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Structural analysis of the interaction between the bacterial

cell division proteins FtsQ and FtsB

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23

24 **Abstract**

25 Most bacteria and archaea use similar proteins within their cell division

26 machinery, which uses the tubulin homologue FtsZ as its central organiser. In

27 Gram-negative *Escherichia coli* bacteria, FtsZ recruits cytosolic, transmembrane,

28 periplasmic and outer membrane proteins, assembling the divisome that

29 facilitates bacterial cell division. One such divisome component, FtsQ, a bitopic

30 membrane protein with a globular domain in the periplasm, has been shown to

31 interact with many other divisome proteins. Despite its otherwise unknown

32 function, it has been shown to be a major divisome interaction hub. Here, we

33 investigated the interactions of FtsQ with FtsB and FtsL, two small bitopic

34 membrane proteins that act immediately downstream of FtsQ. In biochemical

35 assays we show that the periplasmic domains of *E. coli* FtsB and FtsL interact

36 with FtsQ, but not with each other. Our crystal structure of FtsB bound to the β

37 domain of FtsQ shows that only residues 64-87 of FtsB interact with FtsQ. A

38 synthetic peptide comprising those 24 FtsB residues recapitulates the FtsQ:FtsB

39 interactions. Protein deletions and structure-guided mutant analyses validate

40 the structure. Furthermore, the same structure-guided mutants show cell

41 division defects *in vivo* that are consistent with our structure of the FtsQ:FtsB

42 complex that shows their interactions as they occur during cell division. Our

43 work provides intricate details of the interactions within the divisome and also

44 provides a tantalising view of a highly conserved protein interaction in the

45 periplasm of bacteria that is an excellent target for cell division inhibitor

46 searches.

47

48   **Importance**

49   Cells in most bacteria and archaea divide through a cell division process that is

50   characterised through its filamentous organiser, FtsZ protein. FtsZ forms a ring

51   structure at the division site and starts the recruitment of 10-20 downstream

52   proteins that together form an elusive multi-protein complex termed divisome.

53   The divisome is thought to facilitate many of the steps required to make two cells

54   out of one. FtsQ and FtsB are part of the divisome, with FtsQ being a central hub,

55   interacting with most of the other divisome components. Here we show for the

56   first time how FtsQ interacts with its downstream partner FtsB and show that

57   mutations that disturb the interface between the two proteins effectively inhibit

58   cell division.

59

Introduction

The divisome is a macromolecular complex formed by at least 12 essential proteins and even more non-essential proteins. It effects bacterial cell division through a number of processes, including cell constriction, synthesis of the septal peptidoglycan (PG) wall, and ultimately cell separation (1–5). In *Escherichia coli*, divisome assembly starts with formation of a ring structure localised at midcell, containing the bacterial tubulin-homologue FtsZ in the cytoplasm (6, 7) and anchoring of the ring in the inner membrane by FtsA and ZipA (8, 9). This is followed by recruitment of further cell division proteins FtsEX, FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI, and FtsN in order of their localisation inter-dependence, all of them trans-membrane proteins. The functions of several of these divisome proteins have been deduced, such as the role of FtsEX in transmembrane regulation of septal PG hydrolytic enzymes (10), FtsK's role in XerCD-mediated chromosome decatenation (11) and FtsW/PBP1b/FtsI's proposed role as hybrid septal peptidoglycan synthase with transglycosylase and transpeptidase activities, respectively (12). FtsN has a particularly interesting role as the trigger for septal peptidoglycan synthesis, depending on the assembly of the entire complex, somehow affecting FtsA on the cytoplasmic side of the cell membrane directly as a feedback or even checkpoint mechanism (13). FtsQLB, FtsI and FtsN have only single transmembrane helices anchoring them in the membrane (bitopic membrane proteins) in addition to globular periplasmic domains, where they act on/react to PG synthesis.

FtsQ is considered to play a central, yet enigmatic role in assembly of the divisome through a multitude of interactions as no enzymatic function is known

84   for this protein (14). Two-hybrid analyses have suggested that FtsQ interacts

85   with ~10 cell division proteins of which the interactions with FtsB and FtsL were

86   confirmed biochemically (15). The FtsQBL complex may form independently

87   before its recruitment to midcell by FtsK, where it interacts with the later

88   divisome proteins needed for cell division (16).

89   FtsQ is a particularly attractive target for the development of inhibitors of

90   protein-protein interactions (PPIs) that block bacterial division because (i) it is

91   an essential protein of low abundance (~50-250 copies per cell) (17) (ii) it has

92   multiple interactions in the much more easily accessible periplasm (14, 16), and

93   (iii) it has no obvious eukaryotic homologues (18). Previously, the structure of

94   the periplasmic domain of FtsQ from *E. coli* has been solved by X-ray

95   crystallography (19) and shown to consist of two subdomains, named α and β.

96   Together with the trans-membrane domain (TMD, a single bitopic helix in FtsQ),

97   the α domain is believed to be required for recruitment by FtsK although other

98   interactions have been ascribed to this domain as well (19, 20). The α domain is

99   located directly downstream from the TMD in the sequence of FtsQ and

100   corresponds to the more broadly distributed POTRA domains that have been

101   implicated in transient PPIs in a range of transporter proteins (21). The β

102   domain has been implicated in multiple interactions including those with FtsB

103   and FtsL (19).

104   FtsB and FtsL are small bitopic inner membrane proteins (103 and 121 residues

105   in *E. coli*, respectively). FtsB and FtsL have also been suggested to form a distinct

106   subcomplex prior to localisation to the septum making a strictly sequential

107   recruitment less likely. Both proteins may contain a leucine-zipper motif in their

108   periplasmic domain that together with the TMDs have prompted suggestions

109   that FtsB and FtsL form a heterodimeric or tetrameric complex (22, 23). Recently,

110   an *in vivo* scanning photo cross-linking approach to map interactions of FtsQ

111   with FtsB and FtsL at the amino acid level has been conducted, considering one

112   fifth of all surface exposed residues (24). Two hotspots for the interactions with

113   FtsBL were identified on FtsQ: one in the α domain close to the membrane

114   around residue FtsQ R75, primarily with FtsL, and a more pronounced hotspot in

115   the conserved distal part of the β domain around residue FtsQ Y248, primarily

116   interacting with FtsB. In addition, it was previously shown with purified proteins

117   *in vitro* that artificially dimerised FtsB and FtsL, replacing their TMDs with a

118   heterodimeric coiled coil fragment, bind to the periplasmic portion of FtsQ (25).

119   In those experiments, the periplasmic domain of *E. coli* FtsQ was co-purified with

120   the heterodimerised *E. coli* FtsB and FtsL constructs as a stable trimeric complex.

121   FtsB was also shown to interact with FtsQ in the absence of FtsL, whereas FtsL

122   on its own failed to co-purify with FtsQ.

123   Here we present further quantitative biochemical investigations of the

124   periplasmic interaction between FtsQ, B, L and FtsN, showing that the FtsQB

125   complex is formed with a sub-micromolar dissociation constant as determined

126   by surface plasmon resonance (SPR). SPR does not indicate interactions between

127   FtsB and FtsL but shows a trimeric FtsQLB complex. FtsN interacts

128   independently of FtsB with FtsQ, whereas the interaction of FtsQ with FtsL is not

129   additive with FtsB. In line with these findings, we present a crystal structure of

130   the periplasmic domain of *E. coli* FtsQ in complex with the periplasmic domain of

131   *E. coli* FtsB. The structure resolves residues 64-86 of FtsB that form an α helix

132   and a β-strand, linked by a loop. The region in FtsQ that interacts with FtsB has

133   Y248 at its centre and is at the membrane-distal end of the protein, which is in

134   agreement with the previous crosslink data (24). Mutational analysis coupled

135   with cellular microscopy and also SPR confirmed residues in the interacting

136   region highlighted by the crystal structure that are critical for binding of FtsB to

137   FtsQ, and consequently for functioning of these proteins in cell division.

138

139   **Results**

140   **The periplasmic domain of FtsB binds to FtsQ, but not FtsL**

141   To understand the periplasmic interactions of FtsB with FtsQ and FtsL, we

142   performed surface plasmon resonance (SPR) experiments with purified protein

143   domains (Figure 1A & C). The periplasmic domain of *E. coli* FtsB comprising

144   residues 22-103 binds strongly to *E. coli* FtsQ (residues 58-276) immobilised on

145   the SPR chip, with a major K_d of 0.8 μM. Equally, the periplasmic domain of FtsL

146   binds to FtsQ, however in this experiment FtsL was immobilised and the binding

147   and release were so fast that parameters could not be determined reliably.

