Isolation and characterization of topological specificity mutants of minD in Bacillus subtilis

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Summary

In rod-shaped bacteria such as Bacillus subtilis, division site selection is mediated by MinC and MinD, which together function as a division inhibitor. Topological specificity is imposed by DivIVA, which ensures that MinCD specifically inhibits division close to the cell poles, while allowing division at mid-cell. MinD plays a central role in this process, as it positions and activates MinC and is dependent on DivIVA for its own positioning at the poles. To investigate MinD activities further, we have constructed and analysed a collection of minD mutants. Mutations in the conserved ATPase motifs lead to an inactive protein, possibly unable to oligomerize, but which nevertheless retains some affinity for the cell membrane. Several mutations affecting the mid- to C-terminal parts of MinD led to a protein probably unable to interact with DivIVA, but that could still stimulate division inhibition by MinC. These findings suggest that the ATPase activity of MinD is necessary for all its functions (possibly in part by controlling the oligomerization state of the protein). The other mutations may identify a surface of MinD involved in its interactions with DivIVA and a possible mechanism for control of MinD by DivIVA.

Introduction

A fundamental problem in bacterial cell division is the nature of the mechanism that positions the division septum at the correct place in the cell. In rod-shaped bacteria, such as Bacillus subtilis, it is usually placed midway along the long axis of the cell, so that division gives rise to two daughter cells of equal size. The highly conserved tubulin-like protein called FtsZ, a central component of the division apparatus, forms a ring-like structure at the division site (Lutkenhaus and Addinall, 1997): correct mid-cell placement of the FtsZ ring is under the control of the MinCD/DivIVA system (MinCDE in Escherichia coli; see below). This system allows selection of the mid-point of the cell as the division site in preference to two other potential sites located close to the cell poles. Evidence that the poles are potential division sites comes from the minicell (min)-producing mutants: these mutants divide at approximately the normal frequency, but septation frequently occurs close to the cell poles, giving rise to small, spherical usually anucleate cells (Adler et al., 1967; Reeve et al., 1973; Teather et al., 1974).

MinC and MinD act together to inhibit cell division. Apparently, MinC is the actual inhibitor of assembly of the division apparatus, which functions by preventing FtsZ polymerization (Hu et al., 1999). MinD is required for MinC activity (de Boer et al., 1990; Marston and Errington, 1999) by a mechanism that is not yet clear but may involve enrichment of MinC at the cell periphery (where FtsZ ring assembly occurs) (Hu et al., 1999; Raskin and de Boer, 1999a). In B. subtilis, DivIVA protein is required to target the MinCD inhibitor to the cell poles. It is recruited to mid-cell late in cell division and then attracts MinD (and thus MinC) to this site. After division, DivIVA and MinCD remain at the newly formed poles, thus preventing division from occurring again at these sites. Moreover, by sequestering MinCD away from mid-cell, the next FtsZ ring assembly is free to occur at mid-cell. In the absence of DivIVA, MinCD spreads diffusely over the cell and prevents division from occurring anywhere in the cell, leading to filamentation and cell death (Cha and Stewart, 1997; Edwards and Errington, 1999).

In E. coli, the MinCD division inhibitor is controlled in a somewhat different manner. In this organism, DivIVA is not present, and a quite different protein, MinE, controls the topological specificity of MinCD (de Boer et al., 1989). This is achieved by a remarkable mechanism, in which MinD oscillates from pole to pole with a period of about 50 s in a MinE-dependent manner (Raskin and de Boer, 1999b; Rowland et al., 2000; Fu et al., 2001; Hale et al., 2001). MinC oscillates with the same pattern and frequency as MinD but is a passenger in the oscillation (Hu and Lutkenhaus, 1999; Raskin and De Boer, 1999a). In essence, the MinDE machinery seems to work by ensuring that MinC spends more time at the cell poles than at mid-cell.

MinD is highly conserved among bacteria: for example,
the E. coli and B. subtilis proteins share 44% identical residues. It is a member of a large family of ATPases of diverse functions (Koonin, 1993), which includes ParA and Soj, which are involved in plasmid and chromosome partitioning (Hiraga, 2000), and ArsA, which is the energy transducer for the transport of certain anions (Chen et al., 1986). Sequence comparison of these proteins has allowed the identification of five conserved motifs (Gérard et al., 1998) (Fig. 1), among which three are probably involved in the ATPase activity. The E. coli MinD protein has been purified and shown to be able to bind and hydrolyze ATP (de Boer et al., 1991). Recently, the structures of two MinD proteins from Archaeoglobus fulgidus (Cordell and Löwe, 2001) and Pyrococcus furiosus (Hayashi et al., 2001) were described. These structures appear to confirm the importance of the conserved motifs in ATP binding, but they did not shed much light on the functioning of the protein.

