, protein-protein interaction

INTRODUCTION

Bacterial cell division is a complex process, which requires the spatially and temporally coordinated recruitment of different proteins at the division site. A pioneering genetic approach discovered the key components of the septation machinery, leading to the identification of filamentous temperature-sensitive (fts) mutants.1 Many fts genes are clustered on the chromosome and the corresponding proteins have an essential role in initiating and progressing cell division.2 The first detectable event of cell division is the localization of FtsZ at mid-cell to create a ring-shaped structure, named Z-ring.3 Immediately after Z-ring assembly, FtsA is recruited at the division site.4 The assembly of subsequent cell division proteins is strictly dependent on the presence of both FtsA and FtsZ.3 FtsA is a 45-kDa protein highly conserved in eubacteria, which localizes at the division site in a FtsZ-dependent manner forming in its turn a ring structure at mid-cell.4 FtsA is expressed at a very low level and post-translational modifications seem to regulate its cytosolic or membrane localization, although its precise relationships with the other proteins of the septator are still uncertain.5,6 Many functional, genetic, and biochemical evidences indicate that FtsA directly binds FtsZ and phenotypic analysis of conditional-lethal mutants suggests that also FtsI, FtsL, FtsQ, FtsN, FtsK, and MinCD interact with FtsA, even though these latter studies did not clarify whether these contacts occur directly or indirectly.4,7–15 On the other hand, it has been postulated that FtsA can form homodimers and perhaps polymers. The assembly of a FtsA-ring is suggestive of the formation of a multiproteic higher-order structure.4 Furthermore, the conditionally functional allele ftsA104 can effectively complement the temperature-sensitive mutants ftsA2 and ftsA3, but inefficiently substitutes for the amber mutant ftsA16.9 Finally, the C-terminus of FtsA was found to be essential for self-

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Received 10 December 2001; Accepted 15 April 2002
interaction and recent studies on FtsA from *Bacillus subtilis* confirmed its ability to form homodimers. 16,17

Characterization of cell division proteins was recently boosted by structural studies, which demonstrated that FtsA and FtsZ share overall topological similarities with the eukaryotic cytoskeletal proteins actin and tubulin, respectively. 16,17 Whereas FtsZ is the structural and functional homologue of tubulin in bacteria, 16,17 FtsA was predicted to belong to the actin/hsc70/sugar kinase superfamily on the basis of the presence of the actin-fold, a conserved five-motif structure responsible for ATP-binding. 21 Biochemical and genetic studies demonstrated the ATP-binding activity of FtsA and the essentiality of those ligands, drug-delivery systems, and enzyme inhibitors and the surface of M13 bacteriophages. This powerful technique has been widely applied to develop receptor-specific techniques that converge to identify a self-interacting interface of FtsA on subdomain 1C. On the basis of these data, we propose a model for the homodimerization of FtsA on subdomain 1C. On the basis of these data, we propose a model for the homodimerization of FtsA on subdomain 1C. On the basis of these data, we propose a model for the homodimerization of FtsA on subdomain 1C. On the basis of these data, we propose a model for the homodimerization of FtsA on subdomain 1C.

We chose to investigate the binding features of FtsA using affinity screening of a peptide library displayed on the surface of M13 bacteriophages. This powerful technique has been widely applied to develop receptor-specific docking solutions, by previous genetic evidences, and by a striking similarity with contacts occurring in actin.
with his-FtsA to verify the enrichment for specific binders, while $2 \times 10^{11}$ pfu were used for the subsequent round. At the end of the panning procedures, phages were picked from nonconfluent plaques and individually amplified as described above. Single-strand DNA was extracted by resuspending the bacteriophage pellet in 10 mM Tris-HCl, 1 mM EDTA, 4 M NaCl, pH 8.0, followed by ethanol precipitation and sequenced with −96gIII primer (New England Biolabs) using an ABI Prism 377 DNA Sequencer and BigDye Terminators Cycle Sequencing Ready Reaction Kit with AmpliTaq FS DNA Polymerase (Perkin Elmer-Applied Biosystems).

ELISA

The experiment was carried out as described by Harlow and Lane. Briefly, 100 μg/mL of his-FtsA, his-MurC or 1% BSA were adsorbed o.n. at 4°C on Ni-NTA strips in TBS. After four washes with TBS, strips were saturated for 1 h with 1% BSA and, after a brief wash with TBS, $1 \times 10^9$ pfu/well of bacteriophages were incubated for 1 h at room temperature. Wells were washed six times quickly, one time for 10 min and quietly again six times with TBS, 0.5% Tween 20. Rabbit polyclonal antibodies anti-fd (1: 2500; Sigma) in TBS, 0.5% Tween 20 were incubated for 1 h and then washed three times with TBS, 0.5% Tween 20. HRP-labeled secondary antibodies (1:5000; Boehringer) in TBS, 0.5% Tween 20 were added for 30 min, and excess was removed with three washes in the same buffer conditions. Strips were developed by adding 100 μL of TMB (Biorad), following kinetically the reaction for 20 min and then washed three times in the same buffer. Wells were washed six times quickly, one time for 10 min and quietly again six times with TBS, 0.5% Tween 20. Rabbit polyclonal antibodies anti-fd (1: 2500; Sigma) in TBS, 0.5% Tween 20 were incubated for 1 h and then washed three times with TBS, 0.5% Tween 20. HRP-labeled secondary antibodies (1:5000; Boehringer) in TBS, 0.5% Tween 20 were added for 30 min, and excess was removed with three washes in the same buffer conditions. Strips were developed by adding 100 μL of TMB (Biorad), following kinetically the reaction for 20 min in a Spectra Max 250 spectrophotometer (Molecular Devices) at OD$_{450}$nm. Finally 50 μL of 1 M H$_2$SO$_4$ was added, and strips were read at OD$_{450}$nm.

