Oligomerization of the *Bacillus subtilis* division protein DivIVA

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DivIVA appears to be a mediator of inhibition by MinCD of division at the cell poles in *Bacillus subtilis*. Gel permeation and ultracentrifugation techniques were used to show self-association of DivIVA into a form consisting of 10–12 monomers *in vitro*. Western blot analysis of non-denaturing polyacrylamide gels confirms the presence of similar oligomers in *B. subtilis* cell lysates. These oligomers persist in a *B. subtilis* strain containing the *divIVA1* mutation, in which proper vegetative septum positioning is abolished. In contrast, the *divIVA2* mutation, which has a similar biological impact, appears to produce a protein with different oligomerization properties. The results of the present study suggest that oligomerization of DivIVA is important, but not sufficient for its function in the cell division process.

Keywords: cell division, blue native electrophoresis, analytical ultracentrifugation, sporulation

INTRODUCTION

The process of cell division in both *Escherichia coli* and *Bacillus subtilis*, in common with most bacteria, is initiated by the formation of an FtsZ ring-like structure at the mid-cell site. Selection of this site for cell division is controlled by the Min system. This system is best characterized in *E. coli* where it consists of three proteins: MinC, MinD and MinE. MinC and MinD are inhibitors of septation, and MinE imparts topological specificity, ensuring medial cell division (Rothfield & Zhao, 1996). MinE accumulates at or near the mid-cell site during cell division and the formation of a MinE ring-like structure is dependent on MinD but independent of FtsZ (Raskin & de Boer, 1997). MinE is likely to exist as a mixture of monomers and dimers in the cytoplasm (Zhang *et al.*, 1998). Visualization of MinD in living cells reveals that this protein oscillates rapidly between the cell poles (Raskin & de Boer, 1999). A similar oscillation has been observed for MinE (Fu *et al.*, 2001). The topological specificity of division site placement may therefore not involve the localized action of MinE to counteract MinCD mid-cell division in-

hibition, but instead reflect the ability of MinE to move the division inhibitor away from the mid-cell to the cell poles (Fu *et al.*, 2001).

Although there are many similarities in cell division between *E. coli* and *B. subtilis*, it appears that bacilli employ a different strategy to initiate mid-cell division. *B. subtilis* has MinCD homologues but lacks a MinE counterpart. MinCD inhibition seems to be mediated through the unrelated protein DivIVA. This protein ensures that MinCD specifically inhibits division at the cell poles whilst allowing division at the mid-cell during vegetative growth (Cha & Stewart, 1997; Edwards & Errington, 1997). DivIVA localizes to both cell poles as well as to the mid-cell site (Edwards & Errington, 1997). Localization of DivIVA to division sites is dependent not only on FtsZ, but also on other cell-division proteins, including DivIB, DivIC and PbpB (Marston *et al.*, 1998). DivIVA is targeted to the division sites late in their assembly, where it recruits MinD to prevent another division from taking place near the newly formed cell poles. MinD is required both to pilot MinC to the cell poles and to constitute a functional division inhibitor. Sequestration of MinD and subsequently MinC to the cell poles makes available the next mid-cell sites in the newly formed cells for division. Recent results show that the main role of DivIVA is to retain MinCD at the cell poles after the division is complete. After division nears completion, all of the components of the division

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Abbreviation: GFP, green fluorescent protein.

apparatus except DivIVA seem to disappear from this site. Therefore, it appears that at some point DivIVA must become attached to a more permanent anchor at the mature cell pole (Marston & Errington, 1999).

The same pattern of DivIVA targeting seen in *B. subtilis* was observed when a DivIVA-GFP fusion protein was expressed in *E. coli* (Edwards *et al.*, 2000). The subcellular targeting of DivIVA in *E. coli* is dependent on FtsZ and independent of the MinCD system. However, DivIVA does not seem to be able to substitute for MinE, a topological specificity factor in *E. coli*. Surprisingly, the DivIVA-GFP fusion protein was also directed to nascent division sites in the yeast *Schizosaccharomyces pombe* (Edwards *et al.*, 2000) and it is thus possible that some conserved target for DivIVA exists in both prokaryotes and eukaryotes.

In contrast to *E. coli*, *B. subtilis* can undergo an asymmetric septation during the process of sporulation. It has been shown that at the onset of sporulation, assembly of the FtsZ ring shifts from the mid-cell to both polar sites (Levin & Losick, 1996), the switch being dependent on the transcription factor Spo0A. The FtsZ ring forms initially at both polar sites, implying that MinCD inhibition of FtsZ assembly at both sites must be overcome early in the sporulation process. MinCD-deficient cells that have begun to sporulate can form sporulation-like septa at both the normal asymmetric position as well as at, or near, the mid-cell site (Barák *et al.*, 1998). However, the role of DivIVA in sporulation septation is unclear, although it was proposed that an unknown sporulation factor interacts with DivIVA to make polar sites available for septation (Cha & Stewart, 1997).