MinD appears to be a central component of the division site selection system, as it is an intermediate between the topological specificity factor and the division inhibitor in both B. subtilis and E. coli. This suggests that, in B. subtilis, it has several functions. First, an interaction with DivI, the topological specificity factor, which is responsible for proper MinD localization (Marston et al., 1998), although a direct interaction has not been shown so far. Secondly, an interaction with MinC, the division inhibitor, which depends on MinD for its own localization and activation. Yeast two-hybrid experiments have demonstrated a direct interaction between MinC and MinD (Marston and Errington, 1999) (as in E. coli; Huang et al., 1996). Thirdly, several lines of evidence suggest that MinD associates with the cell membrane: fluorescence microscopy indicates that most of the protein lies close to the cell envelope (Marston et al., 1998), and electron microscopic studies of E. coli MinD suggest an inner

Fig. 1. Sequence alignment of B. subtilis, E. coli, A. fulgidus and P. furiosus MinD proteins (alignment obtained with CLUSTAL_W and edited manually; Thompson et al., 1994). The five conserved motifs (Gérard et al., 1998) are boxed. The amino acid changes are indicated below the alignment (bold letters, Min− mutants; bold and italic letters, Sep− mutants).
membrane localization (de Boer et al., 1991). A fourth possible activity might be inferred from the localization patterns of MinD in both B. subtilis and E. coli. In B. subtilis, the DivIVA protein to which MinD is attracted is targeted to small well-defined spots right at the cell poles (Edwards and Errington, 1997), whereas MinD occupies a much broader zone emanating some distance away from the cell pole (Marston et al., 1998). In E. coli, MinD oscillation appears to be co-operative, with most of the protein molecules being attracted to the same cell pole at any point in the oscillation cycle (Raskin and de Boer, 1999b; Rowland et al., 2000). Both kinds of behaviour suggest that MinD localization involves some kind of self-interaction to form oligomers or polymers. This has not yet been tested directly, although there is evidence for a weak MinD–MinD interaction from yeast two-hybrid studies (A. L. Marston, unpublished).

We have investigated MinD function further by site-directed and random mutagenesis. Mutations affecting the conserved ATPase motifs lead to an inactive protein, which nevertheless retains some affinity for the cell membrane. In contrast, several mutations affecting the middle and C-terminal parts of MinD lead to a protein probably unable to interact with DivIVA but that can still stimulate division inhibition by MinC. These findings suggest that the ATPase activity of MinD is crucial for all its functions. They also define a region of MinD that may be involved in its interaction with DivIVA and suggest a possible mechanism for sequestration of MinD.

Results

MinD localization and activity requires the conserved ATPase motifs

Of the conserved motifs identified by Gérard et al. (1998) (Fig. 1), motifs I and III are modified versions of the classical Walker ‘A’ and ‘B’ ATP-binding motifs (also called the ‘P-loop’ and ‘switch II’ sites). Motif II (switch I site) contains two conserved aspartate residues that are probably involved in the co-ordination of Mg$^{2+}$ (Walker et al., 1982; Cordell and Löwe, 2001; Hayashi et al., 2001). We constructed four mutations in these three motifs. Two of them, K16A (i.e. lysine to alanine at amino acid 16) and D120A are predicted to disrupt ATP binding. Lysine 16 is nearly invariant in all Walker A boxes and interacts with the bound nucleotide (Story and Steitz, 1992). In ArsA, an ATPase homologous to MinD, the equivalent of aspartate 120 indirectly co-ordinates the magnesium ion in the ATP-binding pocket (Zhou et al., 2000). The third mutation, aspartate to alanine at amino acid 38 (D38A), probably decreases the affinity of the protein for Mg$^{2+}$: aspartate 38 is one of the two conserved aspartates in motif II. An equivalent amino acid in ArsA has been shown to be essential for the ATPase activity (Zhou and Rosen, 1999). The fourth mutation, glycine to valine at amino acid 12 (G12V), possibly alters nucleotide hydrolysis: the equivalent mutation in HA-ras p21 results in a protein that can bind GTP but has a greatly reduced rate of hydrolysis (Vogel et al., 1988). Western blot analysis indicated that all four MinD mutant proteins were produced at approximately wild-type levels (data not shown).