Bioinformatic Analysis

Conversion of amino acids of the peptide sequence was performed with SIMPLIFY program (GGG) by changing the local data file with physico-chemical code proposed by Taylor. Multiple sequence alignment was performed with CLUSTALW. Similarity between T8A-11 and FtsA was established by using BLASTP program on E. coli genomic CDS translations.

Correlated mutation analysis was performed with program PLOTCORR. Generation of $5 \times 10^3$ different docking solutions for the homodimerization model of FtsA was achieved with program GRAMM.

Correlated mutation-corrected distance factor (cd) for each individual docking solution was calculated using the formula:

$$ cd = \frac{\sum_{i=1}^{n} d_i \cdot r_i}{n} $$

where $d_i$ is the distance between each pair of residues, $r_i$ is the correlation coefficient calculated by the PLOTCORR program to each pair of positions, and $n$ is the number of correlated pairs.

Functional Analysis of FtsA

ftsAΔ7 was cloned in pBAD24 by a three-point ligation with ftsA fragments 1–378 (EcoRI-XhoI), 399–1260 (XhoI-PstI), and pBAD24 (EcoRI-PstI). Fragments of ftsA were obtained by PCR amplification of the DH5α genomic DNA with the following primers. Fragment 1–378: 5’-CCGCAATCCACCATGATCAGGGCGAGGAGACAG-3’ and 5’-CCGCGCTCGGATGATCTCAATTCGCGACACGC-3’. Fragment 399–1260: 5’-CAGGAATTCGAGATTGGCATTATGACGGA-3’ and 5’-AAAAACTGAGTTAAAACCTTTTGCGCACCCA-3’. Wild-type ftsA was cloned using the same terminal oligonucleotides (first and fourth). Fidelity of the cloned inserts was controlled by sequencing both the DNA strands.

Anti-FtsA polyclonal antibodies α1,FtsA were raised in rabbit with purified E. coli his-FtsA essentially as described by Harlow and Lane. For western blotting approximately $5 \times 10^6$ cells, withdrawn at the indicated times, were loaded onto a 12% SDS-PAGE gel. After resolution, proteins were transferred onto Hybond-ECL filters (Amersham) and treated according to the standard procedure with anti-FtsA antibodies (1:30000). Secondary anti-rabbit HRP-labeled antibodies were added (1:8000), and filters were developed with Enhanced Chemiluminescence (ECL) substrates (Amersham).

Influence of FtsAΔ7 and of FtsA expression on E. coli growth rate was studied in DH5α and in XL10GoldKan (Stratagene), both in complete medium (LB: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0; Difco) and in M63 minimal medium (13.6 g/L KH$_2$PO$_4$, 2 g/L (NH$_4$)$_2$SO$_4$, 5 g/L glucose, 100 μg/mL ampicillin, 100 μg/mL kanamycin, pH 7.0).
0.5 mg/L FeSO₄ \times 7\text{H}_2\text{O}, 0.2 g/L MgSO₄ \times 7\text{H}_2\text{O}, 0.2\% glycerol, 0.5 μg/mL thiamine, 0.1% casaminoacids, pH 7.0, Difco) and almost superimposable results were obtained, thus excluding any possible interference from the genetic background and from the environmental conditions. Precultures were inoculated with cell banks stored at 80°C, grown o.n. at 37°C in the presence of 0.2% glucose, 100 μg/mL ampicillin and subsequently diluted to OD₆₀₀nm = 0.02 in prewarmed medium with 100 μg/mL ampicillin. Cultures were grown at 37°C until they reached OD₆₀₀nm = 0.2, then they were split and either induced with 0.2% arabinose or repressed with 0.2% glucose. At the indicated times samples were withdrawn and processed as specified. Microphotographs were taken with a CCD camera (Bio-rad) connected to an Axioskop HBO 50/AC microscope (Zeiss) at \times 1000 magnifications on samples fixed with 0.75% formaldehyde.

Complementation analysis was carried out by growing strains carrying \textit{ftsA(ts)} alleles in NB medium (Oxoid) supplemented with 50 μg/mL thymine (NBT), with 50 μg/mL ampicillin when required and incubated at permissive (30°C) or at restrictive temperature (42°C). Transforms with pBAD24 derivatives were obtained at 30°C, the isolated colonies were inoculated into NBT at 30°C and then plated both onto LB agar with ampicillin (incubated o.n. at 30°C) and on NBT agar (incubated o.n. either at 30°C or at 42°C). A positive complementation result was considered when growth was visible both at 30°C and at 42°C on NBT plates.

**RESULTS**

**Phage Panning of FtsA**

The Ph.D.-7 phage-display library, which exhibits random linear heptamers fused to pIII coat protein, was challenged with purified \textit{E. coli} polyhistidine-tagged FtsA under two experimental conditions. First, a gradual stepwise increase of nonionic detergent in a high ionic strength milieu led to the identification of T8A bacteriophages [Tween 20-8 rounds-FtsA; Fig. 1(A)]. Second, a one-step rise of a milder detergent in a lower ionic strength buffer selected the N4A phages [NP 40-4 rounds-FtsA; Fig. 1(B)]. In both cases, enrichment of specific FtsA binders was revealed by the steady increase of phages recovered after every round of panning. This feature was strictly dependent on the presence of the heptamers, since in a control experiment the bacteriophage VCSM13, which lacks any random sequence, was not positively selected [Fig. 1(A,B), black bars]. To unequivocally confirm a specific enrichment of anti-FtsA phages, each round of selection in PB was tested by ELISA with BSA (black bars) and with his-FtsA (grey bars) at the corresponding stringency conditions. Experiment was run in triplicate.
selected clones, 90 individual plaques were randomly picked and the amino acids of the variable heptapeptides were deduced from their corresponding nucleotide sequences (Table I). The analysis revealed that the selected phages represented 37 unique clones. To judge their sequences (Table I). The analysis revealed that the selected clones, 90 individual plaques were randomly selected and they were further characterized. Any possible recognition of the polyhistidine tail was ruled out, since they displayed a negligible affinity to histidine-tagged MurC, an unrelated protein harboring the same N-terminal tag (Fig. 2; white bars). It should be noted that only for few phages the relative affinity correlated with their frequency of recovery (e.g., T8A-01. See Fig. 2 and Table I). This was somehow expected: in fact, although ELISA results can provide an estimate of the inherent binding property of each phage, the frequency of recovery is affected by several additional factors, including replication advantage, translation and folding bias, pIII coat stability and retained infection efficiency.