148   Surprisingly, when FtsB was added to immobilised FtsL, no binding could be

149   detected. To investigate this further, FtsB and FtsQ were added together to

150   immobilised FtsL, resulting in measurable binding with a K_d of 11 μM. The lack of

151   binding of FtsL alone to FtsB is surprising because it has been proposed that FtsB

152   and FtsL form a coiled coil/leucine zipper complex with each other and when

153   binding to FtsQ (22, 23). When analysing the propensity of FtsL and FtsB to form

154   coiled coils with COILS (Figure 1D) (26), only *E. coli* FtsB showed significant

155   coiled coil content between residues 29-70 (or 77). 2ZIP leucine zipper

156   predictions (27) were also negative for FtsL but positive for FtsB (not shown).

157   We think given these data it is unlikely that FtsB and FtsL form a 1:1 coiled

158   coil/leucine zipper complex and we could detect no binding of their periplasmic

159   domains biochemically. However, FtsQ and FtsB together do bind to FtsL and the

160   binding is not additive in the sense that if FtsL and FtsB bound independently to

161   FtsQ, the resulting binding would be stronger than their individual binding,

162   which is not the case. To test this further, we investigated the binding of the

163   periplasmic domain of FtsN to immobilised FtsQ as a control (Figure 1B & C). *E.*

164   *coli* FtsN comprising residues 57-319 was bound to immobilised FtsQ using SPR

165   and it bound with a K_d of 2.5 µM. When FtsB and FtsN were bound to FtsQ

166   together, a tighter K_d of 0.12 µM resulted, and the resulting binding could be

167   simulated by assuming additive, that means independent binding of FtsB and

168   FtsN, as shown by the calculated plot in Figure 1B. We conclude that FtsB and

169   FtsL alone bind well to FtsQ, and as a complex FtsB and FtsL bind to FtsQ

170   dependent on each other, not binding to their own, independent binding sites on

171   the surface of FtsQ and/or inducing a conformational change in FtsQ. In contrast,

172   the binding of FtsN and FtsB to FtsQ is independent of each other hence additive.

173   Surprisingly, the periplasmic, soluble domains of FtsB and FtsL do not bind to

174   each other in isolation and only FtsB is predicted to form a coiled coil,

175   presumably with itself.

176   **Crystal structure of the periplasmic FtsQ:FtsB complex**

177   Inspired by the SPR results, we purified the complex between the periplasmic

178   domains of *E. coli* FtsB (residues 22-103) and FtsQ (residues 58-276). The final

179   size exclusion chromatogram of the purification is shown in Figure 2A, showing

180   two peaks. Both peaks yielded the same crystals and peak A, of unexpected high

181   apparent molecular weight is presumed to be a dynamic oligomeric state of the

182   complex. Both peaks A and B elute as a defined complex with 1:1 stoichiometry

183   (Figure 2B). We obtained tetragonal crystals of the complex and solved the X-ray

184   crystal structure to 2.6 Å resolution by molecular replacement with a previous *E.*

185   *coli* FtsQ structure (PDB ID 2VH1, Table 1) (19). The resulting structure shows

186   the previously reported two-domain architecture of FtsQ (α and β domains)

187   largely unchanged. FtsB binds to the C-terminal β domain of FtsQ, presumably

188   furthest away from the membrane (Figure 2C & 5B). Only a C-terminal portion of

189   FtsB was resolved in the crystals, comprising residues 64 to 87 (22-103 were

190   present). FtsB forms a helix, followed by a linker largely in β-strand

191   conformation, leading to the final β-strand that extends the central β-sheet of the

192   FtsQ β domain by binding to its last β-strand (Figure 2C). Analysis of the binding

193   mode of FtsB to FtsQ reveals a number of key interactions that are depicted in

194   the stereographic Figure 2D. The only helix resolved in FtsB, approximately

195   residues 65-75, makes a number of charged interactions with the rest of FtsB

196   and FtsQ, most notably two salt bridges that may be involved in stabilising FtsB's

197   conformation (E68-R79 and R72-E82). Both salt bridges are also involved in

198   binding to FtsQ via D245 and R247. Further charged or polar interactions with

199   FtsQ are mediated by FtsB E65, E69 and N73 (not highlighted in Figure 2D). In

200   the crystals, the resolved part of FtsB forms a tight dimer, the significance of

201   which we have not investigated (Figure 2E). Figure 2E also shows the phased

202   anomalous difference density from a SAD experiment with selenomethionine

203   substituted M77, validating the model building, in order to produce absolute

204   certainty for the chain trace since the resolved FtsB domain is small.

205   **FtsB(64-87) is necessary and sufficient for the FtsQ:FtsB interaction**

206   First, we aimed at an *in vitro* validation of the crystal structure by investigating

207   structure-guided mutant FtsB proteins and their binding to FtsQ (Figure 3A & C).

208   We chose FtsB R72A that forms a salt bridge within FtsB with E82 and is in

209   direct contact with F84A that is positioned next to Y248 on the surface of FtsQ

210   (Figure 2D). Both mutant FtsB proteins showed approximately ten-fold reduced

211   binding to immobilised FtsQ. Even more convincingly, FtsQ Y248W, a somewhat

212   conservative mutation, showed no binding whatsoever when FtsB was tested.

213   FtsQ Y248 (with A253) forms the central hydrophobic patch that FtsB latches

214   onto (Figure 2D). All mutants tested confirmed the binding mode of FtsB to FtsQ

215   as shown by the crystal structure of the complex (Figure 2) and this is further

216   supported by an analysis of sequence conservation across FtsB homologues as

217   depicted in Suppl. Figure S1 that shows strong conservation for residues shown

218   in the crystal structure to interact with FtsQ.

219   Resolving only residues 64-87 in the crystal structure of the FtsQ:FtsB complex

220   raised the question of what the remaining residues present during crystallisation

221   do. For this we went back to SPR and tested two FtsB subdomains comprising

222   residues 22-64 and 64-87, chemically synthesised as peptides (Suppl. Figure S2,

223   replacing methionine 77 with norleucine). SPR analysis unequivocally showed

224   that the region within FtsB N-terminal to the structure, residues 22-64, does not

225   bind to FtsB, whereas the region containing only the ordered parts of the

226   structure, residues 64-88, produced binding curves and binding parameters very

227   similar to the original periplasmic 22-103 construct (Figure 3B & C). The binding

228   of the 64-87 peptide was further investigated by fluorescence polarisation, a

229   solution assay. For this, FITC-labelled FtsB 64-87 peptide was synthesised (Suppl.

230   Figure S2) and the decrease in polarisation measured while FtsQ was added

231   (Figure 3D). The resulting K_d of $\sim$ 9 μM is less tight than what we measured by

232   SPR and since identical reagents were used, gives an impression of the variations

233   caused by using different assay technologies, but confirms in principle that FtsB

234   64-87 binds FtsQ in solution. To further show this we crystallised an acetylated

235   version of this peptide with FtsQ, resulting in essentially the same structure as

236   observed for FtsB 22-103, despite being in a different crystallographic space

237   group (Figure 3E, Table 1). It is noteworthy that the synthesised FtsB 64-87

238   peptide is highly soluble in water, at least to 10 mM.

239   As already mentioned, FtsB binding to FtsQ involves only few and minor changes

240   to the conformation of FtsQ when compared to a previous unbound structure of

241   FtsQ (PDB ID 2VH1, Figure 3F) (19). Two exceptions are that FtsQ Y248 and

242   W256 change their side chain conformations significantly upon binding, which

243   fits well with our data showing that FtsQ Y248 is absolutely critical for the

244   interaction with FtsB. The tyrosine side chain of Y248 is protruding from the

245   main structure whereas upon interaction with FtsB it shifts deeply inward

246   providing aromatic stacking with FtsB F84 and potential hydrogen bonding to

247   FtsB R72.

248   We conclude that binding of the periplasmic domain of FtsB to FtsQ, in absence

249   of FtsL, only involves FtsB residues 64-87 as shown by the structure and this

250   interaction can be faithfully reconstituted by using synthesised and water-

251   soluble peptides comprising FtsB residues 64-87.

