The Soluble Periplasmic Domains of *Escherichia coli* Cell Division Proteins FtsQ/FtsB/FtsL Form a Trimeric Complex with Submicromolar Affinity

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Background: The FtsQBL complex plays a key role in bacterial cell division.

Results: Periplasmic domains of FtsQ, FtsB, and FtsL form a trimeric complex with submicromolar affinity. Interactions are focused at the C termini of the subunits.

Conclusion: FtsQ, FtsB, and FtsL form a complex with 1:1:1 stoichiometry.

Significance: Insight into FtsQBL complex formation will facilitate drug design.

Cell division in *Escherichia coli* involves a set of essential proteins that assemble at midcell to form the so-called divisome. The divisome regulates the invagination of the inner membrane, cell wall synthesis, and inward growth of the outer membrane. One of the divisome proteins, FtsQ, plays a central but enigmatic role in cell division. This protein associates with FtsB and FtsL, which, like FtsQ, are bitopic inner membrane proteins with a large periplasmic domain (denoted FtsQp, FtsBp, and FtsLp) that is indispensable for the function of each protein. Considering the vital nature and accessible location of the FtsQBL complex, it is an attractive target for protein-protein interaction inhibitors intended to block bacterial cell division. In this study, we expressed FtsQp, FtsBp, and FtsLp individually and in combination. Upon co-expression, FtsQp was co-purified with FtsBp and FtsLp from *E. coli* extracts as a stable trimeric complex. FtsBp was also shown to interact with FtsQp in the absence of FtsLp albeit with lower affinity. Interactions were mapped at the C terminus of the respective domains by site-specific cross-linking. The binding affinity and 1:1:1 stoichiometry of the FtsQpBpLp complex and the FtsQpBp subcomplex were determined in complementary surface plasmon resonance, analytical ultracentrifugation, and native mass spectrometry experiments.

The Gram-negative bacterial divisome is a dynamic macromolecular complex formed by at least 10 essential and up to 15 accessory proteins that assemble at the midcell plane to affect cell division through a series of defined steps, including cell constriction, synthesis of the septal wall, and ultimately cell segregation (1, 2). Divisome assembly starts with formation of the FtsZ-ring in the cytoplasm and anchoring of the ring in the inner membrane by FtsA and ZipA. This assembly is followed by recruitment of the cell division proteins (FtsK, FtsQ, FtsB, FtsL, FtsW, FtsI, and FtsN), all of them membrane proteins.

FtsQ is considered to play a central, yet enigmatic, role in assembly of the divisome through a multitude of transient interactions (1, 2). Two-hybrid analyses have suggested that FtsQ interacts with ~10 cell division proteins of which the interactions with FtsB and FtsL were confirmed by immunoprecipitation (3). *Escherichia coli* FtsQ is a bitopic membrane protein of 276 residues, including a short cytoplasmic N-terminal domain, a transmembrane (TM)2 segment, and a large periplasmic domain (4). With respect to biogenesis and routing, FtsQ has been extensively characterized (5, 6). FtsQ is considered a particularly attractive target for the development of inhibitors of protein-protein interactions (PPIs) that block bacterial division (7), because of the variety of interactions of FtsQ with key cell division proteins in the relatively accessible periplasm. The low cellular abundance (8) and the lack of eukaryotic homologues contribute to the conceptual suitability of FtsQ as an antibacterial drug target (9). Moreover, FtsQ is a highly conserved protein among cell wall containing bacteria (4). It has been suggested to be one of the six core components of the cell division...
machinery, along with FtsZ, FtsA, FtsK, FtsW, and FtsI (10). Out of 374 strains that have been investigated in a bioinformatics analysis, at least 295 strains express homologues of all three components of the FtsQBL complex (11). Importantly, the structure of the large periplasmic domain of FtsQ has been solved, facilitating structure-based drug design (4).

The periplasmic domain of FtsQ consists of two subdomains, referred to as the α- and β-domain. Together with the TM, the α-domain is believed to be required for recruitment of FtsQ by FtsK to the divisome, although other interactions have been ascribed to this domain as well (12, 13). The α-domain is located directly downstream from the TM and includes a so-called POTRA subdomain that has been implicated in transient association with the host bacterium. Here, we have expressed the soluble components of the FtsQBL complex (11). Importantly, the periplasmic domain of FtsQ has been solved, facilitating structure-based drug design (4).

In studies aimed to develop FtsQ inhibitors, we decided to focus on the characterization of the FtsQBL membrane complex that has been identified as a subcomplex in the division cycle. Consistently, studies have shown interdependencies of FtsQ, FtsB, and FtsL for stability and localization at the divisome (14). The β-domain engages in multiple interactions, including those with FtsB and FtsL (4). In vivo scanning photo-cross-linking approach to map interactions of FtsQ with FtsBL at the amino acid level (13). For extensive coverage of the FtsQ interactome, 50 surface-exposed residues of the periplasmic domain were selected for introduction of a photoprobe meaning that roughly 1 in 5 residues was probed for its molecular contacts. Two hot spots for the interaction with FtsBL were identified as follows: one in the α-domain close to the membrane around residue Arg-75, and one in the conserved distal part of the β-domain around residue Ser-250 (13).

Thermodynamic and structural analysis of the FtsQBL complex is complicated by the fact that it is anchored in the membrane, and overproduction of the full-length proteins is toxic to the host bacterium. Here, we have expressed the soluble periplasmic domains of FtsQ (FtsQp, amino acids 50–276), FtsB (FtsBp, amino acids 25–103), and FtsL (FtsLp, amino acids 64–121) in the cytoplasm separately and in combination to characterize and map their interactions and to provide a template for the development of PPI inhibitors. FtsQp was shown to have a high affinity for dimerized FtsBpLp, and the stable FtsQpFtsBpLp complexes could be purified in large amounts. Interactions were mapped in the C-terminal regions of the three proteins. Surprisingly, FtsBp was found to interact with FtsQp also in the absence of FtsLp, albeit with lower affinity. This could indicate that the association of FtsB and FtsL with FtsQ is hierarchical rather than simultaneous.