### Table I. Sequence and Relative Affinity of FtsA-binding Peptides

<table>
<thead>
<tr>
<th>Φ</th>
<th>Sequence</th>
<th>Freq.</th>
<th>MurC</th>
<th>FtsA</th>
<th>FtsAMurC</th>
<th>Φ/VCSM13</th>
</tr>
</thead>
<tbody>
<tr>
<td>T8A-01</td>
<td>FEKYYWN</td>
<td>25</td>
<td>0.313 ± 0.032</td>
<td>3.762 ± 0.144</td>
<td>12.0</td>
<td>18.5</td>
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<tr>
<td>T8A-49</td>
<td>LQPMWFA</td>
<td>1</td>
<td>0.397 ± 0.034</td>
<td>3.491 ± 0.135</td>
<td>8.8</td>
<td>17.2</td>
</tr>
<tr>
<td>T8A-28</td>
<td>KVFNPW</td>
<td>3</td>
<td>0.249 ± 0.016</td>
<td>1.891 ± 0.047</td>
<td>7.6</td>
<td>9.6</td>
</tr>
<tr>
<td>T8A-11</td>
<td>KLVIPQ</td>
<td>2</td>
<td>0.315 ± 0.021</td>
<td>2.081 ± 0.027</td>
<td>6.6</td>
<td>10.5</td>
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<tr>
<td>N4A-07</td>
<td>LTYRISP</td>
<td>1</td>
<td>0.277 ± 0.027</td>
<td>1.828 ± 0.066</td>
<td>6.6</td>
<td>9.2</td>
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<tr>
<td>N4A-01</td>
<td>LSLPKL</td>
<td>2</td>
<td>0.345 ± 0.037</td>
<td>2.003 ± 0.047</td>
<td>5.8</td>
<td>10.1</td>
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<tr>
<td>T8A-19</td>
<td>QUNLQL</td>
<td>1</td>
<td>0.230 ± 0.009</td>
<td>1.335 ± 0.039</td>
<td>5.8</td>
<td>6.9</td>
</tr>
<tr>
<td>N4A-30</td>
<td>GSYFYFP</td>
<td>1</td>
<td>0.228 ± 0.009</td>
<td>1.185 ± 0.046</td>
<td>5.2</td>
<td>6.2</td>
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<tr>
<td>N4A-04</td>
<td>LVYFMV</td>
<td>1</td>
<td>0.290 ± 0.026</td>
<td>1.423 ± 0.025</td>
<td>4.9</td>
<td>7.4</td>
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<tr>
<td>N4A-21</td>
<td>TTPYTVL</td>
<td>1</td>
<td>0.342 ± 0.015</td>
<td>1.301 ± 0.049</td>
<td>3.8</td>
<td>6.8</td>
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<tr>
<td>N4A-10</td>
<td>LPLNYN</td>
<td>1</td>
<td>0.276 ± 0.009</td>
<td>0.801 ± 0.073</td>
<td>2.9</td>
<td>5.8</td>
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<tr>
<td>N4A-05</td>
<td>HYTSATL</td>
<td>1</td>
<td>0.212 ± 0.017</td>
<td>0.552 ± 0.022</td>
<td>2.6</td>
<td>5.7</td>
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<tr>
<td>N4A-24</td>
<td>WQPNTRP</td>
<td>1</td>
<td>0.280 ± 0.013</td>
<td>0.728 ± 0.031</td>
<td>2.6</td>
<td>5.2</td>
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<tr>
<td>N4A-26</td>
<td>YNPLPT</td>
<td>3</td>
<td>0.275 ± 0.013</td>
<td>0.716 ± 0.064</td>
<td>2.6</td>
<td>5.4</td>
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<tr>
<td>T8A-06</td>
<td>TDRPALS</td>
<td>8</td>
<td>0.301 ± 0.031</td>
<td>0.752 ± 0.054</td>
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<td>5.1</td>
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<tr>
<td>N4A-19</td>
<td>APLESNW</td>
<td>2</td>
<td>0.280 ± 0.022</td>
<td>0.672 ± 0.044</td>
<td>2.4</td>
<td>4.3</td>
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<tr>
<td>N4A-08</td>
<td>IPRTYPL</td>
<td>1</td>
<td>0.272 ± 0.008</td>
<td>0.625 ± 0.010</td>
<td>2.3</td>
<td>3.2</td>
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<tr>
<td>N4A-22</td>
<td>SAPGLH</td>
<td>1</td>
<td>0.267 ± 0.014</td>
<td>0.615 ± 0.038</td>
<td>2.3</td>
<td>4.4</td>
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<tr>
<td>N4A-12</td>
<td>DYHNHLT</td>
<td>1</td>
<td>0.279 ± 0.020</td>
<td>0.586 ± 0.051</td>
<td>2.1</td>
<td>3.8</td>
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<tr>
<td>N4A-16</td>
<td>SFTQSTL</td>
<td>1</td>
<td>0.276 ± 0.019</td>
<td>0.580 ± 0.041</td>
<td>2.1</td>
<td>4.1</td>
</tr>
<tr>
<td>N4A-29</td>
<td>AQAIVQY</td>
<td>1</td>
<td>0.246 ± 0.013</td>
<td>0.517 ± 0.051</td>
<td>2.1</td>
<td>4.5</td>
</tr>
<tr>
<td>T8A-30</td>
<td>HHHVQVN</td>
<td>1</td>
<td>0.297 ± 0.014</td>
<td>0.623 ± 0.064</td>
<td>2.1</td>
<td>4.1</td>
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<tr>
<td>N4A-14</td>
<td>SLCSVPLC</td>
<td>1</td>
<td>0.290 ± 0.030</td>
<td>0.580 ± 0.012</td>
<td>2.0</td>
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<tr>
<td>N4A-13</td>
<td>HTYFPFPM</td>
<td>1</td>
<td>0.190 ± 0.008</td>
<td>0.552 ± 0.024</td>
<td>2.9</td>
<td>5.6</td>
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<td>N4A-27</td>
<td>HVLPLPLH</td>
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<td>0.289 ± 0.009</td>
<td>0.810 ± 0.056</td>
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<td>5.6</td>
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<tr>
<td>N4A-11</td>
<td>LHAOWQDL</td>
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<td>0.286 ± 0.016</td>
<td>0.773 ± 0.045</td>
<td>2.7</td>
<td>5.2</td>
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<tr>
<td>N4A-02</td>
<td>TGNNPFPN</td>
<td>1</td>
<td>0.287 ± 0.026</td>
<td>0.745 ± 0.070</td>
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<td>5.5</td>
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<tr>
<td>N4A-17</td>
<td>LSNTYRP</td>
<td>1</td>
<td>0.274 ± 0.018</td>
<td>0.712 ± 0.038</td>
<td>2.6</td>
<td>5.0</td>
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<tr>
<td>N4A-09</td>
<td>HTPLATA</td>
<td>1</td>
<td>0.287 ± 0.011</td>
<td>0.718 ± 0.037</td>
<td>2.5</td>
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<tr>
<td>N4A-15</td>
<td>HMPNASF</td>
<td>1</td>
<td>0.279 ± 0.017</td>
<td>0.697 ± 0.043</td>
<td>2.5</td>
<td>4.8</td>
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<tr>
<td>N4A-25</td>
<td>YNPYTPL</td>
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<td>0.268 ± 0.024</td>
<td>0.670 ± 0.046</td>
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<td>4.8</td>
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<tr>
<td>T8A-02</td>
<td>VMQATHD</td>
<td>5</td>
<td>0.255 ± 0.013</td>
<td>0.612 ± 0.078</td>
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<tr>
<td>T8A-08</td>
<td>WHTNEYP</td>
<td>3</td>
<td>0.251 ± 0.014</td>
<td>0.603 ± 0.065</td>
<td>2.4</td>
<td>4.8</td>
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<tr>
<td>N4A-03</td>
<td>STHSPES</td>
<td>1</td>
<td>0.300 ± 0.023</td>
<td>0.690 ± 0.061</td>
<td>2.3</td>
<td>3.9</td>
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<tr>
<td>N4A-20</td>
<td>NVNLFFP</td>
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<td>0.276 ± 0.023</td>
<td>0.635 ± 0.022</td>
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<td>N4A-06</td>
<td>QANPLMI</td>
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<td>0.289 ± 0.012</td>
<td>0.636 ± 0.019</td>
<td>2.2</td>
<td>4.2</td>
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<tr>
<td>N4A-23</td>
<td>NTTYTTPT</td>
<td>1</td>
<td>0.277 ± 0.023</td>
<td>0.525 ± 0.038</td>
<td>1.9</td>
<td>4.4</td>
</tr>
</tbody>
</table>