DivIVA is a 19.5 kDa cytoplasmic protein that has sequence similarity to homologues from other Gram-positive bacteria and at a lower level to eukaryotic proteins, including myosin (Edwards *et al.*, 2000). Computer analysis of the DivIVA amino acid sequence suggested that the central region of DivIVA might form an α -helical coiled-coil structure *in vivo* (Edwards *et al.*, 2000). Such structures could be used for oligomerization as in tropomyosin (Lupas, 1996). To examine the possibility that the predicted α -helical coiled-coil structure in DivIVA is involved in protein oligomerization, we have cloned the *B. subtilis* *divIVA* gene into an *E. coli* expression system. The encoded protein has been purified to homogeneity and analysed by analytical ultracentrifugation and blue native polyacrylamide gel electrophoresis. We have also characterized the oligomerization state of three different mutant proteins: DivIVA1, DivIVA2 and DivIVA9.

METHODS

Bacterial strains, plasmids and culture media. All bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, *E. coli* and *B. subtilis* cultures were grown in Luria-Bertani (LB) medium (Ausubel *et al.*, 1987). The *B. subtilis* *divIVA* gene was amplified by PCR using

the primers 5'-ATGGAGGTGGCCATATGCCATTAACGCCAAATG-3' and 5'-GGCCATCCCGGATCCTCACCATCTCC-3', introducing *Nde*I and *Bam*HI sites, respectively (underlined). After excision of the band from an agarose gel, PCR product was purified using the Wizard PCR preps DNA purification system (Promega). The *Nde*I-*Bam*HI cut PCR fragment was inserted between the same sites in the plasmid pET26b(+) to generate pETIVA. Plasmids from different clones were isolated, analysed by restriction endonucleases and those with correct length inserts were further analysed by DNA sequencing.

pUKIVA plasmid derivatives were constructed by recloning *divIVA* mutant genes into the pUK19 backbone by using *Bam*HI and *Xba*I sites.

Expression, purification and electrophoresis of proteins.

Protein overexpression was performed by using *E. coli* strain IB706. Cell cultures were grown in LB medium containing 30 μ g kanamycin ml⁻¹ to an OD₆₀₀ of 0.6, when protein expression was induced by the addition of IPTG to a final concentration of 1 mM. After 3 h further growth, the cells were harvested by centrifugation and frozen at -80 °C. Then 3.5 g cells were resuspended on ice in 10 ml buffer A (50 mM Tris/HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF), lysed by sonication and clarified by centrifugation at 30000 g for 30 min. The soluble cell lysate was applied to a 13 ml Q Sepharose Fast Flow column (Pharmacia Biotech) pre-equilibrated in buffer A. DivIVA9 was eluted with a linear gradient of 0.1–0.5 M NaCl. Fractions containing DivIVA9 were diluted fivefold with buffer A and the procedure was repeated using an FPLC Mono Q HR 10/16 column (Pharmacia Biotech). Fractions containing DivIVA9, which eluted at 300 mM NaCl, were concentrated to a volume of 1 ml and loaded onto a Pharmacia Superose 12 gel filtration column.

Western blot analysis of *B. subtilis* cell lysates fractionated by blue native gel electrophoresis was performed as follows. *B. subtilis* cell cultures were grown in DSM medium (Harwood & Cutting, 1988) at 37 °C until the onset of stationary phase. The cells were harvested by centrifugation and resuspended in buffer A containing 0.25 mg lysozyme ml⁻¹. The cells were lysed by sonication and the suspension clarified by centrifugation at 30000 g for 15 min. An equal amount (75 μ g) of protein from each sample was mixed with glycerol (15%, v/v) and electrophoresed through a blue native 5–18% linear gradient polyacrylamide gel. The proteins were blotted onto a nitrocellulose membrane for immunodetection of DivIVA.