For all these mutant proteins, we tested MinD function and localization as well as MinC localization. The mutations were constructed by polymerase chain reaction (PCR) on a vector (pSG4801) carrying minD and a chloramphenicol resistance gene to allow selection (see Experimental procedures). The mutated copies of minD were introduced into the wild type strain (SG38) or into strains carrying a gfp–minC fusion (strain 2895; here minD is directly downstream of gfp–minC) or a gfp–minD fusion (strain 2896). The latter two recipient strains contain a deletion of minCO at the wild-type locus and the GFP fusions elsewhere on the chromosome driven by a xylose-inducible promoter (P$_{xy}$). Although it does not have minC, strain 2896 allows the examination of MinD localization, which is known to be independent of MinC (Marston and Errington, 1999). Insertion of the plasmid was designed in such a way that only one functional copy of minD was present in the cell, i.e. the mutated copy. In the wild-type background (strains 2867–2869 and 2908), all four mutations produced phenotypes (designated Min–) similar to those of null mutations: minicells accumulated, indicating that division was not inhibited at the poles. All the strains displayed a slightly reduced ability to sporulate (Levin et al., 1992; Lee and Price, 1993; Cha and Stewart, 1997; Barák et al., 1998), and the cell length was also slightly increased, as expected in a minD null mutant strain (data not shown). Thus, all four of these Min– MinD proteins were impaired in their ability to mediate the inhibition of cell division.

Examination of GFP–MinD localization by fluorescent microscopy revealed that none of the mutant proteins was targeted to the cell poles. Instead, they were diffusely distributed along the length of the cell. Figure 2C shows an example of the typical staining pattern for one of the mutants (K16A) compared with the wild type (Fig. 2A); similar results were obtained with all the other mutants (data not shown). Fluorescence intensity line plots across the mutant cells (Fig. 2E) showed that the signal was apparently enriched at the periphery of the cell (indicated by the arrows), suggesting that these mutant proteins retain an affinity for the inner face of the cell membrane, as for the wild-type protein (de Boer et al., 1991; Marston et al., 1998). The transverse bands could correspond to recruitment of the mutant MinD proteins to impending or ongoing cell division events, in which case these proteins may retain some affinity for the division machinery.
More likely, the bands simply represent plates of membrane at division septa, lying perpendicular to the image plane.

Visualization of the intracellular localization of MinC with a GFP–MinC fusion indicated that it was also delocalized and distributed throughout the cell (Fig. 2D; compare with the wild-type pattern of localization in Fig. 2B), similar to minD null mutant strains (Marston and Errington, 1999). These images also showed the accumulation of minicells, as expected for a mutant in which MinD is inactive (Fig. 2A, arrow). Although difficult to detect against the background of dispersed protein, no increase in the GFP signal was evident at the cell periphery (Fig. 2F). Taken together with the Min− phenotype, this suggested that the interaction between MinC and MinD was disrupted in these mutants.

We also introduced mutations in motifs IV and V (V142A and N174A). However, the resulting mutant strains (2870 and 2871) did not have a detectable division placement defect, and localization of MinD and MinC was normal. This indicates that, although the two modified amino acids belong to conserved motifs, they are not essential for MinD activity (data not shown).

Together, these results suggest that ATP binding and possibly hydrolysis are essential for all MinD activities except for its affinity for the membrane.

**Isolation of minD mutants in which division is inhibited throughout the cell**

To investigate the nature of the proposed interaction between MinD and DivIVA involved in MinD targeting to the cell poles, we developed a screening system to isolate minD mutants in which MinD would no longer target to the poles but would retain the ability to activate MinC. As in a divIVA null strain (Cha and Stewart, 1997; Edwards and Errington, 1997), these mutants should grow poorly and be filamentous because cell division would be inhibited throughout the cell. To overcome this difficulty, we made use of strain 2895, in which gfp–minC minD is under the control of the P_xyb promoter. Introduction of a mutagenized minD gene by single cross-over into the chromosome of this strain would lead to a construct in which the mutated copy of minD, which is co-transcribed with gfp–minC, is controlled by P_xyb (a second wild-type copy of minD is present but not expressed). This allows the balanced ratio of MinC and MinD necessary for the proper functioning of the system (Marston and Errington, 1999). A mutation conferring the expected phenotype should lead to colonies growing very poorly in the presence of xylose but that can be rescued when grown without xylose (when they should simply have a classical minicell phenotype).