*Φ*, bacteriophage displaying the selected peptide; sequence, amino acid sequence of the heptapeptides; Freq., frequency of recurrence of each peptide among the sequenced bacteriophages; MurC, average ELISA values (OD<sub>450nm</sub>) ± standard deviation (n = 4) of each phage with MurC as target; FtsA, average ELISA values (OD<sub>450nm</sub>) ± standard deviation (n = 4) of each phage with his-FtsA as target; FtsA/MurC, ratio between the ELISA values of each phage challenged with his-FtsA and his-MurC, respectively; Φ/VCSM13, ratio between the ELISA values to his-FtsA of each recombinant phage (Φ) with respect to the negative control VCSM13. High-affinity binding phages are separated from low-affinity binding phages.
Comparison of the overall amino acid composition of the high-affinity heptapeptides with the corresponding frequencies of the original library revealed that some residues were particularly enriched (W,Y,K,F,V), whereas other were significantly depleted (A,R,S,G) or completely omitted (H,D,C) [Fig. 3(A)]. Conversion of the sequences with dedicated SIMPLIFY program based on physicochemical properties of the amino acids allowed the definition of a degenerate consensus sequence. Briefly, residues were grouped in hydrophobic (Ø: F,Y,W,L,V,I,M), neutral (‡: A,T,G,S), hydrophilic (#: Q,N,K,R,E), prolin residues (P) and then aligned with CLUSTALW. 29,30 Figure 3(B,C) shows that the tripartite consensus sequence is formed by a N-terminal hydrophilic region followed by a hydrophobic core closed at the C-terminus by a prolin residue. Sequences displayed by the low-affinity bacteriophages comply more weakly with the consensus in respect with the high-affinity binders (Table I). The fact that the selected peptides conform to a consensus sequence suggests that they all recognize a unique binding site on FtsA.