Analytical ultracentrifugation. All analytical ultracentrifugation experiments were performed in a Beckman XL-A analytical ultracentrifuge (Beckman-Coulter) using an AN-Ti 60 rotor. The molecular mass averages were determined using the program BIOSPIN and were as follows:

The number average:

$$\langle M_n \rangle = \frac{\sum_i c_i}{\sum_i c_i / M_i}$$

The weight average:

$$\langle M_w \rangle = \frac{\sum_i M_i c_i}{\sum_i c_i}$$

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype, phenotype or relevant characteristics	Source or reference
<i>E. coli</i> strains		
MM294	<i>endA1 hsdR17 supE44 thi-1 recA⁺</i>	Backman <i>et al.</i> (1976)
BL21(DE3)	<i>F⁻ ompT hsdS_B (r_B m_B⁻) gal dcm</i>	Novagen
IB706	pETIVA9 in BL21	This work
IB708	pETIVA2 in BL21	This work
<i>B. subtilis</i> strains		
PY 79	Prototroph	Youngman <i>et al.</i> (1984)
KSS1168	<i>divIVA1 trpC2</i>	Cha & Stuart (1997)
IB703	PY79::pUKIVA9	This work
IB704	PY79::pUKIVA2	This work
Plasmids		
pET26b(+)	Km ^R , <i>lacI</i> ; T7 promoter	Novagen
pETIVA9	Km ^R , <i>lacI</i> ; T7 promoter, <i>divIVA9</i>	This work
pETIVA2	Km ^R , <i>lacI</i> ; T7 promoter, <i>divIVA2</i>	This work
pUK19	Amp ^R Km ^R	Gift from B. Haldenwang
pUKIVA9	Amp ^R Km ^R , <i>divIVA9</i>	This work
pUKIVA2	Amp ^R Km ^R , <i>divIVA2</i>	This work

The z -average molecular mass:

$$\langle M_z \rangle = \frac{\sum_i M_i^2 c_i}{\sum_i M_i c_i}$$

M , molecular mass; c , concentration (mg ml⁻¹).

Light and fluorescence microscopy. Cells were prepared for fluorescence microscopy as described previously (Barák *et al.*, 1996). An Olympus BX60 microscope and MicroImage software was used for microscopy observations, using a filter set for FM 4-64 visualization (550–583 nm excitation and 617–690 nm emission).

RESULTS AND DISCUSSION

Overexpression of *B. subtilis* DivIVA in *E. coli*

To overexpress DivIVA in *E. coli*, the *divIVA* gene was placed under the control of the T7 promoter in plasmid pET26b(+) (see Methods). After transformation of *E. coli* strain MM294, several clones were obtained which were analysed by restriction enzyme cleavage and/or DNA sequencing. Surprisingly, in every case the plasmids had acquired mutations or deletions in the *divIVA* gene even in the absence of induction of *divIVA* expression. In addition to a number of deletions, we have isolated two separate point mutations, *divIVA9* (Glu162Lys) and *divIVA2* (Leu120Pro), along with mutations that introduce stop codons at various positions within the gene. It seems that even low-level expression of *divIVA* is division-inhibitory to *E. coli*. Edwards *et al.* (2000) recently showed that a GFP-DivIVA fusion protein is targeted in *E. coli* to the division sites just as it is in *B. subtilis*. It is likely that DivIVA interacts with the *E. coli* division apparatus in a

way that inhibits growth, so presumably deletions and mutations that negate such inhibition would be selected. Interestingly, deletion mutations in *divIVA* were also acquired when a second copy of *divIVA* was placed under the control of the *spac* promoter in *B. subtilis* (Cha & Stewart, 1997), implying that expression of DivIVA at slightly higher than normal levels is also inhibitory in *B. subtilis*. It is intriguing how such small amounts of protein can influence cell growth. DivIVA probably interacts with one or more components of the division machinery, which is conserved between *B. subtilis* and *E. coli*. A possible partner for such an interaction is FtsA, which has weak homology to eukaryotic actin (Edwards *et al.*, 2000).

Based on the observation that the last seven amino acid residues of the protein are not necessary for the function of DivIVA (Cha & Stewart, 1997), we chose DivIVA9 for our next biochemical experiments. The *divIVA9* gene contains a single base transition at nucleotide 484, which results in a glutamate to lysine amino acid substitution at residue 162 (the third residue from the C terminus) in the protein product. Protein was overexpressed in *E. coli* BL21(DE3) transformed with pETIVA9. SDS-PAGE analysis of cell extracts showed a high level of DivIVA9 production (not shown). Although large amounts of DivIVA9 partitioned into the membrane fraction, sufficient DivIVA9 remained in the soluble fraction to allow protein purification. FPLC purified protein was loaded on a Superose 12 gel filtration column (see Methods). DivIVA9 exhibits a low retention volume, suggesting it exists as a high molecular mass species. To determine the molecular mass of this oligomer, blue native polyacrylamide gradient gel electrophoresis and analytical ultracentrifugation studies were performed.