252   **The FtsQ:FtsB interaction in the context of bacterial cell division**

253   Based on the FtsQ:FtsB structure (Figure 2), we investigated *E. coli* cells

254   harbouring FtsQ and FtsB mutant proteins in order to validate the structure and

255   to understand the contributions of various parts of the interface to the ability to

256   progress cell division. Functioning of the mutants was tested by low-level un-

257   induced expression in *E. coli* strain LMC531, an *ftsQ* temperature-sensitive

258   mutant. Cells grown at the permissive temperature were imaged by phase

259   contrast microscopy (Figure 4A) or spotted on solid medium (Suppl. Figure S3A),

260   followed by incubation at the permissive and non-permissive temperatures.

261   Under these conditions, phase contrast microscopy of LMC531 cells harbouring

262   the empty vector (EV) showed filamentation upon growth in liquid LB medium

263   at the non-permissive temperature whereas positive control cells harbouring a

264   tagged but otherwise wild-type FtsQ construct showed normal cells also at the

265   non-permissive temperature (Figure 4A). Equally, in the spot assay, the negative

266   control cells harbouring the empty vector only grew to high dilutions at the

267   permissive temperature, whereas the positive control showed good growth at

268   high dilution at the non-permissive temperature (Suppl. Figure S3A).

269   Using both assays, mutant FtsQ Y248F showed full complementation, indicating

270   that the hydroxyl moiety in the tyrosine side chain is not required for functioning.

271   In contrast, FtsQ Y248K and even a Y248W substitution were unable to grow on

272   plates at the non-permissive temperature and showed a strongly filamentous

273   phenotype using microscopy at the non-permissive, and even at the permissive

274   temperature, indicating a dominant negative effect on cell division. We also

275   investigated FtsQ D245N and R247Q, conservative mutations probing the role of

276   the polar interactions FtsB is making with FtsQ towards its N-terminal part of

277   the binding region, and these showed no effects. Equally, FtsQ mutants S250A,

278   G251A and W256A showed no obvious effects in the spot assay, ruling out their

279   significance for the FtsB interaction (Suppl. Figure S3A). In order to demonstrate

280   that phenotypes observed were not due to reduced protein levels of the mutated

281   proteins, we performed Western blotting (Suppl. Figure S3B). Given the central

282   role of Y248 and its very strong and selective phenotypes depending on the

283   replacing residue types we also investigated the localisation of a fluorescently

284   labelled version of FtsQ Y248W. This mutant protein showed strong cell division

285   inhibition in both assays but localised normally in cells, being recruited correctly

286   to division sites (Suppl. Figure S4), indicating that only downstream divisome

287   interactions were affected, as predicted.

288   The central role of FtsQ Y248 in the interaction interface suggests that specific

289   mutations at this position are not tolerated because they affect the interaction

290   with FtsB. If this is the case, complementary mutations in this area of the FtsQB

291   complex in FtsB should have a similar effect. To examine this, structure-guided

292   FtsB mutant proteins were tested for functionality by low-level background

293   expression in a conditional *E. coli* FtsB mutant strain NB946 in which the

294   chromosomal *ftsB* gene is under control of an arabinose promoter. Depletion of

295   FtsB occurs when 0.2% L-arabinose is replaced by 0.2% L-glucose in the growth

296   medium leading to filamentation, and eventually cell death, due to the essential

297   nature of FtsB as observed by phase contrast microscopy and a spot assay

298   (empty vector 'EV' negative control, Figure 4B; Suppl. Figure S5). It also

299   confirmed that an HA-tagged FtsB construct that was used as basis for the

300   mutagenesis was able to complement growth and proper cell division in cells

301   grown in the absence of the inducer L-arabinose in contrast to NB946 cells

302   harbouring the empty vector (Figure 4B; Suppl. Figure S5). Mutations were

303   introduced changing conserved residues within the FtsB domain binding to FtsQ

304   and analysed using the microscopy and spot assay described above for FtsQ

305   mutants. FtsB F84A, also compromised in the SPR assay (Figure 3A & C),

306   appeared non-functional, although cell filamentation was relatively mild,

307   suggesting that the aromatic interaction with FtsQ Y248 is critical for FtsB

308   functioning. In agreement with this supposition, changing F84 into tryptophan

309   did not affect FtsB functioning. Y85A was also non-functional with a rather

310   strong filamentation phenotype that was even more pronounced and dominant

311   in a (F84A, Y85A) double mutant (spot assay only, Suppl. Figure S5).

312   The loop between the α-helix and β-strand of the FtsB domain that is resolved in

313   the FtsQB complex structure is connected via two salt bridges, R72-E82 and E68-

314   R79 (Figure 2D). In agreement with the SPR data (Figure 3A & C), both R72A and

315   E82A did not complement FtsB depletion and showed a strong filamentation

316   phenotype suggesting that this salt bridge is essential for interaction or possibly

317   also shaping FtsB into the correct fold for binding. In contrast, FtsB E68A and

318   R79A appeared fully functional, suggesting this second salt bridge is largely

319   dispensable. These findings are further supported by Suppl. Figure S1, which

320   shows that the R72-E82 salt bridge is more conserved than E68-R79. Similarly,

321   FtsB E65 and E69, potentially interacting with FtsQ R196, could be changed into

322   alanine without functional consequences (spot assay only, Suppl. Figure S5).

323   Because of the very low amount of FtsB in cells and the low protein amounts

324   needed to complement in our assays, we have been unable to test protein levels

325   in cells (Figure 4B and Suppl. Figure S5) by Western blotting reliably.

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328

329   **Discussion**

330   Our investigation of the interactions within the FtsQLB complex produced two

331   surprises. It has been proposed that FtsL and FtsB form a coiled coil/leucine

332   zipper heterodimer that binds to FtsQ (22, 23). We show here that at least the

333   periplasmic domains of *E. coli* FtsL and *E. coli* FtsB do not interact, but both

334   proteins bind well to FtsQ, forming a heterotrimeric complex. This has been

335   reported before based on pull-down experiments (25). Based on these data and

336   the lack of coiled coil/leucine zipper prediction for the periplasmic domain of

337   FtsL, we suggest that FtsL binds to both FtsB and FtsQ in the complex, at least in

338   *E. coli*. It is worth noting that FtsL proteins from other organisms do show weak

339   coiled coil/leucine zipper predictions and FtsL from *Bacillus subtilis* shows

340   strong prediction. It is conceivable that different interaction modes exist

341   between these small proteins.

342   The second surprise was that the structure of the complex between the

343   periplasmic domains of *E. coli* FtsB and FtsQ contained FtsB residues 64-87 only.

344   We showed that this subdomain is indeed necessary for the interaction but is

345   also sufficient, suggesting that the remaining residues within FtsB are disordered

346   in the crystals and do not bind to FtsQ.

347   Overall, the interaction interface between FtsQ and FtsB observed here in the

348   structure (Figure 2) corresponds to the main interaction site that was identified

349   by *in vivo* site-specific photo crosslinking (24). In that study, 50 surface-exposed

350   positions in the periplasmic domain of *E. coli* FtsQ were changed into Bpa using

351   amber suppressor technology for photon-induced crosslinking of FtsQ with its

352   binding partners. Using this scanning approach, the strongest crosslinking to

353   FtsB was observed at Y248Bpa and S250Bpa. Moreover, paired cysteine

354   mutagenesis enabled the formation of a disulfide bond between FtsQ S250C and

355   FtsB V88C. Both findings are in line with our structure. Although FtsB 88V is not

356   included in the structure, the last resolved residue, L87, is immediately

357   juxtaposed to FtsQ S250 and Y248 in FtsQ is the central residue of the

358   hydrophobic patch on FtsQ that binds FtsB. Similarly, functional analysis of the

359   FtsQ Bpa mutants showed that they all complemented the function of FtsQ in an

360   FtsQ Ts mutant grown at the non-permissive temperature, except for Y248Bpa,

361   highlighting again the crucial nature of this position that does not allow even

362   small alterations. It is also notable that the orientation of the Y248 side chain is

363   different in the unbound FtsQ (PDB ID 2VH1) (19) as compared with the

364   FtsQ:FtsB complex described here (Figure 3F).

365   Of note, FtsQ S250 and Y248 are part of a conserved region in the interaction

366   interface with FtsB that stretches from Y243 to W256 (compare Figure 2C & D

367   with Figure 5B). Single substitutions in this motif, D245N, R247Q, G251A,

368   W256A (this study), G255C and S250C (24) did not affect functioning of FtsQ

369   consistent with the observed tolerance of FtsQ towards Bpa substitutions. In an

370   independent study, FtsQ amino acids 257 to 276 were shown to be dispensable

371   for function whereas shorter truncations were less stable, possibly because FtsB

372   and/or FtsL were not recruited (28).