Because peptide displayed by phage libraries has been widely used to infer protein ligands of target molecules,22,23 we searched the databases for similarity with the high-affinity peptide sequences. 31 Conventional BLASTP search on *E. coli* database did not retrieve either known interacting partners of FtsA, nor other proteins involved in cell division. Only when T8A-11 sequence (KLWVIPQ) was submitted, FtsA itself appeared among the best hits.31 The degree of homology with the parental sequence is remarkable: 62.5% of identity and 78.5% of similarity with residues 126 –133 (RVLHVIPQ). This region of FtsA is located on subdomain 1C and is exposed on the surface of the molecule.19 This evidence strongly suggested that residues 126–133 are involved in FtsA homodimerization, because sequence of T8A-11 was affinity selected to interact with FtsA.

Correlated Mutation Analysis

The identification by phage panning of amino acids 126–133 as putative determinants of FtsA homodimerization did not give information on which residues are contacted by these amino acids and how the two molecules of FtsA are arranged in the homodimer. To shed light on these issues, a correlated mutation analysis of FtsA orthologues was performed. Correlated mutations arise in protein sequences to compensate substitutions that independently occur during evolution. As residues in physical contact are subjected to a high selective pressure to compensate for substitutions, this analysis predicts protein contacts, both for inter-domain docking and for intermolecular interactions.34,37

Multiple sequence alignment of orthologous FtsA proteins was analyzed using PLOTCORR program and the obtained amino acid pairs were compared with their respective position in the crystal structure of FtsA from *T. maritima*.19,32 A particularly notable correlation (average correlation coefficient $r = 0.62$) was found between a group of residues encompassing the ATP-binding site and amino acids 138–151 of the loop connecting S6–S7 strands in subdomain 1C (Table II). Remarkably, the region of homology with peptide T8A-11 flanks at the immediate N-terminus this loop [Fig. 4(A,B)]. Therefore, correlated mutation analysis converged to identify subdomain 1C as an interacting interface of FtsA dimerization. Multiple sequence alignment of the orthologous FtsA proteins of this region displays a good compliance with the peptide consensus motif, which appears as one of the best conserved in evolution outside the ATP-binding site, thus supporting its functional relevance (Fig. 5). Taken together, all these evidences indicate that residues 126–133 (homologous to T8A-11 peptide) and 138–151 (correlated mutation pairs) of subdomain 1C have a role in FtsA self-interaction.

A Model for the Homodimerization of FtsA

On the basis of this information, we propose a model for the homodimerization of FtsA. In the model plotted in Figure 4(C), the first FtsA molecule (blue) inserts its subdomain 1C into the groove of the second monomer (red) formed by the intersection of the upper flat surface of subdomain 1A, the loops surrounding helix H9 of subdo-
The arrangement orients residues 126–133 (yellow) of the upper monomer in close proximity to the apical strands S12–S13 of the second monomer, while amino acids 138–151 (red spheres), lying in the loop connecting S6–S7, face the correlated pairs in subdomain 1A of the lower FtsA molecule (green spheres) close to the ATP-binding site. To probe the accuracy of the proposed model, 5 alternative docking solutions were generated with the program GRAMM and analyzed with two different approaches. First, the distance between the correlated residues was corrected by the correlation coefficient obtained by the program PLOTCORR to give more weight to pairs of amino acids with a higher correlation coefficient. Finally, a “correlated mutations-corrected distance” factor \( cd \) for each individual solution was calculated (see Materials and Methods). Figure 6(A) shows the distribution of the \( cd \) values, where the low \( cd \) value of the proposed model (black arrow) indicates a good correlation with contacts predicted by calculation of correlation coefficients.

A second evaluation of accuracy was performed by using the harmonic average factor \( Xd \). This factor estimates not only physical contacts between residues, but also performs a continuous measurement of proximity taking into account.

### TABLE II. Correlated Mutation Analysis

<table>
<thead>
<tr>
<th>Subdomain 1C</th>
<th>Active site</th>
<th>Correlation Coefficient ( r )</th>
</tr>
</thead>
<tbody>
<tr>
<td>D138</td>
<td>S43</td>
<td>0.548</td>
</tr>
<tr>
<td></td>
<td>R300</td>
<td>0.892</td>
</tr>
<tr>
<td></td>
<td>G336*</td>
<td>0.892</td>
</tr>
<tr>
<td>Q140</td>
<td>H87</td>
<td>0.616</td>
</tr>
<tr>
<td></td>
<td>S192*</td>
<td>0.515</td>
</tr>
<tr>
<td>G142</td>
<td>I15</td>
<td>0.504</td>
</tr>
<tr>
<td></td>
<td>G213</td>
<td>0.515</td>
</tr>
<tr>
<td>K144</td>
<td>G236</td>
<td>0.507</td>
</tr>
<tr>
<td>P146</td>
<td>G37</td>
<td>0.641</td>
</tr>
<tr>
<td></td>
<td>G39</td>
<td>0.578</td>
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<tr>
<td></td>
<td>S43</td>
<td>0.618</td>
</tr>
<tr>
<td></td>
<td>G45</td>
<td>0.795</td>
</tr>
<tr>
<td></td>
<td>M46</td>
<td>0.674</td>
</tr>
<tr>
<td></td>
<td>D242</td>
<td>0.796</td>
</tr>
<tr>
<td></td>
<td>R300</td>
<td>0.687</td>
</tr>
<tr>
<td></td>
<td>G336*</td>
<td>0.687</td>
</tr>
<tr>
<td>V147</td>
<td>G236</td>
<td>0.618</td>
</tr>
<tr>
<td>L149</td>
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<td>0.513</td>
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<tr>
<td></td>
<td>G45</td>
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<td></td>
<td>D47</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>L334</td>
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</tr>
<tr>
<td></td>
<td>G336*</td>
<td>0.734</td>
</tr>
<tr>
<td>G151</td>
<td>I35</td>
<td>0.529</td>
</tr>
<tr>
<td></td>
<td>G37</td>
<td>0.690</td>
</tr>
<tr>
<td></td>
<td>S43</td>
<td>0.550</td>
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<tr>
<td></td>
<td>G45</td>
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<td>M46</td>
<td>0.502</td>
</tr>
<tr>
<td></td>
<td>A64</td>
<td>0.515</td>
</tr>
<tr>
<td></td>
<td>E69</td>
<td>0.508</td>
</tr>
</tbody>
</table>

\( * \)Correlation coefficients \( r \), corresponding to pairs of residues present in subdomain 1C related to those surrounding the ATP-binding site. Values were obtained by using the program PLOTCORR. Asterisks indicate that amino acids S192 and G336 are targeted in lethal conditional mutants \( ftsA40 \) and \( ftsA102 \), respectively.