### Experimental Procedures

**Growth Conditions**—E. coli strain BL21 (DE3) variants were grown in TY medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl/liter) with shaking at 200 rpm at 37 °C. When indicated, glucose was used at 0.4% (22 mM), ampicillin at 100 μg/ml (286 μM), spectinomycin at 50 μg/ml (93 μM), and chloramphenicol at 30 μg/ml (116 μM).

**Plasmid Constructions for Periplasmic Domains**—Standard PCR and cloning techniques were used for DNA manipulation. The sequence encoding FtsQp (residues 50–276), preceded by a hexahistidine tag, was cloned into a PET16b vector using forward primer Ncol-His6-ftsQp (CAGCCcatggGCAGCATC-ATCATCATGAAATATCCGCAACGCC) and reverse primer HindIII-ftsQp (GGTCAagcttCATTGTTGTTCTGCC-TGTG) to produce His6-ftsQp. From this construct, His6-ftsQp was cloned into a PET16b vector using forward primer Ncol-ftsQp (TATAccatggGCAGGATATCCGCAACGCC) and reverse primer HindIII-ftsQp.

The sequence encoding the e5-coil (24), preceded by a tobacco-etched virus protease cleavage site (TEV), was cloned into MCS-1 of a pCDFDuet vector (Novagen) using forward primer BamHI-TEV-e5 (ATAGgatccGGGTATACATGACTATACCCGCG and reverse primer XhoI-ftsLp), His6-ftsLp (forward primer Eco39I-His6-ftsLp (TATATagcgctGCAGCGAGCGTGAATCGCAGG), and reverse primer Sall-ftsLp (ATAGtgcagTTATGTTGTTGTTGCC), directly following His6-TEV-e5, resulting in His6-TEV-e5-ftsLp. From this construct e5-ftsLp (forward primer Ncol-e5-ftsLp, TATAccatggGCAGGATATCCGCAACGCC), and reverse primer Sall-ftsLp (ATAGtgcacTTACATGACTATCCCGCAACGCC) and reverse primer Sall-ftsLp, His6-ftsLp (forward primer BamHI-ftsLp), His6-ftsLp (forward primer BamHI-ftsLp, TATAccatggGCAGGATATCCGCAACGCC, and reverse primer Sall-ftsLp), and His6-ftsLp (forward primer Ncol-ftsLp, TATAccatggGTATACATGACTATCCCGCAACGCC, and reverse primer Sall-ftsLp) were constructed.

The sequence encoding the k5 coil (24), preceded by a TEV protease cleavage site and a hexahistidine tag, was cloned into MCS-2 of a pCDFDuet vector (Novagen) using forward primer Ndel-His6-TEV-k5 (TATATagctgGCGACGGACCATCACC-ATCATCACCGAGCTGAGAAGATCGTCGCCGAAAGTATCCGCCCTTTGAAAG), and reverse primer BglII-Eco47III-k5 (ATAGagatctCTAAGCGCAGGCTATAGCGCAGG), and reverse primer Sall-ftsLp, and His6-ftsLp (forward primer BamHI-ftsLp), TATAccatggGCAGGATATCCGCAACGCC, and reverse primer Sall-ftsLp, and His6-ftsLp (forward primer Ncol-ftsLp, TATAccatggGTATACATGACTATCCCGCAACGCC, and reverse primer Sall-ftsLp) were constructed.
Spel-His<sub>6</sub>-ftsL<sub>p</sub>, TATAactagCTGACCGCTCAGCGC, and reverse primer Xhol-ftsL<sub>p</sub>, and ftsL<sub>p</sub> (forward primer Ndel-ftsL<sub>p</sub>, TATAactagCTGACCGCTCAGCGC, and reverse primer Xhol-ftsL<sub>p</sub>) were constructed. All three plasmids were derived from the constructs described above by subcloning. Plasmids carrying DNA sequences encoding the coiled coils e5 and k5 were kindly provided by Thierry Vernet and André Zapun (24). An Avi tag, hexahistidine tag, and TEV protease cleavage site were introduced at the N terminus of FtsQ<sub>p</sub> in two steps, using forward primer AHT-ftsQ<sub>p</sub> 1 (CGCGAGAAATCAGATGCAGAAGAAACCTGTAATTCCAGGGTGAAATGCAGAACTGCAGCTGC) and reverse primer HindIII-ftsQ<sub>p</sub> in the first step and forward primer AHT-ftsQ<sub>p</sub> 2 (TACCCTATGGGCAATCATCACACATCACAGGTCTTGAACGACATCCTGGAAGCGCAGAAAATC) and reverse primer HindIII-ftsQ<sub>p</sub> in the second step. The product was cloned into the pET16b expression vector to give pET16b-Avi-ftsQ<sub>p</sub>.

Construction of His<sub>6</sub>-FtsQ<sub>p</sub> Cross-linking Mutant Expression Vectors—The periplasmic domains of the amber codon mutants were amplified from the respective full-length FtsQ<sub>shs</sub> constructs (13) and cloned into the pET16b vector. This was done either by PCR using forward primer Ncol-His<sub>6</sub>-ftsQ<sub>p</sub> and reverse primer p29SEN Rv (ACCCCGTACTGCGCAGG) (for K59tag and Q76tag) or by subcloning into pET16b-His<sub>6</sub>-ftsQ<sub>p</sub> using KpnI and HindIII (for V127tag, Q60tag, and S250tag).