373   In the FtsQ:FtsB structure, FtsB Y85 is oriented towards FtsQ L226, a position

374   that was shown to crosslink very strongly to FtsB previously (24). In accordance

375   with these data, it has been shown that FtsB truncated at Y85 is unable to

376   complement and interact with FtsQ whereas FtsB truncated at D90 is fully

377   functional (29), and this is also supported by our data showing that FtsB 64-88

378   binds as well as the entire periplasmic domain comprising residues 22-103

379   (Figure 3B & C).

380   The three residues upstream of FtsB R79 have been changed individually into

381   cysteine (S76C, M77C and T78C) in a previous study (24) without any effect on

382   FtsB functioning, arguing that the precise sequence of the loop that connects the

383   FtsB α-helix and β-strand is not very relevant for FtsB functioning.

384   Finally, we would like to speculate on the nature of the FtsQ:B complex when in

385   the membrane and with FtsL and N. Here, we found that FtsQ and FtsB together

386   form molecular species bigger than a 1:1 complex (Figure 2A & B). In order to

387   understand this better, we also performed size exclusion chromatography with

388   multiple angle light scattering (SEC-MALS) and analytical ultracentrifugation

389   (AUC, Figure 5A, Suppl. Figure S6A). Size estimates from those experiments for

390   FtsB, FtsQ and FtsQ:B are summarised in Suppl. Figure S6B. FtsB alone occurred

391   in several species, including some high molecular weight oligomers. FtsQ was

392   almost exclusively monomeric and the FtsQ:FtsB complex showed two species,

393   most likely corresponding to 1:1 and 2:2 complexes. Taken this into account and

394   the previous structure of the coiled coil segment of FtsB comprising residues 28-

395   60 (PDB ID 4IFF) (30), a hybrid model of the entire periplasmic FtsQ:FtsB

396   complex can be assembled (Figure 5B). In this model, the FtsB coiled coil

397   segment, disordered in our crystal structure, forms a homodimer, as observed in

398   the 4IFF crystal structure (that, admittedly, was artificially dimerised by fusing it

399   to a coiled coil dimerisation domain) (30). FtsQ only binds to the C-terminal part

400   of FtsB, containing residues 64-87 as demonstrated here by the structure and

401   other experiments. Because FtsB is a dimer, this means that the complex will

402   recruit two FtsQ molecules, explaining the observed 2:2 stoichiometry and a

403   measured molecular mass of 68-69 kDa. The model also predicts that the

404   transmembrane segments of FtsB are dimerised or in very close proximity, in

405   contrast to those of the two FtsQ molecules, that could be further apart. As

406   shown, FtsL binds together with FtsB to FtsQ, most likely binding to surfaces on

407   FtsB and FtsQ simultaneously. It is not clear from looking at sequence

408   conservation on the FtsQ surface where those binding surfaces are located since

409   the FtsB binding covers almost perfectly a highly conserved patch on FtsQ

410   (Figure 5B, blue patch), although we would like to speculate that FtsL might bind

411   to the second interaction hotspot on FtsQ around residue FtsQ R75 (24). Equally,

412   FtsN binding, demonstrated here biochemically, involves binding to FtsQ

413   independently of FtsB and the location of that binding site is currently unknown.

414   With our crystal structure we conclude that it is a valid representation of the

415   complex formed during cell division and relates to previous data regarding FtsQ

416   and FtsB. The complex represents an exciting target for structure-based design

417   of protein interaction inhibitors shutting down cell division based on peptides or

418   peptide mimetics as the FtsQ:FtsB interaction surface is small, the 64-87 FtsB

419   peptide is water soluble and the interaction is easily interrupted by single

420   mutations. In future, it may be possible to biochemically add more components

421   of the divisome as we report here direct interactions of FtsQ also with FtsL and

422   FtsN, and to investigate the structure and function of divisome subcomplexes in

423   the current absence of any reports of isolated or over-expressed divisome holo

424   complexes, with only one study showing high-molecular weight divisome

425   complexes by Western blotting (31).

426 **Materials and Methods**

427 *Surface plasmon resonance (SPR)*

428 SPR was performed using a Biacore T200 instrument using CM5-sensor chips

429 (GE Healthcare). Both reference control and analyte channels were equilibrated

430 in HBS-N buffer (0.01 M Hepes pH 7.4, 0.15 M NaCl) at 20 °C. FtsQ (58-276) or

431 FtsL (58-121) were immobilised onto chip surfaces through amide coupling

432 using the supplied kit to reach RU values (resonance units) of ~1000 and 3300,

433 respectively.

434 Triplicate SPR runs were performed in 10 mM Hepes, pH 7.4, 150 mM NaCl,

435 0.4 % (w/v) glycerol with analytes injected for 120 s followed by a 600 s

436 dissociation in 1:2 dilution series with initial concentrations of FtsB (22-103),

437 FtsB (64-88) of 80 µM; FtsB (22-64) of 200 µM; FtsB-F84A and FtsB-R72A of 68

438 µM; FtsN (58-320) and FtsN (58-320) + FtsB (22-103) of 40 µM each for

439 experiments where FtsQ was the ligand. For runs where FtsL (58-121) was

440 attached to the chip surface, initial concentrations of FtsQ (58-276) and FtsB

441 (22-103) were 40 µM each either alone or in combination. The surface was

442 regenerated with 10 mM HCl for 60 s. After reference and buffer signal

443 correction, sensogram data were fitted using KaleidaGraph (Synergy Software)

444 and Prism (GraphPad Software).

445 *Cloning, protein expression and purification*

446 FtsQ (58-276) and FtsN (57-319) from *Escherichia coli* strain K12 (FtsQ:

447 FTSQ_ECOLI; FtsN: FTSN_ECOLI) were amplified using PCR from genomic DNA,

448 cloned into the NdeI/BamHI sites of plasmid pHis17 and expressed as a C-

449 terminal His$_6$-tag in *E. coli* (see Table 1 for exact protein sequences).

450   FtsB (22-103) and FtsL (58-121) from *E. coli* (FtsB: FTSB_ECOLI; FtsL:

451   FTSL_ECOLI) were amplified using PCR from genomic DNA, cloned into the

452   NdeI/BamHI sites of plasmid pET15b and expressed as a N-terminal His_6-tag in *E.*

453   *coli* (see Table 1 for exact protein sequences).

454   Recombinant proteins were expressed in *E. coli* C41(DE3) cells, which were

455   grown in 2xTY media with ampicillin (100 mg/L) at 37 °C with 200 rpm shaking

456   to an OD_{600} of 0.6. Cultures were then shifted to 20 °C and induced by the

457   addition of 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (*IPTG),* before

458   overnight incubation. Selenomethionine-substituted FtsB (22-103) cell cultures

459   were grown to early log phase (A_{600} = 0.6) at 37° C in M9 minimal medium,

460   supplemented with 0.4 % glucose, 2 mM $MgSO_4$. 100 mg/L of DL-

461   selenomethionine (Generon), 100 mg/L of lysine, threonine, phenylalanine and

462   50 mg/l of leucine, isoleucine and valine were added as solids. Fifteen minutes

463   later, protein expression was induced with 0.5 mM IPTG and grown overnight at

464   20 ^{0}C.

465   *Protein purification*

466   For crystallisation experiments, bacterial cell pellets containing over-expressed

467   FtsQ (58-276) and FtsB (22-103) were mixed and resuspended in 50 mM Tris,

468   150 mM NaCl, 10 % glycerol, pH 8.0. Cell lysis was carried out at 25 kPSI using a

469   cell disruptor (Constant Systems) and the lysate was clarified by centrifugation

470   at 20,000 rpm for 30 min at 4° C. The supernatant was passed over a HisTrap™

471   HP column (GE Healthcare). The column was equilibrated with 25 mM Tris, 150

472   mM NaCl, 10 % glycerol, 0.5 mM DTT, pH 8.0. Proteins were eluted with 300 mM

473   imidazole in the same buffer. Peak fractions were concentrated and loaded onto

474   a HiLoad Sephacryl S300 16/60 column (GE HEalthcare) equilibrated in 25 mM

475   Tris, 100 mM NaCl, 2 mM DTT, pH 7.4. Purified FtsQB complex was concentrated

476   to 6 mg/mL using centrifugal concentrators (Vivaspin, Sartorius) for immediate

477   use. For SPR and SEC-MALS assays, FtsB, FtsL, FtsQ and FtsN proteins were

478   purified separately using the same protocol, then concentrated to 5-10 mg/mL

479   before freezing in liquid nitrogen and storage at -80°C.