minD was randomly mutagenized by PCR and cloned in pSG1301 for integration by single cross-over in strain 2895 (see Experimental procedures). Of about 1500 transformants screened in the presence of xylose, four seemed to grow very poorly and appeared to be filamentous when examined by microscopy. All four candidates (Sep− mutants) carried mutations that would produce substitutions in the middle or C-terminal parts of MinD: A123P (minD12, strain 2912); S148T (minD16, strain 2916); N165Y (minD15, strain 2915); and D185A (minD11, strain 2911). The structure of the integration in each mutant was checked by PCR and appeared to be correct for all the strains except 2912. We used homologous recombination to construct a derivative of the minD12 mutant in which the plasmid sequences were removed (see Experimental procedures). The resultant strain (2927) also carried gfp–minC minD12 driven by P_xyb.

![Fig. 2. Examples of the localization of GFP–MinD and GFP–MinC in a Min− mutant. Cells were grown to exponential phase (OD600 of 0.6–0.7) in S medium containing 0.5% xylose. Samples were taken for fluorescence microscopic imaging of the GFP. A, C and E. Dispersed membrane distribution of GFP–MinD(K16A) (C, strain 2888) compared with wild-type GFP–MinD (A, strain 2893). (Note that these strains do not have minC and therefore produce minicells – arrowed in A.) B, D and F. Diffuse cytoplasmic distribution of GFP–MinC in a minD17(K16A) mutant background (D, strain 2880) compared with a wild-type (minD+) background (B, strain 2886). E and F. Fluorescence intensity line plots across the cells in (C) and (D) respectively (at positions corresponding to the white lines in C and D). Scale bar (B) = 2 μm.](image-url)
but it had a structure slightly different from the other mutant strains because only one copy of \textit{minD} remained. This derivative proved to have the genetic structure expected and the correct \textit{minD} DNA sequence. Western blot analysis showed that, when grown in the presence of xylose, all the Sep$^-$ mutants produced the mutated MinD protein at approximately wild-type levels (data not shown).

\textit{Sep$^-$} mutants are probably affected in DivIVA interaction

To confirm that division was inhibited in the mutants, we determined the cell length frequency distribution of exponentially growing cultures of strains 2911, 2915, 2916 and 2927 in the presence of xylose (in these conditions, the mutant MinD protein is produced). As shown in Fig. 3B-E, the cell length was increased in all Sep$^-$ mutant strains compared with a strain carrying the equivalent construct producing a wild-type MinD protein (Fig. 3A, strain 2885). The filamentous phenotype was relatively mild in strain 2911 (D185A; Fig. 3B) but was particularly striking in strains 2915 (N165Y; Fig. 3C) and 2927 (A123P; Fig. 3E), indicating that some of the substitutions have a stronger effects than others. We also observed a small number of minicells, indicating that division was not totally inhibited at the poles (as is typical of \textit{divIVA} null mutants; Reeve \textit{et al.}, 1973; Edwards and Errington, 1997). These observations would be consistent with the production of mutant MinD proteins that allow inhibition of division but are not properly topologically regulated.

To examine the topological regulation of the division inhibitor in the Sep$^-$ mutants, we analysed MinC localization using the \textit{gfp–minC} fusion that these strains carry under the control of \textit{Pxyl}. Fluorescence microscopy of exponentially growing cells in the presence of xylose showed that GFP–MinC was not properly targeted to the cell poles, although prominent bands of GFP were evident at irregular intervals along the length of the cell filaments, probably corresponding to sites where residual cell divisions had occurred or were occurring (Fig. 4). Some of the strong bands were in doublets, probably corresponding to sites of minicell formation, or were localized in minicells, as mentioned above (e.g. arrows in Fig. 4C and D). The signal was reminiscent of that of GFP–MinD in these mutants (see below) and strikingly different from that of GFP–MinC in the Min$^-$ mutants (compare with Fig. 2D), consistent with MinC still being under the control of MinD. In addition to the strong bands, some fainter bands of GFP–MinC were evident at intermediate positions, possibly corresponding to potential division sites (arrowheads). We reported previously that MinCD has an affinity for division sites independent of DivIVA (Marston and Errington, 1999). Presumably, this reflects an interaction between MinCD and the division machinery associated with the activity of MinCD in preventing division. (The irregular spacing of these bands could result from the loss of regulation of FtsZ ring formation as a result of repeated failure of rings to form and mature into sites of active division.) In general, the localization pattern of the mutants, particularly the release of the protein from the completed cell poles, was similar to that of GFP–MinC in a \textit{divIVA} null mutant (Marston and Errington, 1999),
strongly suggesting that MinD retained its ability to interact with MinC but was impaired in its interaction with DivIVA.