Construction of Single Strand DsbA Expression Vectors—DNA encoding the DsbA signal sequence (ssDsbA) was amplified from MC4100 genomic DNA. DNA encoding His<sub>6</sub>-FtsQ<sub>p</sub>, His<sub>6</sub>-e5-FtsB<sub>p</sub>, and His<sub>6</sub>-k5-ftsL<sub>p</sub> was amplified from the respective plasmids described above. The signal sequence was introduced at the N termini of the specific genes by overlap PCR, and the resulting products were cloned into pET16b (ftsQ<sub>p</sub>) or pCDFDuet (ftsB<sub>p</sub> and ftsL<sub>p</sub>) expression vectors. DNA encoding FtsQ<sub>P</sub>, His<sub>6</sub>-FtsB<sub>p</sub>, e5-FtsB<sub>p</sub>, and k5-ftsL<sub>p</sub> was amplified from the respective plasmids described above. The resulting PCR products were cloned directly after the DsbA signal sequence in the existing ssDsbA fusion plasmids. All combined FtsB<sub>p</sub>/FtsL<sub>p</sub> expression vectors were obtained by subcloning.

Pulldown of Protein (Complexes) from E. coli Lysate—E. coli BL21 (DE3) cells harboring one of the pCDF-ftsB<sub>L</sub><sub>p</sub> variants and/or one of the pET16b-ftsQ<sub>p</sub> (Novagen) variants were grown in 25 ml of growth medium to an A<sub>600</sub> of 0.8 when protein expression was induced by adding isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 1 mM. After 2 h of induction, the cultures were cooled on ice, and the cells were harvested (10,000 × g, 15 min, 4 °C) and resuspended in 6 ml of binding buffer (50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole, 2 mM DTT, and 1 mM PMSF). The cells were lysed by two passages through a One Shot cell disruptor (Constant Systems) at 1.3 kbar. After centrifugation at 13,000 × g (15 min, 4 °C) to remove the cell debris, the lysate was cleared by ultracentrifugation at 293,000 × g (45 min, 4 °C). The supernatant was diluted with 6 ml of binding buffer and incubated (agitated) with 250 μl of Ni<sup>2+</sup>-nitrotriacetic acid (NTA)–agarose beads (50% suspension in ethanol, Qiagen) for 1.5 h at 4 °C. The beads were washed three times with 6 ml of 50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole (pH 8.0) followed by elution with 1 ml of 50 mM sodium phosphate, 300 mM NaCl, 400 mM imidazole (pH 8.0). At larger scale, the purification was done using an AKTA FPLC system (GE Healthcare) equipped with a HiTrap TALON crude column (GE Healthcare) using buffer containing 5, 20, and 100 mM imidazole (pH 8.0) for binding, washing, and elution, respectively. The protein was concentrated to a volume of 0.5–1.5 ml (Vivaspin 20, 10,000 MWCO, GE Healthcare) and purified by size exclusion chromatography using a Superdex 200 HR 10/30 column (GE Healthcare) in buffer containing 50 mM sodium phosphate, 150 mM NaCl, and 10% (v/v) glycerol (pH 8.0). Fractions containing the target protein were pooled and concentrated (Vivaspin 20, 10,000 MWCO, GE Healthcare) to a volume of 0.5–1.5 ml (10,000 × g, 4 °C).

Disuccinimidyl Glutarate Cross-linking—Disuccinimidyl glutarate (Thermo Scientific) dissolved in acetone was added in concentrations between 0.05 and 2.0 mM to 50 μl of purified protein complex (FtsQ<sub>p</sub>B<sub>L</sub><sub>p</sub> or FtsQ<sub>p</sub>B<sub>L</sub><sub>p</sub> 0.8 mg/ml) in HEPES buffer (50 mM HEPES (pH 8.0), 150 mM NaCl, 10% (v/v) glycerol). The final concentration of acetoneitrile was 2% (v/v). The reaction mixture was incubated for 2 h on ice, after which the reaction was quenched by addition of Tris-HCl (pH 8.0) to a concentration of 20 mM and incubation for 30 min on ice. Samples were analyzed by SDS-PAGE (12% (w/v) acrylamide gel) and subsequent Coomassie G-250 staining.

Cross-linking with Bis(succinimidyl)-3-azidomethyl Glutarate (BAMG) and Digestion—BAMG was synthesized as described previously (25). Protein complexes (FtsQ<sub>p</sub>B<sub>L</sub><sub>p</sub> or FtsQ<sub>p</sub>B<sub>L</sub><sub>p</sub>) were cross-linked in 6.6 ml of HEPES buffer with 0.4 mM BAMG at a protein concentration of 0.38 mg/ml. After 1 h the reaction was quenched by adding 1 M Tris-HCl (pH 8.0) to a final concentration of 50 mM. Subsequently, the proteins were concentrated and washed twice with HEPES buffer on 0.5 ml of Amicon Ultra 10-kDa cutoff centrifugal filters (Millipore). Protein complexes were completely denatured by adding urea to a final concentration of 6 M. The solution was diluted six times by the addition of 100 mM ammonium bicarbonate and digested with trypsin (Trypsin Gold, Promega, Madison, WI) overnight at 37 °C at a 1:50 (w/w) ratio of enzyme and substrate. Peptides were desalted on C18 reversed phase TT3 top tips (Glygen, Columbia, MD), eluted with 0.1% TFA in 50% acetonitrile. Peptide complexes were completely denatured by adding urea to a final concentration of 6 M. The solution was diluted six times by the addition of 100 mM ammonium bicarbonate and digested with trypsin (Trypsin Gold, Promega, Madison, WI) overnight at 37 °C at a 1:50 (w/w) ratio of enzyme and substrate. Peptides were desalted on C18 reversed phase TT3 top tips (Glygen, Columbia, MD), eluted with 0.1% TFA in 50% acetonitrile.

Enrichment and Analysis of Cross-linked Peptides—Cross-linked peptides were enriched by diagonal strong cation exchange (SCX) chromatography. Between the primary and secondary SCX runs, fractions were treated with tris(2-carboxyethyl)phosphine to reduce the azide group in the BAMG-derived moiety of cross-linked peptides to an amine group, leading to the required change in chromatographic behavior of target peptides, as described previously (26). Cross-linked peptides were analyzed by LC-MS/MS using Fourier transform ion cyclotron resonance mass spectrometry using Mascot Distiller (Matrix Science, London, UK) for data processing as described previously (26).