480   *Crystallisation,* data collection and structure determination

481   Crystallisation conditions were found using our in house high-throughput

482   crystallisation platform (32), by mixing 200 nL *SeMet*-substituted FtsBQ or wild

483   type FtsQB solution at 6 mg/mL, with 200 nL of 1920 different crystallisation

484   reagents in MRC vapour diffusion sitting drop plates. Crystals were grown at

485   19 °C by vapour diffusion in 0.15 M potassium thiocyanate, 20 % PEG 550 MME,

486   0.1 M sodium cacodylate pH 6.5.

487   For the FtsQ (58-276)/FtsB (64-88 peptide) complex crystallisation, FtsB

488   synthetic peptide was added to 4 mg/mL FtsQ protein solution with molar ratio

489   of 1 FtsQ:4 FtsB. Crystals were grown at 19 °C by vapour diffusion in 0.057 M

490   potassium thiocyanate, 10 % PEG 550 MME, 0.1 M sodium cacodylate pH 6.5.

491   25 % (v/v) glycerol was used as a cryo-protectant. Conditions yielding crystals

492   were optimised, and crystals from either the initial screens or subsequent

493   optimisations were selected for data collection. Diffraction images were collected

494   from single frozen crystals at beamlines I03, I04 and I04-1 at Diamond Light

495   Source (Harwell, UK) as indicated in Table 1. Diffraction images were processed

496   with XDS (33) and further processed with the CCP4 package of programs (34).

497   Initial phases were determined by molecular replacement using PHASER with a

498 previous *E. coli* FtsQ structure as search model (PDB ID 2VH1) (35). Iterative

499 model building and refinements were carried with MAIN, REFMAC and PHENIX

500 (36–38). Data and model statistics are summarised in Table 1.

501 *FtsB (64-87) peptide synthesis, purification and characterisation*

502 Peptides were synthesised by Fmoc-based solid-phase peptide synthesis on

503 H-rink amide ChemMatrix® resin (Merck). The peptide sequence was assembled

504 using an automated synthesiser (Syro II, MultiSynTech). For amino acid coupling

505 4 equivalents (eq) of the Fmoc protected amino acids (Iris Biotech) according to

506 the initial loading of the resin were mixed with 4 eq of 1-[bis(dimethylamino)-

507 methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate

508 (HATU) and 8 eq *N,N*-diisopropylethylamine (DIPEA) and added to the resin for

509 40 min. In a second coupling step, the resin was treated with 4 eq of the Fmoc-

510 protected amino acid mixed with 4 eq benzotriazole-1-yl-oxy-tris-pyrrolidino-

511 phosphonium hexafluorophosphate (PyBOP) and 8 eq 4-methylmorpholine

512 (NMM) for 40 min. After double coupling a capping step to block free amines was

513 performed using acetanhydride and DIPEA in *N*-methyl-2-pyrrolidinone (NMP)

514 (1:1:10) for 10 min. Fmoc deprotection was performed using 20 % piperidine in

515 dimethylformamide (DMF) for 5 minutes, twice. After each step the resin was

516 washed 4 times with DMF. For the crystallisation experiment of FtsB 64-87 with

517 FtsQ, the peptide was acetylated with Ac_2O final Fmoc deprotection. The

518 fluorescently labelled peptide for affinity measurements was *N*-terminally

519 coupled with 8-(9-fluorenylmethyloxycarbonyl-amino)-3,6-dioxaoetanoic acid as

520 stated above, and subsequently fluoresceinisothiocyanat (FITC) was coupled

521 manually using 8 eq DIPEA for 1.5 h, twice. Final cleavage was performed with

522   94 % trifluoroacetic acid (TFA), 2.5 % 1,2-ethanedithiole (EDT), 2.5 % H_2O and

523   1 % triisopropylsilane (TIPS) for 1.5 h, twice. The cleavage solutions were

524   combined and peptides were precipitated with diethyl ether (Et_2O) at -20 °C for

525   10 min. Peptides were resolved in water/acetonitrile (ACN) 5:5 and purified by

526   reversed-phase high performance liquid chromatography (HPLC; Nucleodur C18

527   column [Macherey-Nagel]; 10×125 mm, 110 Å, 5 μm particle size) using a flow

528   rate of 6 mL/min (A: ACN with 0.1 % TFA, B: water with 0.1 % TFA). Obtained

529   pure fractions were pooled and lyophilised. Peptide characterisation was

530   performed by analytical HPLC (1260 Infinity [Agilent Technology]; flow rate of

531   1 mL/min, A: ACN with 0.1 % TFA, B: water with 0.1 % TFA) coupled with a mass

532   spectrometer (6120 Quadrupole LC/MS [Agilent Technology]) using electro

533   spray ionisation (Eclipse XDB-C18 column [Agilent], 4.6×150 mm, 5 μm particle

534   size). Analytical HPLC chromatograms recorded at 210 nm are shown in Suppl.

535   Figure S2. Quantification of acetylated peptide was performed by HPLC-based

536   comparison (chromatogram at 210 nm) with a reference peptide and

537   quantification of fluorescein labelled peptide was performed using the extinction

538   coefficient ε=77.000 $M^{-1}cm^{-1}$ of fluoresceinisothiocyanat (FITC) in 100 mM

539   sodium dihydrogen phosphate; pH 8.5.

540   *Fluorescence Polarisation Assay (FP)*

541   To determine the affinity of the FtsB peptide to FtsQ, a 0.1 mM DMSO solution of

542   the FITC-labelled peptide was dissolved in 10 mM Hepes pH 7.4, 150 mM NaCl,

543   0.1% Tween-20 to yield a 40 nM peptide solution. A 3-fold dilution of FtsQ (15

544   μL per well) was presented in a 384-wellplate (black, flat bottom [Corning]) and

545   incubated with the peptide solution (5 μL, final peptide concentration 10 nM).

546   The final protein concentration ranged from 800 µM – 0.5 nM. After incubation

547   for 1 h at room temperature, fluorescence polarisation was measured using a

548   Tecan Spark 20M plate reader with λ_{ex} = 485 nm and λ_{ex} = 525 nm. K_d-values

549   were determined by nonlinear regression analysis of dose-response curves using

550   GraphPad Prism software.

551   *Size exclusion chromatography with multi-angle light scattering (SEC-MALS)*

552   The masses in solution of FtsB (22-103), FtsQ (58-276) both single and in a

553   complex were estimated using an online Dawn Heleos II 18 angle light scattering

554   instrument (Wyatt Technologies) coupled to an Optilab rEX online refractive

555   index detector (Wyatt Technologies). Protein samples (100 µL) were resolved on

556   a Superdex S-75 10/300 analytical gel filtration column (GE Healthcare), pre-

557   equilibrated with 25 mM Tris pH 7.4, 100 mM NaCl, 1 mM DTT at 0.5 mL/min.

558   Protein concentration was determined from the excess differential refractive

559   index based on dn/dc of 0.186 mg/mL. The light scattering and protein

560   concentration at each point across the peaks in the chromatograph were used to

561   determine the absolute molecular mass from the intercept of the Debye plot

562   using Zimm's model as implemented in the ASTRA version 5.3.4.20 software

563   (Wyatt Technologies). In order to determine the inter-detector delay volumes,

564   band broadening constants and the detector intensity normalisation constants

565   for the instrument, we used BSA as a standard prior to sample measurements.

566   *Analytical Ultracentrifugation*

567   Samples of FtsB (22-103), FtsQ (58-276) alone or mixed together at

568   concentrations of 1 mg/mL were subjected to velocity sedimentation at 50,000

569   rpm at 20 °C in 25 mM Tris pH 7.4, 100 mM NaCl, 1 mM DTT using 12 mm double

570   sector cells in an An50Ti rotor using an Optima XL-I analytical ultracentrifuge

571   (Beckmann). The sedimentation coefficient distribution function, c(s), was

572   analysed using the SEDFIT program, version 15.0 (39). The partial-specific

573   volumes (v-bar), solvent density and viscosity were calculated using SEDNTERP

574   software (SEDNTERP: Thomas Laue, University of New Hampshire).