The correlated pairs in subdomain 1A of the lower FtsA molecule (green spheres) close to the ATP-binding site.

To probe the accuracy of the proposed model, \( 5 \times 10^3 \) alternative docking solutions were generated with the program GRAMM and analyzed with two different approaches. First, the distance between the correlated residues was corrected by the correlation coefficient obtained by the program PLOTCORR to give more weight to pairs of amino acids with a higher correlation coefficient. Finally, a “correlated mutations-corrected distance” factor \( cd \) for each individual solution was calculated (see Materials and Methods). Figure 6(A) shows the distribution of the \( cd \) values, where the low \( cd \) value of the proposed model (black arrow) indicates a good correlation with contacts predicted by calculation of correlation coefficients.

A second evaluation of accuracy was performed by using the harmonic average factor \( Xd \). This factor estimates not only physical contacts between residues, but also performs a continuous measurement of proximity taking into account.

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Fig. 3. Sequence analysis of the high-affinity binding peptides. (A) Relative amino acid abundance in the high-affinity heptapeptides directed against FtsA. Composition of the 10 heptapeptides was compared with amino acid frequency of Ph.D.-7 library. The fold increase (black bars) and fold decrease (white bars) are shown. Cysteine (2A), aspartic acid and histidine (2B) residues are completely absent in the selected peptides (grey bars). (B) FtsA-binding peptides were aligned after sequence transformation with SIMPLIFY program. Ø: F,Y,W,L,V,I,M (hydrophobic, boxed); ‡: A,T,G,S (neutral); #: Q,N,K,R,E (hydrophilic); P: prolin. The degenerate consensus is indicated (bottom). (C) Distribution of amino acid residues at each position of the alignment shown in (B). Hydrophobic residues (Hpb: F,Y,W,L,V,I,M; white bars) are enriched at positions 5–8, hydrophilic residues (Hpl: N,Q,K,R,E; black bars) at positions 3–4, whereas prolin (Pro: light grey bars) at position 9. Neutral residues (Neu: A,T,G,S) are indicated as dark grey bars.
the distance of both the correlated pairs and all pairs of positions. Briefly, the distances between pairs of residues were grouped in bins of 4 Å for each of the previous $5 \times 10^3$ docking solutions, obtaining two different distributions of binned data for the indicated correlated pairs and for all pairs of positions. The difference between the two distribu-

Fig. 4. A model for FtsA homodimerization. (A) Amino acid sequence of E. coli FtsA. Residues of subdomain 1C are underlined. Region homologous to T8A-11 peptide is bolded with a yellow background. Correlated pairs of amino acids belonging to subdomain 1C are depicted with a red background, whereas their counterparts have a green background. (B) Ribbon plot of FtsA crystal structure. The structure is divided into four subdomains 1A, 1C, 2A, 2B. Positions of strands S6–S7 and S12–S13 are indicated. The correlated amino acid pairs in the loop connecting the S6–S7 strands are depicted as red spheres, whereas their counterparts surrounding the active site are depicted as green spheres. The ATP molecule is drawn in blue. The region homologous to peptide T8A-11 is represented as yellow backbone. (C) Ribbon plot of the proposed model of a FtsA homodimer. The first molecule of FtsA (blue, top) inserts its subdomain 1C into the cleft made by subdomains 2B, 2A, and 1A of the second molecule (red, bottom). The region of the first monomer homologous to peptide T8A-11 (yellow backbone) contacts the loop connecting strands S12–S13 of the second monomer, while the correlated mutation residues on loop between strands S6–S7 (red spheres) are located in close proximity with their counterparts on the facing monomer (green spheres).
tions was calculated bin by bin and normalized to increase weight of closer distances (see Materials and Methods). The higher the \( X_d \) value, the lower is the distribution of distances of the correlated pairs. Therefore, the actual protein-protein arrangement tends to show a \( X_d \) value higher than the majority of the docking solutions. Figure 6(B) shows that the \( X_d \) value for the proposed model (black arrow) is located among the highest scores of all the 5 \( \times \) 10^3 alternative docking solutions, confirming once more a good accuracy of the dimerization model.

### Protein-Protein Interaction Residues

Prediction of amino acids involved in protein-protein interactions allows characterizing protein domains and discriminating among docking models. To probe the accuracy of our model, we performed an analysis to detect protein–protein interaction residues in the framework of a neural network. This system combined the evolutionary information accumulated in sequence profiles derived from family alignments with structural data of the surface
patches identified as sets of neighbor residues exposed to solvent.\textsuperscript{35,36} In brief, the neural network was trained with a large set of dimer-forming pairs of known interacting proteins extracted from the SPIN database, and it used as input both 3D coordinates from Protein Data Bank entries and multiple sequence alignments from the HSSP database (see Materials and Methods). The output consisted of a list of surface residues predicted as interacting determinants of the protein (positive contacts; Table III). Once these residues were mapped on the homodimerization model, some interesting features were immediately evident. First, approximately two thirds of the contact residues are distributed on two regions matching exactly the model proposed for the homodimerization of FtsA (Fig. 7; gold spheres). These regions are located on the interface between subdomains 2B-1A (Region I) and 1A-1C (Region II) of the facing monomers [Fig. 7(B)]. More strikingly, two residues among the amino acids predicted to be involved in protein-protein interactions are precisely V130 and I131, which belong to the T8A11-like sequence RVLHVIPQ (Fig. 7, magenta spheres). In conclusion, an independent approach converged to support the reliability of the predicted model for the homodimerization of FtsA.