Identification of Cross-linked Peptides—For nomination by Mascot (version 2.3.02) (27) of candidate cross-linked peptides (28), a database of all possible cross-linked species was calcu-
lated (29) from forward and reversed sequences of FtsB, FtsL, and FtsQ, based on a maximum of three missed tryptic cleavages per peptide. The database was interrogated with MS/MS data of cross-linked peptide-enriched SCX fractions at 15 ppm precursor mass tolerance and 0.015 Da mass tolerance for fragment ions. Methionine oxidation was applied as a variable modification. No Mascot threshold score was taken into account for cross-link candidate generation.

Validation and Assignment of Cross-linked Peptides—Candidate cross-linked peptides generated by Mascot were validated by the Yeun Yan software tool as described previously (26). In short, for proposed candidate cross-linked peptides, Yeun Yan calculates the masses of possible b and y fragments, b and y fragments resulting from water loss (b0, y0) and ammonia loss (b*, y*), fragment ions resulting from cleavage of the amide bonds of the cross-link, and b, b0, b*, y, y0, and y* fragments resulting from secondary fragmentations of such cleavage products.

An ions score is calculated to provide a measure for the degree of matching of the experimental MS/MS spectrum with the theoretical spectrum. The YY score is calculated according to the equation YY score = \( \left( \frac{f_{\text{assigned}}}{f_{\text{total}}} \right) \times 100 \), in which \( f_{\text{assigned}} \) is the total number of matching fragment ions at 15 ppm mass accuracy, and \( f_{\text{total}} \) is the total number of fragment ions in the spectrum with a minimum of 8 and a maximum of 40, starting from the fragment ion of highest intensity. For each precursor ion selected for MS/MS, no more than one candidate cross-linked peptide or decay peptide, i.e. a cross-linked peptide candidate with either one or both composing peptides from the reversed database, is assigned, out of possible other candidates nominated by Yeun Yan. The highest scoring candidate for a particular precursor ion is assigned if it fulfills all of the following criteria: (i) the YY score is at least 50 and is higher than the YY score of possible other candidates for that precursor; (ii) the sum of the unambiguously assigned y is higher than that of all possible other candidates for that precursor; and (iii) the number of unambiguously assigned y ions to each of its composing peptides is at least one and is the same as or higher than the number of unambiguously matching y ions to each of the composing peptides in possible other candidates for that precursor. The false discovery rate (FDR) is defined by FDR = \( \left( \frac{FP}{TP + FP} \right) \times 100 \), in which \( FP \) is the number of decay peptide MS/MS spectra fulfilling the criteria for assignment, and \( TP \) is the number of assigned target peptide spectra.

Native Mass Spectrometry—All protein samples were buffer exchanged to an MS-compatible buffer (150 mM ammonium acetate (pH 7.4)) using 5000 MWCO centrifugal filter units (Millipore). Samples were loaded into gold-coated borosilicate acetate (pH 7.4)) using 5000 MWCO centrifugal filter units (Millipore). Samples were load into gold-coated borosilicate capillaries prepared in-house. Individual purified protein samples were run on an LCT (Micromass, Uppsala, Sweden) was used in all experiments. NeutrAvidin (fisher) was coupled to the surface of the active and reference channel of a Series S CM5 sensor chip (GE Healthcare) using the BIAcore amine coupling protocol (34). Immobilization and interaction studies were conducted at 25 °C in 20 mM Na2HPO4, 300 mM NaCl, 5% (v/v) glycerol, and a flow rate of 60 µl/min. FtsQp was enzymatically biotinylated in vitro according to the protocol of the BirA biotin-protein ligase standard reaction kit (Avidity, Aurora, CO) and captured on the NeutrAvidin surface in a manual run. In this case, because we aimed to measure protein-protein interactions, we immobilized 100 response units of FtsQp on the chip, giving a theoretical \( R_{\text{max}} \) of 100 RU's and 50 RU's for FtsBpLp and FtsBp, respectively. We applied double referencing to the data using both a reference channel and a blank measurement to correct the results.

Photo-cross-linking and Purification of His6-FtsQp—E. coli BL21 (DE3) cells harboring vectors pEVOl-pBpF (35), pCDF-E5-FtsQp-k5-ftsLp, and one of the pET16b-His6-FtsQp variants were grown in 25 ml of growth medium. When an \( A_{600} \) of ~0.5 was reached, p-benzoyl-phenylalanine was added to 0.5 mM and l-arabinose to 0.2%. After 30 min of continued growth, isopropyl-1-thio-β-D-galactopyranoside was added to 1 mM, and the cells were grown for a further 2 h, harvested by centrifugation (10 min, 10,000 × g), and resuspended in 25 ml of PBS. The cell suspension was exposed to 1.5 J/cm2 of 365-nm light (taking ~5 min) in 12 × 12-cm dishes in a Bio-Link BLX-365 (Vilber Lourmat). The cells were harvested and resuspended in 6 ml of 100 mM NaH2PO4, 1 M NaCl, 10 mM imidazole, 8 M urea (pH 8.0, NaOH) containing cOmplete EDTA-free protease inhibitor (Roche Applied Science). The cells were disrupted by a single passage through a One Shot cell disruptor (Constant Systems) at 2.14 kbar. After centrifugation at 10,000 × g for 10 min at 4 °C, the supernatant was centrifuged at 293,100 × g for 45 min at 4 °C. To the resulting supernatant 50 µl of Ni2+-NTA-garose (Qiagen) was added, and the suspension was incubated at ambient temperature (agitated) for 2 h. The Ni2+-NTA-garose beads were washed three times with 5 ml of 100 mM NaH2PO4, 1 M NaCl, 20 mM imidazole, 8 M urea (pH 8.0, NaOH), followed by elution with 80 µl of 100 mM NaH2PO4, 1 M NaCl, 300 mM imidazole, 8 M urea (pH 8.0). Proteins eluted from the Ni2+-NTA-garose beads were separated by SDS-
TABLE 1

| System                                      | FtsQp construct | FtsBp construct | FtsLp construct |
|---------------------------------------------|-----------------|-----------------|-----------------|
| FtsQpBpLp                                    | FtsQp           | e5-FtsBp        | His₅-TEV-k5-FtsLp |
| FtsBpLp                                     | FtsQp           | e5-FtsBp        | His₅-TEV-k5-FtsLp |
| FtsQpBp                                     | FtsQp           | His₅-FtsBp      | His₅-FtsLp       |
| FtsQp                                       | His₅-FtsQp      |                 |                 |
| FtsBp                                       |                 |                 |                  |
| Avi-FtsQp                                    | Avi-His₅-TEV-FtsQp |                |                 |

PAGE, followed by Western blotting for detection of adducts. FtsQp, FtsBp, and FtsLp were detected using affinity-purified polyclonal rabbit antibodies.