575   *Plasmids, strains and growth conditions for complementation assays*

576   *E. coli* strains BL21(DE3), LMC531 (*ftsQ1*[Ts]) (40), and NB946 (41) were grown

577   in LB medium with shaking at 200 rpm. When indicated, strains LMC531 and

578   NB946 were grown in GB1 medium (4.83 g of $K_2HPO_4 \cdot 3H_2O$, 2.95 g of KH_2PO_4,

579   1.05 g of $(NH_4)_2SO_4$, 0.10 g of $MgSO_4 \cdot 7H_2O$, 0.28 mg of $FeSO_4 \cdot 7H_2O$, 7.1 mg of

580   $Ca(NO_3)_2 \cdot 4H_2O$, 4 mg of thiamine, 0.4% glycerol [v/v], 0.2% casamino acids

581   [w/v] per litre at pH 7.0). When required, 0.2% L-arabinose, 0.2% L-glucose, 100

582   μg/mL ampicillin, 30 μg/mL chloramphenicol, 25 μg/mL kanamycin, and 50

583   μg/mL spectinomycin were added to the culture medium.

584   Standard PCR and cloning techniques were used for DNA manipulation.

585   p29SENX-SH8FtsQ encoding full length FtsQ with an amino-terminal StrepII-

586   His8 dual tag (24) was used as template for mutagenesis in *SH8ftsQ* using

587   overlap extension PCR.

588   The DNA sequence encoding full length FtsB with a carboxy-terminal HA tag and

589   the DNA sequence encoding full length FtsL with an amino-terminal FLAG tag

590   were cloned by PCR and transferred into the pCDF plasmid (Novagen) resulting

591   in pCDF-FtsB-HA FLAG-FtsL. Mutations in *ftsB-HA* were introduced by overlap

592   extension PCR.

593   Plasmids pTHV037-mNG-FtsQ was constructed by cloning *ftsQ* into pTHV037 (42).

594   *ftsQ* was amplified from *E. coli* genomic DNA with primers containing restriction

595   sites for *Eco*RI and *Nco*I, also used to digest pTHV037. A triple asparagine linker

596   was introduced immediately downstream the *Eco*RI site. Plasmid and insert were

597   ligated with T4 DNA ligase (NEB, Ipswich, MA). Finally, the plasmid was amplified

598   by circular PCR to incorporate the Y248W mutation and obtain the plasmid

599   pTHV037-mNG-FtsQY248W.

600   *Functionality and detection of FtsQ and FtsB mutants*

601   To assess functioning of FtsQ mutants by microscopy, *E. coli* LMC531 cells

602   harbouring one of the p29SENX-SH8FtsQ mutants were grown overnight at 28 °C

603   in LB medium supplemented with 100 μg/mL ampicillin. The same medium was

604   inoculated 1:100 with the overnight cultures and incubated for 5 h at 28°C or

605   42°C. To assess functioning of FtsB mutants by microscopy, *E. coli* NB946 cells

606   harbouring one of the pCDF-FtsB-HA FLAG-FtsL mutants were grown overnight

607   at 37 °C in LB medium supplemented with 25 μg/mL ampicillin, 20 μg/mL

608   kanamycin and 0.2% L-arabinose. Then, LB medium supplemented with the

609   indicated antibiotics and 0.2% L-glucose or 0.2% L-arabinose was inoculated

610   1:100 with the overnight cultures and incubated for 5 h at 37 °C. The cells were

611   fixed by addition of formaldehyde to 3% at room temperature for 15 min,

612   harvested by centrifugation (13,000 x g, 2 min) and resuspended in PBS for the

613   examination of cell morphology by phase-contrast microscopy. To determine the

614   expression of mutant FtsQ under these conditions, cells were harvested from the

615   cultures by centrifugation (13,000 x g, 2 min) and the pellet was resuspended in

616   2X SDS sample buffer and incubated for 5 min at 96 °C. The crude cell lysates of

617   LMC531 were separated on 12% SDS-PAGE gels and analysed by Western

618   blotting using anti-FtsQ and anti-FtsB affinity-purified rabbit polyclonal

619   antibodies.

620   To assess functioning of FtsQ mutants by complementation of growth on solid

621   medium, *E. coli* LMC531 cells harbouring one of the p29SENX-SH8FtsQ mutants

622   were grown overnight at 28 °C in LB medium with 100 µg/mL ampicillin. The

623   same medium was inoculated 1:100 with the overnight cultures, incubated for

624   10 h at 28°C and then diluted 1:100 in GB1 medium with 100 µg/mL ampicillin

625   for overnight growth at 28°C. The same medium was inoculated 1:100 with the

626   overnight cultures and incubated for 5 h at 28 °C or 42 °C. The cultures were ten-

627   fold serially diluted and 4 µl of each dilution was spotted on GB1 agar medium.

628   The growth was assessed after 21 h incubation at 28°C or 18 h at 42 °C.

629   To assess functioning of FtsB mutants by complementation of growth on solid

630   medium, *E. coli* NB946 cells harbouring one of the pCDF-FtsB-HA mutants were

631   grown overnight at 37 °C in LB medium supplemented with 25 µg/mL ampicillin,

632   20 µg/mL kanamycin and 0.2% L-arabinose. The same medium was inoculated

633   1:100 with the overnight cultures and incubated for 10 h at 37 °C and then

634   diluted 1:100 in the same GB1 medium for overnight growth at 37 °C. The same

635   medium was inoculated 1:100 with the overnight cultures and incubated for 5 h

636   at 37 °C. The bacterial suspensions were diluted and spotted as described above

637   on GB1 agar medium supplemented with the indicated antibiotics and either

638   0.2% L-arabinose or 0.2% L-glucose and growth was analysed after 18 h

639   incubation at 37°C.

640   *Fluorescence microscopy*

641   Temperature sensitive FtsQ strain LMC531 (40) containing plasmid pTHV037-

642   mNG-FtsQ or pTHV037-mNG-FtsQY248 was grown in LB overnight at 30 °C

643   supplemented with 100 µg/mL ampicillin. The day after, samples were diluted

644   (1:500) in 25 mL of the same medium and grown at 30 °C until OD_{600} was 0.25.

645   Cells were then diluted 1:10 in cultures at 30 or 42 °C. When OD_{600} was 0.05,

646   samples were induced with 15 µM IPTG for 2 mass doubling times. Non-induced

647   samples were used as control. Cells were immobilised on 1 % agarose (43) and

648   imaged with a Nikon Eclipse T1 microscope (Nikon Plan Fluor 100x/1.30 oil ph3

649   DLL objective) coupled to an EMCCD camera (Hamamatsu Flash 4.0).

650

Acknowledgements

658

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796   and is essential for cell division. Mol Microbiol **51**:645–657.
797

798   **Figure legends**

799   **Figure 1. The periplasmic domains of *E. coli* FtsQ and FtsB form a stable**

800   **complex *in vitro*.** A) Surface plasmon resonance (SPR) experiments

801   investigating the interactions of the periplasmic domains of *E. coli* FtsQ, FtsB and

802   FtsL. FtsB binds to FtsQ. FtsL binds to FtsQ, and FtsL and FtsB together also bind

803   to FtsQ, although synergistically, not independently. FtsB and FtsL do not bind to

804   each other in isolation. Note that the proteins mentioned last were immobilised.

805   B) SPR investigation of the interaction of the periplasmic domain of *E. coli* FtsN

806   with FtsQ. A control, showing that binding of FtsB and FtsN to FtsQ is additive,

807   meaning they bind independently to FtsQ, different to how FtsB and FtsL bind. C)

808   Summary table showing quantitative analysis of the SPR data presented in

809   panels A) and B). D) Coiled coil predictions of full-length *E. coli* proteins FtsB,

810   FtsL and FtsQ, calculated with COILS (26). Note that only FtsB shows significant

811   coiled coil prediction, between residues ~29-70 (or 77). This makes it unlikely

812   that FtsB and FtsL form a canonical heteromeric coiled coil and we show in panel

813   A that they do not interact *in vitro* on their own. Please note that also predictions

814   with 2ZIP (27) were negative for FtsL (but positive for FtsB) (not shown).

815   **Figure 2. Crystal structure of the complex between the periplasmic**

816   **domains of *E. coli* FtsB and FtsQ.** A) Size exclusion profile showing elution of

817   the FtsQB complex. The complex elutes as two peaks (see Figure 5A and Suppl.