To confirm that the phenotype observed in the Sep− mutants resulted from an improper localization of the mutant MinD protein, we examined GFP–MinD localization directly for MinD-S148T and MinD-N165Y. Plasmids carrying minD with the corresponding mutations were recovered from the chromosomes of strain 2915 and 2916 (see Experimental procedures). They were then introduced by single cross-over integration into strain 1981, carrying a gfp–minD fusion under the control of Pxyl. The resulting strains were grown to exponential phase in the presence of xylose, and the cells were examined by fluorescence microscopy. As expected, GFP–MinD-S148T and GFP–MinD-N165Y produced a relatively delocalized signal with only weak targeting or retention of the protein at the cell poles (Fig. 5A and B). As for GFP–MinC, stronger internal bands were present, probably corresponding to nascent division sites that had escaped inhibition by MinCD, and some fainter internal bands (arrowheads), probably corresponding to potential division sites at which inhibition of division was actively occurring. Again, the localization was very similar to that of wild-type GFP–MinD in a divIVA null mutant (Fig. 5C). As for GFP–MinC, these observations would be consistent with the mutant MinD proteins retaining some affinity for the division apparatus and only being defective in their interaction with DivIVA.

Taken together, these results indicate that the inhibition of cell division throughout the cell in the Sep− mutants results from improper localization of MinD, leading to localization and activation of the division inhibitor MinC not only at the poles but also elsewhere in the cell. The results also showed that it is possible to separate the activities of MinD involved in its positioning from those involved in the interaction with MinC.

Discussion

Phenotype of minD mutants predicted to be affected in nucleotide binding or hydrolysis

We have constructed several mutant strains carrying mutations in the ATPase domain of MinD. In all these mutants, MinD seems to be totally inactive and delocalized, although it retains some affinity for the cell membrane. The interaction with MinC, which appears no longer to be associated with the cell membrane, is probably disrupted. Several mutations in the ATP-binding

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domain of E. coli MinD (among which lysine to alanine at amino acid 16) have been shown to affect the capacity of the protein to activate MinC (de Boer et al., 1991), but the localization of these mutant proteins has not been reported. The inability of our mutants to activate MinC could reflect the failure to position and concentrate this protein at the membrane, which is thought to be the basis for MinC activation by MinD (Hu et al., 1999; Raskin and De Boer, 1999a). Three of the mutations that we introduced are likely to interfere with nucleotide binding, consistent with this being essential for MinD activity. The fourth substitution, G12V, was designed to alter nucleotide hydrolysis. When the same substitution was introduced into Soj, a distant relative of MinD, the protein still localized, but it no longer showed any dynamic behaviour (Quisel et al., 1999). There are several possible explanations for the fact that MinD-G12V is delocalized and inactive. First, nucleotide binding might be impaired in this protein, in which case this mutant is analogous to the other three Min mutants. Secondly, MinD-G12V might be a hyperactive ATPase, in which case MinD would mainly be in the ADP-bound state. This would imply that MinD-ADP is unable to localize. Thirdly, this protein might bind ATP but be defective in hydrolysis. In this case, MinD-ATP would be unable to localize to the poles, possibly because it cannot self-assemble (see Introduction and below). According to either of the latter models, nucleotide hydrolysis, and not only nucleotide binding, would be necessary for MinD activity. It should also be noted that we cannot exclude the possibility that the mutations we have constructed simply disrupt the three-dimensional structure of the protein in a relatively non-specific manner.