**Purification of FtsQpBpLp from the Periplasm—**1.5 OD units of cells were collected (1100 × g, 10 min), and the supernatant was discarded, and the cell pellets were resuspended in 10 μl of medium. 20 μl of CHCl₃ was added, and the cells were incubated at room temperature for 15 min. 200 μl of 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole was added; the cells were spun down (6000 × g, 20 min), and 180–190 μl of the upper phase was collected. The proteins were purified from the periplasmic fractions using TALON beads (GE Healthcare) according to the pulldown procedure described above.

**Results**

**Expression and Purification of FtsQp, FtsBp, and FtsLp Complexes—**To facilitate analysis of the interactions in the FtsQBL divisome subcomplex, we removed their membrane anchors and expressed only the soluble periplasmic domains of these proteins individually and together in different combinations in the cytosol of *E. coli*. Unless stated otherwise, the domain that was least abundant upon combined expression was expressed in His₅-tagged form for affinity purification to maximize the elution of fully assembled complexes. The mutants used are listed in Table 1. FtsQp and FtsBp could be expressed individually to high levels and purified by affinity chromatography when fused to an N-terminal hexahistidine tag (data not shown). FtsLp could only be expressed to detectable amounts upon fusion to an N-terminal hexahistidine tag (data not shown). Probably, this is due to the higher AT content at the 5’-end of the gene encoding His₅-FtsLp, which has been shown before to improve expression (13). Although His₅-FtsLp was expressed in reasonable amounts, it appeared aggregation-prone during purification (data not shown). Previously, it was shown that the full-length versions of FtsB and FtsL are interdependent for stable expression (11, 16, 18, 36, 37). Simultaneous expression of FtsBp and FtsLp indeed resulted in detectable accumulation of both proteins, but FtsBp did not co-purify with tagged FtsLp (Fig. 1, A, lane 5, and B, lane 3), indicating that the affinity between these domains is low under these conditions. Probably, the TM segments of the full-length FtsB and FtsL proteins are required for efficient interaction. The TMs may extend the predicted coiled coil structures in the respective periplasmic domains that are located directly adjacent to the membrane (11, 18, 38). To compensate for the absence of potentially interacting TM segments in our constructs, we fused artificial coils of opposite charge to the N terminus, a strategy that has proven successful to dimerize the soluble domains of DivIC and FtsL from *Streptococcus pneumoniae* that show some similarity to FtsB and FtsL of *E. coli*, respectively (24, 39). The coils were designed such that the pairing residues connect directly to the leucine zipper domains.

Co-expression of the coil constructs allowed efficient and specific co-purification of FtsBp with tagged FtsLp, (Fig. 1, A, lane 10, and B, lane 6) and vice versa (data not shown), indicating formation of an artificially constrained dimer. The positive effect of complex formation on expression of FtsLp is again consistent with the earlier mentioned interdependency of stability.

Efficient co-expression of the artificially constrained dimer FtsBpLp constructs with FtsQp was observed (Fig. 1A, lane 16). Without optimization of culture or induction conditions, up to 35 mg of FtsQpBpLp complex could be purified from 1 liter of culture. Importantly, FtsQp was very specifically and efficiently co-purified with the FtsBpLp dimer indicating that the periplasmic domains fold properly and interact to form a stable soluble complex (Fig. 1A, lane 20, and B, lane 12). Moreover, correct folding of FtsQp upon overexpression in the *E. coli* cytosol can be expected because a similar approach was used for crystallization studies (4).

Surprisingly, FtsQp was also co-purified with FtsBp (with or without coil) but not with FtsLp (with or without coil) (Fig. 1A, lanes 25 and 30, and B, lanes 15 and 18; with coil not shown). This indicates that FtsQp has affinity for FtsBp even in the absence of FtsLp but FtsQp has no detectable affinity for FtsLp in the absence of FtsBp. To verify these interactions in a physiologically relevant context, we expressed the same domains in the periplasm by fusing them to the DsbA signal peptide. This hydrophobic signal peptide recruits the signal recognition particle ensuring co-translational targeting to and translocation across the inner membrane, similar to the corresponding full-length membrane proteins (40, 41). Although expression levels were lower, probably due to the limited capacity of the signal recognition particle and secretory machinery, the pulldown experiments yielded the same FtsQpBpLp and FtsQpBp complexes of properly processed subunits (data not shown). Apparently, the soluble periplasmic domains of these proteins are able to fold and interact upon release in the periplasm, their natural environment, as they do in the cytoplasm underscore the relevance of the observed interaction.

**Analysis of the Interactions in the FtsQpBpLp Complex by Site-directed Cross-linking—**In addition to the demonstrated assembly of the FtsQpBpLp complex in the cytosol and periplasm, we wanted to verify that the overall conformation is similar to the corresponding domains in the context of the full-length membrane-anchored complex. Recently, we identified amino acid residues in full-length FtsQ that interact with FtsB and FtsL in the FtsQBL complex using an in vivo scanning photo-cross-linking approach (13). Using the same approach in cells that overexpress the soluble complex, we now probed the interaction of FtsQp with FtsBpLp from a selection of positions that either did (Lys-59, Gln-76, Thr-236, and Ser-250) or did not (Val-127 and Thr-144) show cross-linking to FtsB and FtsL in the full-length complex (13). With the exception of Lys-59, which was significantly less abundantly cross-linked to
Interactions of the Periplasmic Domains of FtsQ/FtsB/FtsL

FtsL$_p$, cross-linking from all diagnostic positions was consistent with that observed with the full-length proteins, indicating correct complex formation (Fig. 1C). The absence of the N-terminal domains of FtsQ, FtsB, and FtsL might affect the interaction of Lys-59 in the membrane-proximal regions of the FtsQ$_p$B$_p$L$_p$ complex, either because of the lack of potentially interacting TM segments, or because of incomplete folding of the extreme N-terminal residues of the FtsQ$_p$ protein (11, 42).