\textbf{ftsAΔ7, a Deletion Mutant Lacking Amino Acids 126–133}

Functional role of residues 126–133 of FtsA was examined by producing an in frame site-directed deletion mutant lacking this region of the protein (Fig. 8). We placed the internally deleted gene \textit{ftsA}\textsubscript{Δ7} under the control of a PBAD promoter in a pBAD24 vector (pBAD-FtsA\textsubscript{Δ7}). This expression system is elective for both overexpression studies and complementation analysis, because transcription from PBAD promoter can be finely tuned over a wide range of inducer concentrations or completely silenced by the repressor.\textsuperscript{26} To assess whether the deletion caused an alteration in protein structure such that it became completely unfolded or degraded, we analyzed the effects of overexpression of FtsA\textsubscript{Δ7} in comparison with wild-type FtsA (pBAD-FtsA). In time-course experiments, FtsA and FtsA\textsubscript{Δ7} showed a comparable expression profile when analyzed by Western blotting (Fig. 9). The expression was confirmed to be tightly controlled, because basal level and repressed state were under the detection threshold (lanes 1 and 8). This experiment shows that protein degradation was not significant, allowing further characterization of the cellular effects produced by the recombinant proteins. The overexpression of FtsA and FtsA\textsubscript{Δ7} had a dramatic impact on cell growth. Induction of the two proteins promptly caused a significant drop of the growth rate, which ultimately ended in a complete arrest [Fig. 10(A)]. This behavior was expected for overexpression of wild-type FtsA, because its cellular ratio can not be altered without blocking cell division.\textsuperscript{7} To determine whether this phenotype was mediated by a bacteriostatic or a bactericidal mechanism, we quantitatively estimated the killing effect of the expression of the two FtsA proteins by plating serial dilutions of

\begin{table}
\centering
\caption{Residues of FtsA Predicted to be Involved in Protein-Protein Interaction\textsuperscript{1}}
\begin{tabular}{llllll}
\hline
Region I & Region II & Not I or II \\
\hline
V10 (4) & V79 (1) & I36 (2) & M71 (1) & N34 (5) & C90 (1) \\
L81 (3) & V130* (3) & L358 (2) & L362 (3) & Q61 (2) & N92 (3) \\
I131* (2) & L383 (6) & D369 (2) & A371 (2) & K230 (4) & V231 (1) \\
Y385 (7) & Q372 (7) & Y375 (2) & P233 (1) & A329 (3) \\
\hline
\end{tabular}
\textsuperscript{1}Amino acids obtained from the protein interaction analysis were arranged in three regions according to their spatial distribution in the proposed model for FtsA dimerization. Region I comprises residues facing subdomain 2B of the accompanying FtsA molecule, while Region II groups amino acids proximal to subdomain IC of the attendant monomer. Asterisks on bolded V130 and I131 indicate their position within the T8A11-homologous region. Numbers in parentheses indicate neural network reliability index for positive contacts.
\end{table}
the induced and of the repressed cultures. Figure 10(B) shows that viability of cells expressing FtsA and FtsA/H9004 decreased steadily with induction time, reaching a difference in respect with their repressed negative controls up to 10⁶ colony-forming units. Effects of the overexpression of FtsA/H9004 matched those of FtsA also as far as cell phenotype is concerned. Under microscopic examination, induction of FtsA/H9004 gave rise to long nonseptate filamentous cells [Fig. 10(C,b)], which were almost indistinguishable from cells expressing the wild-type protein [Fig. 10(C,c)]. As negative controls, cells transformed with pBAD24 and with the same vector bearing the gene encoding for MBP were analyzed in parallel, confirming that lethality and filamentation were exclusively associated with the expression of FtsA and FtsA/H9004 (data not shown).

The experiments shown in Figure 10 demonstrated that expression at high level of FtsA/H9004 led to cellular effects similar to those obtained with the wild-type protein. These results can be explained only by assuming that FtsA/H9004 is properly folded in vivo, because deletion of amino acids 126–133 preserved, if not even slightly increased, the cellular toxicity of FtsA overexpression.

Fig. 7. Mapping of FtsA residues predicted to be involved in protein-protein interaction. (A) Ribbon plot of the crystal structure of FtsA. Residues involved in protein-protein interaction are depicted as gold spheres. Amino acids V130 and I131, also predicted as interacting residues, are drawn as magenta spheres indicating their position within the T8A11-homologous region (yellow backbone). The ATP molecule is depicted in blue. (B) Proposed model for the homodimerization of FtsA. The predicted interacting residues of both monomers are colored in gold and grouped in two regions, marked as I and II, respectively. The position of amino acids V130 and I131 and of the T8A11-homologous region are indicated as in (A).