MinD mutants affected in interaction with DivIVA

According to our model for the operation of the Min system in B. subtilis, DivIVA anchored at the cell poles interacts with MinD to recruit it (and consequently MinC) to the polar regions. If this model were correct, it should be possible to obtain mutations in minD that leave intact its ability to interact with MinC and promote inhibition of division but block its interaction with DivIVA and, therefore, the restriction of division inhibition to the cell poles. To our knowledge, this class of mutation has not been obtained with the E. coli system, but we were able to obtain them for B. subtilis. Four different Sep– mutants were isolated and, in each case, the phenotype was consistent with the model: the localization patterns observed for both MinD and MinC fusions to GFP were similar to those described previously for divIVA null mutants (Marston et al., 1998; Marston and Errington, 1999). The similarity included both the loss of retention of MinCD at the cell poles and the residual enrichment at nascent and potential division sites (Marston et al., 1998; Marston and Errington, 1999). Formally, we cannot exclude a model in which MinCD leaves the cell poles in the mutants because it has an increased general affinity for the division machinery. However, as the interaction with the division machinery is thought to take place through MinC, at least in E. coli (de Boer et al., 1990; Hu et al., 1999), and the mutations lie in minD, we do not currently favour this model.

The Sep– mutants all had substitutions in the mid- to C-terminal part of the protein, raising the possibility that this constitutes a domain or surface involved in the putative interaction with DivIVA. The sequence of
B. subtilis MinD could readily be aligned with that of E. coli and with those of the two organisms for which MinD structures have recently been solved: A. fulgidus (Cordell and Löwe, 2001) and P. furiosus (Hayashi et al., 2001) (Fig. 1). Comparison of the alignments and the structures suggests that the secondary structural elements are highly conserved. On this basis, three of the substitutions we found (corresponding to A. fulgidus residues labelled Ser-144, Leu-159 and Ile-176) lie in or near helices H5 and H6 [according to the nomenclature of Cordell and Löwe (2001)] (Fig. 6). These residues lie near each other on one side of the protein and may therefore comprise part of a region involved in the interaction with DivIVA. Two of the residues are actually located in loops just adjacent to the helices. This is not uncommon for amino acids involved in protein–protein interactions (for example, see Williams et al., 1997), but the relatively unconserved nature of these loops makes it difficult to make firm predictions about the precise configuration of the relevant residues in the B. subtilis MinD protein.

The substitution at A123 (corresponding to Ala-119 of A. fulgidus MinD in Fig. 6) is not far distant in three-dimensional terms from the other substitutions, again suggesting a potential region of contact with DivIVA. However, this substitution is particularly interesting, because it is highly conserved, lying in the ‘switch II’ region of the protein (Fig. 1). In the P. furiosus structure, the equivalent residue (A121) is thought to have a role in the catalysis of ATP hydrolysis because the backbone N of this residue makes a hydrogen bond with a water molecule that is aligned in such a way that it could make an in-line nucleophilic attack on the γ-phosphate of ATP (Hayashi et al., 2001). We suggest that DivIVA may use a contact with this alanine to regulate nucleotide hydrolysis. By analogy with the regulation of actin polymerization (Schmidt and Hall, 1998), we speculate that DivIVA could act as a ‘capping’ protein to stabilize the self-assembly (possibly ATP dependent) of MinD oligomers at the cell pole. This would provide a mechanism for sequestering the MinCD inhibitor to the cell poles. In extensive time lapse experiments (M. El Karoui, unpublished), we have not detected an oscillatory behaviour for MinD, as has been described for E. coli (Raskin and de Boer 1999b; Rowland et al., 2000). The distinction between the two systems may lie in the MinE protein, which differs from DivIVA in being mobile, but which we suggest could also use nucleotide hydrolysis to control MinD assembly/disassembly to bring about oscillation. In vitro experiments with the purified proteins will be required to test this model but, so far, the B. subtilis proteins have proved refractory to purification in an active form.

Fig. 6. Structure of MinD from A. fulgidus showing various secondary structural features, and with residues corresponding to those affected by the Sep– mutations of B. subtilis minD labelled. H5 and H6 refer to α-helices as defined by Cordell and Löwe (2001).
**Experimental procedures**