818   Figure S6A for SEC-MALS and AUC data on the same complex) that both

819   produced crystals and are most likely related to oligomerisation or dimerisation

820   of FtsB. C) Crystal structure of the complex determined to 2.6 Å resolution by

821   molecular replacement. Crystallographic data are listed in Table 1. Only residues

822   64 to 87 of FtsB are resolved in the structure. FtsB forms a short helix, a

823   connecting loop and a β-sheet that aligns in an antiparallel orientation with the

824   last strand of the β domain of FtsQ. D) Stereo plot of FtsB 64-87 in stick

825   representation, showing key residues involved in interactions with FtsQ, and

826   also internal contacts that are important for FtsB to adopt this particular

827   structure. Coloured from N- to C-terminus blue to red. Inset shows the same

828   orientation as a ribbon plot. E) In the crystals, FtsB forms a tight dimer that

829   buries hydrophobic residues, including methionine 77. To be certain about the

830   register of the amino acids of FtsB, we replaced M77 with SeMet and performed

831   a SAD experiment (Table 1). The resulting phased anomalous difference density

832   highlights the only methionine in FtsB in the correct location, validating our

833   interpretation.

834   **Figure 3. Validation of the crystal structure.** A) SPR experiments showing that

835   FtsB mutants R72A and F84A display significantly compromised binding to FtsQ,

836   as does FtsQ mutant Y248W to FtsB, which showed no binding. The crystal

837   structure of the complex of FtsB and FtsQ implicates FtsB F84 and FtsQ Y248 in

838   forming the binding interface. FtsB R72 is involved in a key salt bridge with FtsB

839   E82 and its interruption seems to abrogate binding between FtsB and FtsQ as

840   well. Note that these and other mutants were also investigated *in vivo* as

841   described in Figures 4 and Suppl. Figures S3-S5. B) SPR experiments

842   investigating the role of the FtsB residues that were not resolved in the crystal

843   structure, as only residues 64-87 were visible, 22-63 were not. FtsB binding to

844   FtsQ only requires residues from 64 onwards until 87 and it is to be concluded

845   that the remainder of the protein in the crystals is disordered. C) Summary table

846   quantifying the SPR data in panels A) and B). D) Corroborating the point that

847   only FtsB residues 64-87 are needed for the interaction between FtsB and FtsQ, a

848   fully synthetic peptide was produced (see Suppl. Figure S2) and its binding to

849   FtsQ investigated by fluorescence polarisation as the peptide also carried an

850   FITC moiety at the N-terminus. Fitting of the binding curve yielded a K_d of 9.5 μM,

851   similar to the values obtained with recombinant FtsB 22-103 and 64-88 in B)

852   and C). E) In fact, using a fully synthetic peptide of FtsB 22-87 (without FITC)

853   produced crystals that, although in a different space group, show exactly the

854   same structure and arrangement as observed before when adding the entire

855   periplasmic domain of FtsB (22-103) to FtsQ periplasmic domain. F)

856   Superposition of un-bound *E. coli* FtsQ periplasmic domain (PDB ID 2VH1) (19)

857   and our FtsQ structure in complex with FtsB. Overall, there are only small

858   deviations, but Y248 dramatically changes its sidechain conformer (and the

859   entire loop 247-252 changes conformation slightly), as does W256.

860   **Figure 4. Mutating residues implicated in FtsB and FtsQ complex formation**

861   **impairs cell division**. A) FtsQ mutants. The FtsQ temperature-sensitive *E. coli*

862   strain LMC531 (40), harbouring a plasmid for the expression of mutant SH_8FtsQ

863   was grown for 5 h at the permissive temperature (28 °C) or at non-permissive

864   temperature (42 °C). Cells were analysed by phase-contrast microscopy. EV:

865   empty vector/plasmid. B) FtsB mutants. The *E. coli* FtsB depletion strain NB946

866   (41), harbouring a plasmid for the expression of mutant FtsB-HA derivatives was

867   grown under non-depleting conditions in the presence of 0.2% L-arabinose

868   (Ara+) and under depleting conditions in the presence of 0.2% L-glucose (Glu+).

869   Cells were analysed by phase-contrast microscopy. EV: empty vector/plasmid.

870   Mutated residues are highlighted in Figure 2D.

871 **Figure 5. Model of the periplasmic complex between *E. coli* FtsB and FtsQ.**

872 A) Size exclusion chromatography with multiple angle light scattering (SEC-

873 MALS) of the complex of FtsB and FtsQ. FtsB forms large oligomers on its own.

874 FtsQ is monomeric alone but together, FtsB and FtsQ most likely form a 2 + 2

875 complex. Analytical ultracentrifugation (AUC) was also used to investigate the

876 same complex with very similar results (Suppl. Figure S6). B) Residues 22-64 of

877 FtsB were shown to not interact with FtsQ (Figure 3B & C). A previous crystal

878 structure (PDB ID 4IFF) (30) showed residues 28-60 to be able to form a coiled

879 coil arrangement with each other and we think it is likely that this interaction

880 leads to the dimerisation of the FtsB and FtsQ complex into the observed 2 + 2

881 stoichiometry. Residues 22-64 within FtsB link its single transmembrane helix to

882 the FtsQ-interacting domain in FtsB and also produce the putative dimer as

883 shown by forming a homodimeric coiled coil. It was previously shown that a

884 region around R75 in FtsQ links to FtsL (24). FtsQ is shown in surface

885 representation with sequence conservation colour coded (blue: most conserved,

886 red: least conserved). It is clear from the plot that the FtsB binding region

887 (residues 64-87) covers most of the highly conserved patch on the β domain of

888 FtsQ.

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891

892   **Figure legends Supplementary Figures**

893   **Supplementary Figure S1.** Sequence conservation-based colouring of FtsB

894   surface, shown in stereo. Around 100 sequences similar to *E. coli* FtsB (database

895   ID FTSB_ECOLI) were identified using BLAST and sequence conservation was

896   determined from a CLUSTAL OMEGA multiple sequence alignment with

897   CONSURF (http://consurf.tau.ac.il/2016/). FtsB residues 64-87 are shown as a

898   stick model coloured by conservation from blue (most conserved residues in the

899   alignment) to red (least conservation). FtsQ is shown as a grey molecular surface.

900   All residues involved in major interactions with FtsQ are conserved in FtsB, in

901   addition to internal interactions, such as R72 - E82.

902   **Supplementary Figure S2.** Table: peptide characteristics (molecular formula,

903   one-letter code sequence, molecular weight, calculated and detected m/z ratios

904   for multi charged masspec ions ($[M+nH]^{n+}$). Abbreviations: Ac: acetyl; FITC:

905   fluoresceinisothiocyanat fluorophore; PEG: 8-amino-3,6-dioxaoctanoyl linker;

906   Nle: norleucine amino acid replacing methionine 77. **A)** HPLC chromatogram at

907   210 nm of peptide used for co-crystallisation with FtsQ (Figure 3D), gradient:

908   20-50 % ACN in 10 min and mass spectra of corresponding peptide. **B)** HPLC

909   chromatogram at 210 nm of FITC PEG peptide for fluorescence polarisation

910   (Figure 3D), gradient: 30-60 % ACN in 10 min, starting at 3 minutes and mass

911   spectra of corresponding peptide**.**

912   **Supplementary Figure S3.** A) Spot assay of FtsQ mutants used in Figure 4A. B)

913   Western blot showing expression levels of FtsQ mutants used in A).

914   **Supplementary Figure S4.** Fluorescence microscopy localisation of FtsQ

915   (Y248W) mutant protein in *E. coli* cells. Phase contrast images, fluorescent

916   images and fluorescence profiles per cell (from 0 to 200 AU) plotted against

917   normalised cell length (from 0 to 100 %) of ts FtsQ LMC531 strain expressing

918   wild type FtsQ or FtsQY248W fused to mNeonGreen (mNG) at 30 or 42°C. *n*

919   indicates number of cells. The graphs show the average total fluorescence per

920   cell plotted against the normalised cell length. Filaments have therefore a higher

921   fluorescence per cell. In addition, the expression of the proteins is higher at the

922   non-permissive temperature. Scale bar represents 2 μm.

923   **Supplementary Figure S5.** Spot assay showing FtsB mutants used in Figure 4B.