To further characterize the FtsQ$_p$B$_p$L$_p$ complex and map interaction sites not only in FtsQ but also in FtsB and FtsL, we used an independent bifunctional cross-linking strategy followed by peptide fragment fingerprinting to identify the cross-linked peptides. Of note, mapping of cross-linked amino acids is known to be problematic due to the relative paucity and complexity of cross-linked peptides. To address this issue, we used the recently developed amine-reactive cross-linker BAMG that introduces an azide group in the cross-linked peptides to enable enrichment of the cross-linked adducts by diagonal strong cation exchange chromatography. For analysis by mass spectrometry, a custom-made mass reference database containing all possible cross-linked peptides within the complex was calculated from both the forward and reverse protein sequences. Hits with one or both composing peptides with a
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TABLE 2

List of significant BAMG cross-linked peptides

| No. | Mass Da | Peptide A | Peptide B | Protein A | Protein B | Assigned spectra (number) |
|-----|---------|-----------|-----------|-----------|-----------|--------------------------|
| FtsQpBpLp |        |           |           |           |           |                          |
| 1   | 2492.4002 | XQPWDELK | LPLX1LVLTGER | FtsQ-Lys-113 | FtsQ-Lys-59 | 2                        |
| 2   | 3001.5655 | LQMQRHVPDSQENIVVQX | LVPDASXR | FtsL-Lys-121 | FtsB-Lys-93 | 86                       |
| 3   | 3294.6159 | LQMQRHVPDSQENIVVQX | LVPDASXR | FtsL-Lys-121 | FtsQ-Lys-183 | 4                        |
| 4   | 1715.8988 | LVPDASXR | EM*GQLMADX | FtsB-Lys-93 | FtsQ-Lys-183 | 3                        |
| 5   | 2203.1089 | LVPDASXR | EM*GQLMADX | FtsB-Lys-93 | FtsQ-Lys-183 | 1                        |
| 6   | 2008.9492 | EM*GQM*LAXDR | GDTMXR | FtsQ-Lys-183 | FtsQ-Lys-218 | 3                        |

In short, FtsQpBpLp was purified by Co2⁺/NTA affinity chromatography followed by gel filtration. Conditions to obtain partial cross-linking were tested using the commercially available cross-linker disuccinimidyl glutarate, which has the same spacer length and cross-link efficiency as BAMG. Based on these experiments, a BAMG concentration of 0.4 mM was used to cross-link the pure FtsQpBpLp complex. To identify juxtaposed lysine residues, the BAMG-treated complex was subjected to trypsin digestion, and cross-linked peptides were enriched by diagonal strong cation exchange chromatography as detailed under “Experimental Procedures.” The isolated peptides were subjected to LC-MS/MS analysis, and cross-linked peptides were identified using the custom-made database of all possible intra- and intermolecular cross-linked peptides in the complex.

In the FtsQpBpLp complex, a total of 99 cross-linked spectral matches were identified, representing two intramolecular and four intermolecular cross-links (Table 2). In general, intermolecular cross-linking appears focused in the C terminus of the complexes. However, it is difficult to compare the BAMG cross-links with these models, which the first one was considered more plausible (43). It is questionable, and they were not investigated in more detail. Annotated MS/MS data are shown in supplemental Table S1 for each of the six identified cross-links. No decoy sequences were found with the applied criteria for identification, indicating a very low false discovery rate. Also, no false positives were detected in the FtsQpBpLp complex (Table 2).

Analysis of the Subunit Stoichiometry of FtsQpBpLp (Complexes) by Native Mass Spectrometry and Analytical Ultracentrifugation—The subunit stoichiometry in the FtsQBL complex is not known. Based on bioinformatics analysis and protein docking studies a hexameric (FtsQpBpLp) model was proposed, of which the first one was considered more plausible (43). It is difficult to compare the BAMG cross-links with these models, because the cross-linked residues of FtsBp (Lys-93) and FtsLp (Lys-121) are located in the flexible C termini of the proteins and were not included in the models. We therefore used native mass spectrometry to determine the exact subunit stoichiometry in the FtsBpLp, FtsQpBpLp, and FtsQpBpLp complexes.
The native MS spectra are shown in Fig. 3, and the measured and theoretical masses are presented in Table 3. In the individually expressed samples, FtsQp and FtsBp were detected with masses of 26.9 and 10.3 kDa, both consistent with monomeric species lacking the N-terminal methionine residue. In the constrained FtsBpLp dimer, a 25.0-kDa species was observed that presumably represents the dimer lacking both N-terminal methionines. However, the two individual proteins, FtsQp and FtsBp, are still more abundant in the spectrum.

In the FtsQpBp sample, only monomeric FtsQp (26.9 kDa) and FtsBp (10.3 kDa) could be observed. These results may indicate that when just two proteins are co-purified, the resulting complex is either not formed or is prone to dissociation during sample handling required for mass spectrometric analysis. More interestingly, in the co-purified FtsQpBpLp sample, native MS gave rise to a multifaceted spectrum in which ions of the 1:1:1 stoichiometry of the subunits in the predominant globular complex. The minor peak at 3.4 S has a calculated mass of 123.1 kDa, perhaps representing dimers of the ternary complex in the main peak. All the constituent proteins appear to be in complex as there are no species sedimenting at the respective coefficients seen for the individual proteins (Fig. 4).