**Bacterial strains and plasmids**

*Bacillus subtilis* strains and plasmids used in this study are listed in Table 1. All cloning was carried out in *E. coli* MC1061 (Wertman et al., 1986), except for vector pSG4820, in which case *E. coli* DH5α (Gibco BRL) was used. To construct plasmid pSG4801, a 0.7 kb DNA fragment containing the minD gene was amplified by PCR using primers AM1 (5′-gagagagactgagggtgaggc-3′) and AM2 (5′-gattctttgcttgat 3′), introducing Xhol and EcoRI sites respectively. The EcoRI–Xhol fragment was inserted between these sites in vector pSG1301. pSG4815 and pSG4816 were obtained by digesting chromosomal DNA from strains 2915 and 2916 with Acc65I, ligating in diluted conditions and transforming into *E. coli*. To construct pSG4820, a 1.4 kb DNA fragment containing the minC and minD genes was amplified by PCR from strain 2912 using primers AM15 (5′-ctcgtcacacagcttcg-3′) and OM27 (5′-ttgac gatattttacttctgtactctgaatg-3′), introducing Xhol and EcoRI sites respectively. The EcoRI–Xhol fragment was inserted between these sites in pSG1729.

**General methods**

*Bacillus subtilis* cultures were grown in S medium supplemented with tryptophan (20 μg ml⁻¹) and hydrolysed casein as described by Marston et al. (1998). When necessary, 0.5% xylene was added. *B. subtilis* strains were transformed according to the method of Anagnostopoulos and Spizizen (1961) as modified by Jenkinson (1983). Transformants were selected on Oxoid nutrient agar containing, as necessary, chloramphenicol (5 μg ml⁻¹) or spectinomycin (50 μg ml⁻¹). DNA manipulations and *E. coli* transformations were carried out by standard methods (Sambrook et al., 1989).

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**Table 1. B. subtilis strains and plasmids.**

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</tr>
<tr>
<td>2911</td>
<td>As SG4811, minD::pSG4811 (minD cat) (G12V)</td>
<td>pSG4811—SG38 (CmR)</td>
</tr>
<tr>
<td>2912</td>
<td>As SG4812, minD::pSG4812 (minD cat) (A123P)</td>
<td>pSG4812—SG38 (CmR)</td>
</tr>
<tr>
<td>2913</td>
<td>As SG4813, minD::pSG4813 (minD cat) (N165Y)</td>
<td>pSG4813—SG38 (CmR)</td>
</tr>
<tr>
<td>2916</td>
<td>As SG4814, minD::pSG4814 (minD cat) (S148T)</td>
<td>pSG4814—SG38 (CmR)</td>
</tr>
<tr>
<td>2918</td>
<td>As SG4815, minD::pSG4815 (minD cat) (N165Y)</td>
<td>pSG4815—SG38 (CmR)</td>
</tr>
<tr>
<td>2919</td>
<td>As SG4816, minD::pSG4816 (minD cat) (S148T)</td>
<td>pSG4816—SG38 (CmR)</td>
</tr>
<tr>
<td>2927</td>
<td>As SG4817, minD::pSG4817 (minD cat) (S148T)</td>
<td>pSG4817—SG38 (CmR)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSG1301</td>
<td>bla cat. Integration plasmid</td>
<td>Stevens et al. (1992)</td>
</tr>
<tr>
<td>pSG1293</td>
<td>bra amyE3 spc Pxyf-gfp amyE5</td>
<td>Lewis and Marston (1999)</td>
</tr>
<tr>
<td>pSG1790</td>
<td>bra amyE3 spc Pxyf-gfp–minD amyE5</td>
<td>Marston et al. (1998)</td>
</tr>
<tr>
<td>pSG1736</td>
<td>bra amyE3 spc Pxyf-gfp–minD amyE5</td>
<td>Marston and Errington (1999)</td>
</tr>
<tr>
<td>pSG4801</td>
<td>bra cat minD3</td>
<td>This work</td>
</tr>
<tr>
<td>pSG4801mut</td>
<td>bra cat minDmut</td>
<td>Random mutagenesis</td>
</tr>
<tr>
<td>pSG4804</td>
<td>bra cat minD1 (K16A)</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>pSG4805</td>
<td>bra cat minD2 (D38A)</td>
<td>Site-directed mutagenesis</td>
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<tr>
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<td>bra cat minD3 (D120A)</td>
<td>Site-directed mutagenesis</td>
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<tr>
<td>pSG4807</td>
<td>bra cat minD4 (V142A)</td>
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<td>pSG4808</td>
<td>bra cat minD5 (N174A)</td>
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<tr>
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<td>bra cat minD6 (G12V)</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>pSG4815</td>
<td>bra cat minD15 (N165Y)</td>
<td>Plasmid rescued from strain 2915</td>
</tr>
<tr>
<td>pSG4816</td>
<td>bra cat minD16 (S148T)</td>
<td>Plasmid rescued from strain 2916</td>
</tr>
<tr>
<td>pSG4820</td>
<td>bra amyE3 spc Pxyf-gfp–minD amyE5</td>
<td>This work</td>
</tr>
</tbody>
</table>