924   **Supplementary Figure S6.** A) Analytical ultracentrifugation (AUC) of FtsQ, FtsB

925   and in complex. B) Summary table of SEC-MALS (Figure 5A) and AUC data (panel

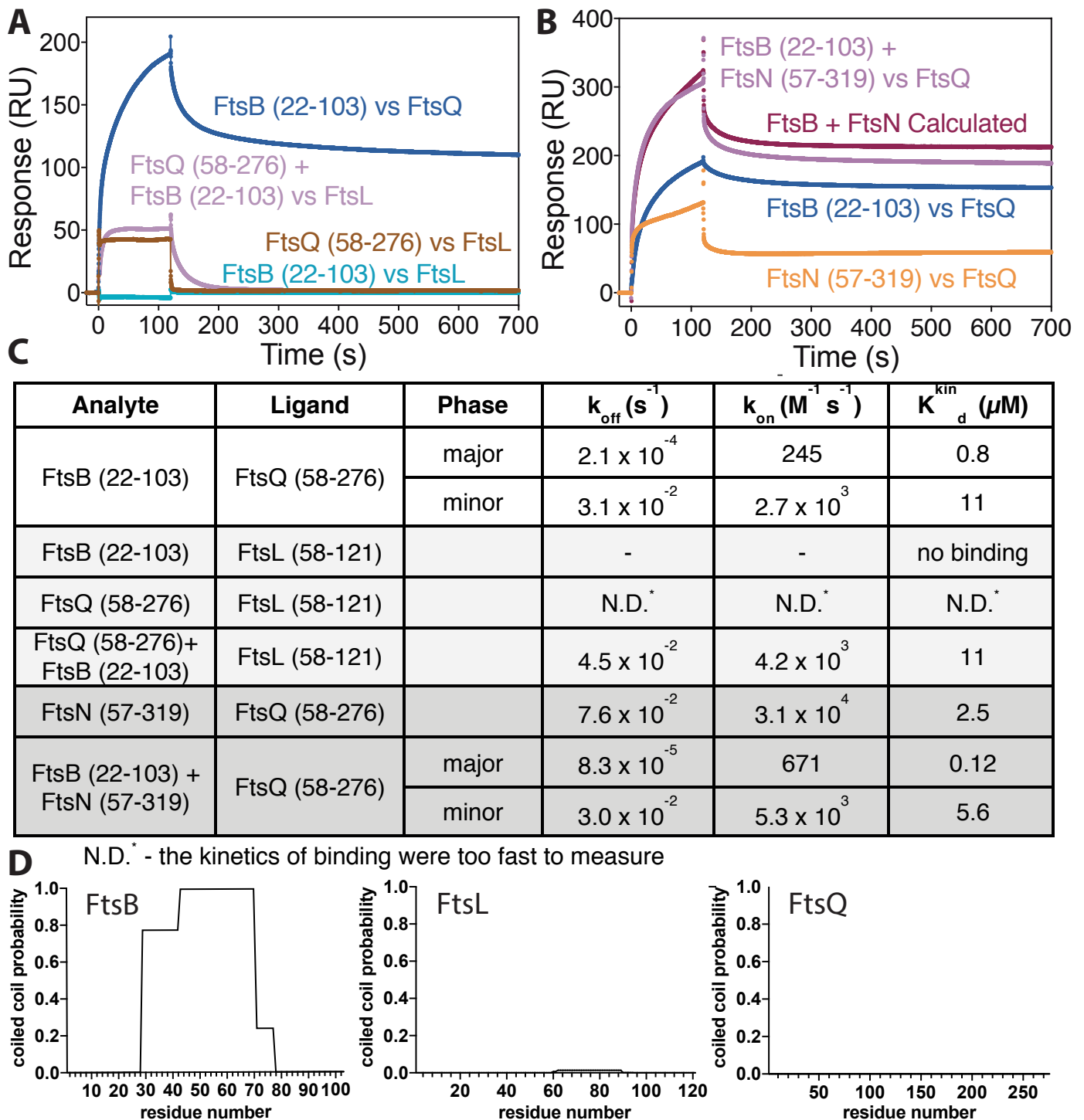926   A) describing the properties of FtsQ, FtsB and their complexes.

Figure 1.

A FtsB (22-103) vs FtsQ; FtsQ (58-276) + FtsB (22-103) vs FtsL; FtsQ (58-276) vs FtsL; FtsB (22-103) vs FtsL

B FtsB (22-103) + FtsN (57-319) vs FtsQ; FtsB + FtsN Calculated; FtsB (22-103) vs FtsQ; FtsN (57-319) vs FtsQ

C

Analyte	Ligand	Phase	k_{off} (s^{-1})	k_{on} (M^{-1} s^{-1})	K_d^{kin} (μM)
FtsB (22-103)	FtsQ (58-276)	major	2.1×10^{-4}	245	0.8
		minor	3.1×10^{-2}	2.7×10^{3}	11
FtsB (22-103)	FtsL (58-121)		-	-	no binding
FtsQ (58-276)	FtsL (58-121)		N.D.*	N.D.*	N.D.*
FtsQ (58-276)+ FtsB (22-103)	FtsL (58-121)		4.5×10^{-2}	4.2×10^{3}	11
FtsN (57-319)	FtsQ (58-276)		7.6×10^{-2}	3.1×10^{4}	2.5
FtsB (22-103) + FtsN (57-319)	FtsQ (58-276)	major	8.3×10^{-5}	671	0.12
		minor	3.0×10^{-2}	5.3×10^{3}	5.6

D N.D.* - the kinetics of binding were too fast to measure

FtsB; FtsL; FtsQ

Figure 2.

Figure 3.

C

Analyte	Ligand	Phase	$k_{off} (s^{-1})$	$k_{on} (M^{-1} s^{-1})$	$K_d^{kin} (\mu M)$
FtsB (22-103)	FtsQ (58-276)	major	2.1×10^{-4}	245	0.8
		minor	3.1×10^{-2}	2.7×10^{3}	11
FtsB (64-87)	FtsQ (58-276)	slow	1.0×10^{-3}	559	1.8
		fast	1.1×10^{-2}	1.7×10^{4}	0.7
FtsB (22-64)	FtsQ (58-276)		-	-	no binding
FtsB-R72A	FtsQ (58-276)		1.5×10^{-2}	577	26
FtsB-F84A	FtsQ (58-276)		1.4×10^{-2}	365	38
FtsB (22-103)	FtsQ Y248W		-	-	no binding

Figure 4.

Figure 5.

Tables

Table 1. Crystallographic data

Statistics			
Sample	***E. coli periplasmic FtsQB complex***	***E. coli periplasmic FtsQB complex: SeMet***	***E. coli periplasmic FtsQB complex: FtsB peptide***
NCBI database IDs	FtsQ: FTSQ_ECOLI FtsB: FTSB_ECOLI	FtsQ: FTSQ_ECOLI FtsB: FTSB_ECOLI	FtsQ: FTSQ_ECOLI FtsB: FTSB_ECOLI
Constructs	Q: M-58-276-GSH$_6$ B:MGSSHHHHHHSSGLVP RGSHM-22-103	Q: M-58-276-GSH$_6$ B:MGSSHHHHHHSSGLVP RGSHM-22-103	Q: M-58-276-GSH$_6$ B: 64-87 (synthetic, acetylated, M77 replaced with norleucin)
Method	molecular replacement	selenium SAD	molecular replacement
Data collection			
Beamline / source	Diamond I04-1	Diamond I03	Diamond I04
Wavelength (Å)	0.92819	0.97980	0.97950
Crystal / helical			
Space / point group	$P4_12_12$	$P4_12_12$	C2
Cell (Å)	93.3, 93.3, 106.0	92.5, 92.5, 105.2	117.2, 48.0, 131.6, 112.4°
Data			
Resolution (Å)	2.6	2.8	2.8
Completeness (%)[1]	99.5 (99.5)	100.0 (99.9)	98.5 (99.7)
Multiplicity[1]	12.8 (13.4)	14.0 (13.8)	3.2 (3.3)
(I) / σ(I) [1]	22.6 (2.1)	33.3 (3.9)	10.6 (1.8)
R_{merge}[1]	0.082 (1.702)	0.078 (1.130)	0.077 (0.744)
R_{pim}[1]	0.024 (0.477)	0.021 (0.314)	0.051 (0.488)
CC1/2	0.999 (0.821)	1.000 (0.944)	0.998 (0.686)
Anomalous correlation		0.833 (0.010)	
selenium sites		1	
Refinement			
R / R_{free}[2]	0.2376 / 0.2595		0.2299 / 0.2770
Models	FtsQ 58-260 FtsB 64-87 4 waters		2x FtsQ 58-260 2x FtsB 64-87 0 waters
Bond length rmsd (Å)	0.005		0.012
Bond angle rmsd (º)	0.970		1.637
Favoured (%)[3]	99.5		99.5
Disallowed (%)[3]	0.5		0.5
MOLPROBITY score	99th percentile		98th percentile
PDB IDs	**XXXX**		**XXXX**

[1] Values in parentheses refer to the highest recorded resolution shell.
[2] 5% of reflections were randomly selected before refinement.
[3] Percentage of residues in the Ramachandran plot (PROCHECK 'most favoured' and 'additionally allowed' added together).