364 -cgcgatgagcat cggtgcgtgcatgtcctggcagaggtatgccgtatt- 411
FtsA

122 -RDEH RVLVIPQ EYAI - 137

FtsAΔ7

364 -cgcgatgagcat ctc gagtatgcggatt- 389′

Fig. 8. Sequences of wild-type FtsA and of FtsAΔ7. Top: DNA and amino acid sequence of wild-type FtsA from E. coli in the region homologous to T8A-11. Amino acids and nucleotides which are deleted in the mutant FtsAΔ7 protein are bolded. Bottom: The heptapeptide homologous to T8A-11 is deleted in FtsAΔ7. Hyphens indicate the new notation of nucleotides and amino acids in FtsAΔ7. The newly inserted XhoI site is underlined.
Amino Acids 126–133 Are Essential

To assess the functional relevance of amino acids 126–133 we evaluated the ability of ftsAΔ7 to complement either a thermosensitive missense ftsA allele (ftsA2; D2 strain) or an amber mutation of ftsA in a temperature-sensitive suppressor background (ftsA16; OV16 strain). These two strains were transformed with pBAD24 carrying wild-type ftsA or ftsAΔ7. Their ability to grow at permissive (30°C) and nonpermissive temperature (42°C) was tested by inducing expression of FtsA and FtsAΔ7 at physiological level (0.02% arabinose) and at overexpressed level (0.2% arabinose). The result of complementation assay is summarized in Table IV. As expected, wild-type FtsA was able to rescue the conditional-lethal phenotype of D2 and OV16 strains at the non-permissive temperature only when expressed at low levels, because its overexpression caused filamentation and lethality. On the contrary, FtsAΔ7 failed to recover the lethal phenotype of both strains when grown under restrictive conditions at any expression level, thus demonstrating that amino acids 126–133 are essential for FtsA activity and hence for E. coli cell division.

**DISCUSSION**

In this work we propose a model for the self-interaction of FtsA, based on the similarity of peptides selected by phage panning with a subdomain region of FtsA itself and on the analysis of the correlated mutations occurring in the orthologous sequences of FtsA. The accuracy of this model was validated with different approaches. First, a large number of alternative docking solutions was probed, confirming that the proposed model is in substantial agreement with the position of the putative contact points of the two facing molecules. In addition, mapping of residues predicted as determinants of inter-molecular contacts provided support for the proposed relative orientation of the two FtsA molecules in the homodimer. Finally, the functional relevance of region 126–133 of subdomain 1C was unequivocally demonstrated by the lethal phenotype of the ftsAΔ7 mutant.

It is worthy to be mentioned that our analysis did not retrieve the 27 C-terminal residues of FtsA, which had been demonstrated by genetic and functional approaches to have an essential role in FtsA homodimerization. This discrepancy is probably due to the flexible nature of the terminal part of FtsA, which also made impossible its solution in the crystal structure. Nevertheless, position of the C-terminal tail is most likely oriented on the same direction and in close proximity to subdomain 1C. Therefore, this subdomain and the extreme C-terminal part of FtsA could both contribute to the formation of the interacting interface.

Despite the different orientation of subdomain 1C, the mutual disposition of the FtsA molecules in the proposed model shares many similarities with the arrangement of the actin monomers in the polymerized form. In fact, subdomain 1C of one monomer of FtsA occupies in the homodimer the space corresponding to the structurally related subdomain 1B of actin. Moreover, sets of amino acids that are proposed in our model to directly contact the facing FtsA molecules have corresponding functional counterparts in actin. For instance, the loop connecting S11–S12 strands of subdomain 2B in actin, which is structurally equivalent to the loop linking S12–S13 strands in FtsA (contacted by the T8A11-homologous motif), was demonstrated to be involved in actin polymerization by site-directed mutagenesis, e.g., actin mutant G245DK has a negligible polymerization activity, and by analysis of crystal structures of the actin molecule. On the other hand, the loop connecting strands S4–S5 in actin subdomain 1B, corresponding to the loop between strands S6–S7 in subdomain 1C of FtsA (the correlated mutation pairs), was directly implicated in actin self-interaction by covalent derivatization studies—polymerization is severely affected by targeting H40, Q41 and K50—by characterization of temperature-sensitive mutants (R37A, R39A, K50A, D51A) and by stereoplot analysis of the actin three-dimensional structure. As in actin, the proposed model is consistent with the assembly of FtsA units in polymers. In particular, the disposition of the FtsA molecules in the polymerized form is reminiscent of the ribbon structure in actin, where extensive contacts organize adjacent monomers with an alternate symmetry along a screw axis.

Amino acid counterparts of the correlated mutations on subdomain 1C encompass the ATP-binding site on the facing monomer. Genetic analysis provided a wide set of lethal-conditional alleles of ftsA, which contributed to define functional domains of the protein. It is noteworthy that among them ftsA40 and ftsA102 display point mutations that target residues belonging to the correlated pairs. In particular, ftsA40 (S192L) encodes a temperature-sensitive protein, whereas ftsA102 (G336D) encodes a nonfunctional protein (see Table II). Although these two amino acids are located close to the active site, they are not directly involved in nucleotide binding. Rather, muta-
tions occurring at these positions are expected to alter the geometry of the active site, indirectly reducing the affinity for ATP, as it was demonstrated for ftsA102.\textsuperscript{6} Conversely, coordinated changes of these positions in the orthologous proteins with amino acids which reside in the facing subdomain 1C suggest that a mutual interplay can exist between homodimerization and nucleotide binding. Moreover, crystal structure of FtsA from \textit{T. maritima} revealed
that the connection of subdomain 1C with the rest of the protein is flexible, with an angle of rotation of 13.5°. Although flexibility could not be exclusively related to ATP-binding, van den Ent and Löwe proposed that binding of the nucleotide may fix subdomain 1C in a specific orientation for intermolecular docking.

ACKNOWLEDGMENTS

The authors thank J. Lowther for essential contribution in the production of polyclonal antibodies directed against FtsA; C. Righetti for providing purified his-MurC and for accurate technical support; F. Faggioni for invaluable sequencing work; and B. Jansson, C. Moser, N. Frandsen, S. Ferri, and M. Altieri for extensive helpful discussions. Vector pMFV12 expressing N-terminal his-FtsA was kindly provided by M.J. Ferrándiz, and the technical support in complementation assays from M. Casanova was greatly appreciated.

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