a. Note that *minD::pSG4804 (minD1 cat) (K16A)*, for example, means that the non-replicating plasmid pSG4804 has integrated into the minD gene of the recipient strain by single cross-over (selected using the chloramphenicol resistance gene, cat) and that the site of crossing over was such that the *minD1allele* (which carries the K16A amino acid change) is expressed, rather than the wild-type allele.

Site-directed mutagenesis of minD

Plasmid pSG4801 DNA (0.3 ng ml−1) was amplified by PCR (with Pfu polymerase), using one primer containing the desired mutation and a reverse primer (not complementary) with its 5′ extremity precisely one base before the 5′ end of the mutagenic primer. A linear product of the size of the plasmid was purified on a 0.7% agarose gel. Approximately 100 ng of the purified PCR product was incubated with ATP (3 mM), 1 unit of T4 polynucleotide kinase and 1 unit of DNA ligase in ligase buffer (total volume 30 μl) overnight at room temperature. A 15 μl sample of the reaction was used to transform E. coli strain MC1061. The presence of the mutation was checked by DNA sequencing using a departmental service. The resulting plasmids were introduced by single cross-over into the relevant strains, and the integrations were checked by PCR and sequencing.

Random mutagenesis of minD

A 0.7 kbp fragment containing the minD gene was amplified using primers AM1 and AM2 with Taq polymerase in mutagenic conditions. The balance of the dinucleotides was altered (200 μM dGTP and dATP and 1 mM dCTP and dTTP). The concentration of magnesium chloride was increased (6.25 mM) and the following programme was used: 1 min at 95°C, 1 min at 45°C and 1 min 30 s at 72°C for 30 cycles. The mutagenized EcoRI–XhoI DNA fragment was ligated to EcoRI–XhoI-digested pSG1301 and transformed into E. coli strain MC1061. All the ampicillin-resistant transformants were collected and inoculated into 100 ml of 2YT medium. Cells were grown overnight, harvested and used to isolate plasmid DNA (with Plasmid II, Promega) and the plasmid was digested with EcoRI and HindIII. The relevant strains were grown overnight, either in S medium containing 0.5% xylose or on nutrient agar plates with the appropriate antibiotic and 0.5% xylose. The following morning, each strain was inoculated into warm S medium containing 0.5% xylose to give a final OD600 of 0.1. All cultures were grown at 30°C until they reached an OD600 of 0.7–0.8, when samples were taken for microscopy. For cell length analysis, the strains were grown as described above, except that samples were taken when the cultures reached an OD600 of 0.4, and the cells were ethanol fixed as described previously (Hauser and Errington, 1995).

Subcellular localization experiments and cell length analysis

The relevant strains were grown overnight, either in S medium without xylose or on nutrient agar plates with the appropriate antibiotic and 0.5% xylose. The following morning, each strain was inoculated into warm S medium containing 0.5% xylose to give a final OD600 of 0.1. All cultures were grown at 30°C until they reached an OD600 of 0.7–0.8, when samples were taken for microscopy. For cell length analysis, the strains were grown as described above, except that samples were taken when the cultures reached an OD600 of 0.4, and the cells were ethanol fixed as described previously (Hauser and Errington, 1995).

Microscopy

The subcellular localization patterns of GFP–MinD and GFP–MinC were examined in live cells on agarose slides (1.2% in water) as described by Glaser et al. (1997) For cell length analysis, ethanol-fixed cells were also examined on agarose slides. Images were acquired and analysed with a Princeton Instruments Micromax 1300Y/HS CCD camera and METAMORPH version 3.6 software. Final images were assembled using Adobe PHOTOshop version 5.5.

Acknowledgements

This work was supported by a grant from the Biotechnology and Biological Sciences Research Council. M.E.K. was the recipient of an EMBO long-term fellowship, reference number ALTF 209-1999. J.E. is the recipient of a BBSRC Senior Research Fellowship. We thank Alice Taylor for DNA sequencing, and Adele Marston for strains and helpful discussions.

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