The mass for the major species in the FtsQpBpLp complex (2.3 S) is 65.1 kDa, perhaps representing a dimer of dimers. In contrast to the FtsQpBpLp sample, minor species of 9.9 kDa (0.7 S) and 33.9 kDa (1.5 S) are detected in the FtsQpBp sample, with similar sedimentation coefficients to those of FtsBpLp (0.7 S) and FtsQpLp (1.4 S) subunits when centrifuged alone. In the absence of FtsQp, FtsBp, and FtsLp, can form a heterodimer (1.3 S), as observed in MS, with another species at 2.1 S, representing a strained FtsBpLp dimer, a 25.0-kDa species was observed that presumably represents the dimer lacking both N-terminal methionine residues.

Theoretical and experimental masses of the proteins and protein complexes that were identified by native mass spectrometry are presented in Table 3. In the individually expressed samples, FtsQp and FtsBp were detected with masses of 26.9 and 10.3 kDa, both consistent with monomeric species lacking the N-terminal methionine residue. In the constrained FtsBpLp dimer, a 25.0-kDa species was observed that presumably represents the dimer lacking both N-terminal methionines. However, the two individual proteins, FtsQp and FtsBp, are still more abundant in the spectrum.

In the FtsQpBp sample, only monomeric FtsQp (26.9 kDa) and FtsBp (10.3 kDa) could be observed. These results may indicate that when just two proteins are co-purified, the resulting complex is either not formed or is prone to dissociation during sample handling required for mass spectrometric analysis. More interestingly, in the co-purified FtsQpBpLp sample, native MS gave rise to a multifaceted spectrum in which ions of the 1:1:1 stoichiometry of the subunits in the predominant globular complex. The minor peak at 3.4 S has a calculated mass of 123.1 kDa, perhaps representing dimers of the ternary complex in the main peak. All the constituent proteins appear to be in complex as there are no species sedimenting at the respective coefficients seen for the individual proteins (Fig. 4).

The values in parentheses are the \( s_{20,w} \) sedimentation coefficients, corrected for viscosity and density of the solvent, relative to that of water at 20 °C.

The mass for the major species in the FtsQpBpLp complex (2.3 S) is 65.1 kDa, perhaps representing a dimer of dimers. In contrast to the FtsQpBpLp sample, minor species of 9.9 kDa (0.7 S) and 33.9 kDa (1.5 S) are detected in the FtsQpBp sample, with similar sedimentation coefficients to those of FtsBpLp (0.7 S) and FtsQpLp (1.4 S) subunits when centrifuged alone. In the absence of FtsQp, FtsBp, and FtsLp, can form a heterodimer (1.3 S), as observed in MS, with another species at 2.1 S, representing a tetrameric species. Consistent with the native MS data presented above and the SPR data (see below), the combined AUC results indicate that the affinity of FtsQp is higher for FtsBpLp than for FtsBpLp alone.

**Biosensor Studies of FtsQBL Interactions**—Both the native MS and AUC data suggest a higher affinity interaction in the FtsQpBpLp complex than in the FtsQpBp complex. To determine the

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**TABLE 3**

| Sample | Species | Theoretical massa | Theoretical mass - methioninea | Experimental |
|--------|---------|-------------------|-------------------------------|--------------|
| FtsQp  | FtsQp   | 27,070.6          | 26,939.4                     | 26,943.02    |
| FtsBp  | FtsBp   | 10,431.2          | 10,300.0                     | 10,300.91    |
| FtsQpBp| FtsQpBp | 38,755.4          | 38,493.0                     | 38,500.78    |
| FtsBpLp| FtsBpLp | 51,552.9          | 51,159.3                     | 51,173.34    |
| FtsQpBp| FtsQpBp | 26,247.7          | 26,116.5                     | 26,118.80    |
| FtsBp  | FtsBp   | 26,247.7          | 26,116.5                     | 26,118.80    |
| FtsLp  | FtsLp   | 12,797.5          | 12,666.3                     | 12,666.60    |

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**TABLE 4**

| Sample | \( s_{20,w} \) | Massa |
|--------|-----------------|-------|
| FtsQp  | 1.4 (3.0)       | 31.4  |
| FtsBp  | 0.7 (1.8)       | 12.2  |
| FtsBpLp| 1.3 (2.8)       | 28.0  |
| FtsQpBp| 2.1 (5.5)       | 57.4  |
| FtsQpLp| 0.7 (1.4)       | 9.9   |
| FtsBpLp| 1.5 (3.2)       | 33.9  |
| FtsQpBp| 2.3 (5.0)       | 65.1  |
| FtsBp  | 1.9 (4.1)       | 51.0  |
| FtsLp  | 3.4 (7.4)       | 123.1 |

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* a Date were calculated from protein sequence with ProtParam webtool (50).

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**FIGURE 3.** Analysis of subunit stoichiometry in purified FtsQpBpLp (sub-)complexes by native mass spectrometry \((n = 1)\).
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![FIGURE 5. Immobilization strategy of FtsQp for biosensor analysis. To mimic the native membrane topology of FtsQ(4), Avi-FtsQp was site-specifically biotinylated and immobilized (IM) on a CMS sensor chip decorated with NeutrAvidin (NA) (8).](image)

interaction parameters in more detail, we performed SPR-based biosensor experiments using FtsQp tethered to the chip surface as ligand and FtsBp or FtsBpLp dimer as analyte in the flow solution. Importantly, not only the affinities, but also the kinetics and thermodynamics of binding can be analyzed by SPR.

In previous FtsQp labeling experiments, we observed that the affinity of FtsQp for FtsBp is diminished upon random chemical modification of its lysine residues.3 Therefore, we chose to introduce an Avi-tag at the N terminus of FtsQp to enzymatically biotinylate the protein for subsequent tethering to a CMS-sensorchip functionalized with NeutrAvidin. An added advantage of this strategy is the presumably uniform orientation of the protein, with the membrane-proximal domain near the chip surface and the interaction hot spots near the C terminus protruding toward the flow that contains its binding partners (Fig. 5). The results showed that FtsQp has an affinity of 70.3 ± 6.4 nM for FtsBP1p (pKD = 7.15 ± 0.04), whereas its affinity for FtsBP alone is 2 orders of magnitude lower at 22.3 ± 1.7 µM (pKD = 4.65 ± 0.04) (Table 5). This difference is consistent with AUC and native MS data that already suggested a weaker affinity of FtsBP for FtsQp in the absence of FtsBp (Figs. 3 and 4). In addition, we found that the kd of FtsBP1p was much lower than the kd of FtsBp.

### Discussion

To better understand the critical role of FtsQ, FtsB, and FtsL in bacterial cell division, it will be important to define the structural organization and stoichiometry of FtsQBL complexes. In this study, we have co-expressed the soluble periplasmic domains of E. coli FtsQ, FtsB, and FtsL (referred to as FtsQp, FtsBp, and FtsLp respectively) yielding a stable 1:1:1 trimeric complex with predominant interactions between the C-terminal regions of the respective proteins.

For complex formation, FtsBp and FtsLp had to be supplied with N-terminal coils of opposite charge to force their dimerization. Most likely, this compensates for the absence of the TM regions that are required for functioning of FtsB and FtsL and may contribute to their interaction (18, 39, 44). Dimerization appeared essential for stable expression of FtsLp, but FtsBp expression was not affected by the absence of FtsLp or FtsQp (this study) in contrast to full-length FtsB that showed a breakdown product just below FtsB when expressed without FtsL (18).

### Table 5

| Parameter          | FtsBp | FtsBpLp |
|--------------------|-------|---------|
| KD (µM)            | 22.3 ± 1.7 | 70.3 ± 6.4 |
| pKD               | 4.65 ± 0.04 | 7.15 ± 0.04 |
| kd (min)           | 3.0 ± 3 | 8.9 ± 1.2 |

Although the membrane-proximal leucine zipper motifs in E. coli FtsB and FtsL are required for optimal heterodimerization (19), they appear insufficient for stable complex formation in the absence of the TM regions consistent with the corresponding complex in S. pneumoniae (24). Vice versa, the interaction between the TMs was shown to be insufficient for FtsB-FtsL interaction (18), suggesting that both the TM and membrane-proximal domains contribute to this interaction. Although FtsQ enhances the interaction of FtsB and FtsL, FtsQp does not restore the interaction of FtsBp and FtsLp without the fused coils. Presumably, interactions between the TMs in combination with those in the periplasmic domains are needed to form a stable FtsQBL complex.

Purification of the FtsQpBpLp complex allowed the identification of juxtaposed lysine residues by BAMG cross-linking. In general, the data confirm and extend the interaction interfaces between the C termini of FtsQ and FtsB deduced from site-directed thiol and photo-cross-linking (13). FtsBp-Lys-93 was cross-linked to FtsQp-Lys-218, which is spatially adjacent to the

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3 M. Glas, A. Fish, J. J. P. de Esch, and J. Luijtenk, unpublished data.
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Recently, it has been proposed that FtsQBL may function as a conformational switch to derepress septal peptidoglycan synthesis by PBP3 and FtsW and couple it to FtsA-mediated contraction of the Z-ring at the cytoplasmic side of the inner membrane (21, 23). It is tempting to speculate that the conformational flexibility in the C termini of FtsB and FtsL is relevant to this interaction given the relatively modest affinity and the low cellular abundance of the endogenous full-length versions. However, the corresponding full-length proteins are confined, hence concentrated, in the inner membrane. Irrespective of this consideration, FtsLp clearly failed to co-purify with FtsQp and had a strong tendency to aggregate upon separate expression (Fig. 1B, lanes 3, 9, and 18). It has been shown previously that FtsB and FtsL do not form a subcomplex when FtsQ is depleted from cells, whereas FtsB and FtsL associate independent of localization to the septum (i.e. in the absence of FtsK) provided that FtsQ is expressed (3). Combined, these data indicated that within the FtsQBL complex a hierarchical assembly order exists in which FtsQ interacts first with low affinity to FtsB. This may alter the conformation of FtsB and potentially also FtsQ to increase the affinity for FtsL that subsequently binds to both FtsQ and FtsB through interactions that are focused at the C terminus of the subunits. Of note, the FtsQpBp complex cannot interact with FtsLp as such but requires an artificial dimerization strategy to pre-associate it with FtsBp, a role that in the full-length proteins may be fulfilled by the TM regions and adjacent leucine zipper domains. As a result of the FtsLp association, the interaction between FtsQp and FtsBp appears to become more robust as demonstrated by the native MS experiments. FtsQp subcomplexes are detected in the FtsQpBpLp sample but not in the FtsQpBp sample itself, suggesting that FtsQpBp is a short-lived intermediate in the assembly process. Consistently, the AUC and BAMG data indicate that the FtsQpBp complex has a tendency to form higher order structures.

In conclusion, our data indicate that the periplasmic domains of FtsQ, FtsB, and FtsL form a 1:1:1 complex for which the TM segments are not strictly required. The ease of purification of the FtsQpBpLp complex will accelerate the elucidation of structural features of the complex. Attempts will be made to extend the complex with soluble domains of upstream and downstream divisome components, in particular FtsN. Finally, the expression and interaction data presented here will facilitate the development of inhibitors that block formation of this critical divisome assembly both through structure-based design and high throughput protein interaction assays.
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Author Contributions—M. G. was involved in all experiments and preparation of the manuscript. H. B. v.d. B. v. S. designed and constructed vectors for expression of mutant proteins. S. H. M. designed, performed, and analyzed the experiments shown in Fig. 4 and Table 4. L. d. J. and W. R. designed, performed, and analyzed the experiments shown in Fig. 2. Table 2, and supplemental Table S1. A. J. R. H. and F. L. designed, performed, and analyzed the experiments shown in Fig. 3 and Table 3. G. M. K. provided technical assistance. A. F. performed initial analysis on subunit stoichiometry. T. d. B. contributed to experimental design and provided critical advice. W. B., I. J. P. d. E., and J. L. conceived and coordinated the study. All authors reviewed the results and approved the manuscript.

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