Fig. 1. Electrophoretic analysis of DivIVA proteins. (a) 5–18% gradient blue native gel electrophoresis. Lanes: 1, molecular mass marker – ferritin dimer (880 kDa) and ferritin monomer (440 kDa); 2, 100 µg cell lysate from induced *E. coli* IB706 strain; 3, DivIVA9 fraction after FPLC Mono Q HR 10/16 column; 4, DivIVA9 fraction after Superose 12 gel filtration column; 5, molecular mass marker – BSA dimer (132.4 kDa) and monomer (66.2 kDa). (b) Western blot analysis of DivIVA proteins fractionated by blue native 8–12% gradient gel electrophoresis. DivIVA was detected by incubation with the primary antiserum diluted 1:2000, followed by alkaline phosphatase-conjugated anti-mouse IgG diluted 1:1000. Lanes: 1, DivIVA9 protein (1 µg) expressed and purified from *E. coli*; 2–4, 75 µg cell lysates from *B. subtilis* strains PY79 (wild-type) (2), KSS1168 (*divIVA1*) (3), IB703 (*divIVA9*) (4); 5, 6 µg cell lysate from the *E. coli* IB708 (*divIVA2*) expression strain after 3 h induction with 1 mM IPTG; 6, 6 µg cell lysate from the *E. coli* IB706 (*divIVA9*) expression strain after 3 h induction with 1 mM IPTG. Molecular mass markers were ferritin monomer (440 kDa), BSA dimer (132.4 kDa), BSA monomer (66.2 kDa) and soybean trypsin inhibitor (21.5 kDa). The protein oligomers corresponding to 10–12 mer (A) and 5–6 mer (B) are marked by arrows.

Blue native gel electrophoresis

The negative charge of protein-bound Coomassie blue G-250 dye is the main determinant of sample electrophoretic mobility in this method, which is suitable for determining the apparent molecular masses of proteins with a pI below 8.6 (Schägger *et al.*, 1994). The theoretical pI of DivIVA is 4.6, thus DivIVA was resolved by blue native gel electrophoresis with a 5–18% linear acrylamide gradient. The electrophoretic mobility of protein standards is linearly dependent on $\log M_r$, allowing the molecular mass of DivIVA9 to be estimated at 200 kDa, corresponding to a decamer (Fig. 1a).

Analytical ultracentrifugation

Analytical ultracentrifugation is a preferred method of determining absolute molecular mass since, unlike gel filtration, it does not depend on the assumption that the macromolecule under investigation is spherical (Harding, 1994; Laue & Stafford, 1999). To explore oligomerization of DivIVA9, sedimentation equilibrium studies were carried out at loading concentrations of 0.1, 0.25 and 0.5 mg ml⁻¹. The number average molecular mass M_n , the weight average molecular mass M_w and the z -average molecular mass M_z were determined (Roarke & Yphantis, 1969). Each of these molecular mass averages is weighted increasingly towards the higher oligomers, so that the number average will reflect the lower order molecular mass oligomers, whereas the z -average molecular mass reflects the higher order oligomers. The oligomeric state of DivIVA9 increases from a value expected for a dimer, to one corresponding

to a 10–12 mer (Fig. 2a). To assess the stoichiometry of oligomerization further, two species plots of $1/M_w$ vs $1/M_z$ were made (Roarke & Yphantis, 1969). The two plots for the highest loading concentrations of DivIVA9 suggest that the lowest observed molecular mass corresponds to a dimer whereas the highest is consistent with a 10–12 mer (Fig. 2b). Since the molecular mass averages approach an asymptotic value, it can be concluded that higher order aggregates of DivIVA9 do not exist under the experimental conditions. These results, and those from blue native gel electrophoresis, are in good agreement. It appears therefore that DivIVA9 forms several oligomers *in vitro*, the largest of which is a 10–12 mer.

DivIVA mutant proteins oligomerize differently

The purified DivIVA9 protein was also used to raise a murine polyclonal antiserum that had a high specificity as revealed by Western blotting (Fig. 1b). To ascertain whether DivIVA forms the same oligomer *in vivo*, we performed Western blot analyses of *B. subtilis* cell lysates fractionated by blue native gel electrophoresis. It is evident that DivIVA from wild-type *B. subtilis* cell lysates forms the same oligomer as the purified DivIVA9 protein from *E. coli* (Fig. 1b; lanes 1 and 2). It is important to emphasize that with this technique we are examining the soluble DivIVA fraction. Western blots of SDS-PAGE gels revealed that up to 40% of DivIVA was associated with the membrane fraction (not shown). It is possible that membrane-associated DivIVA is assembled into the ring-like structure that has been observed

Fig. 2. (a) Molecular mass averages of DivIVA9 derived from analytical ultracentrifugation at a rotor speed (Beckman AnTi rotor) of 15000 r.p.m. The molecular mass averages generated are: number average, M_n (filled circles); $M_{y,1}$, the smallest observable species (filled squares); molecular mass average, M_w (open triangles) and z-average molecular mass, M_z (open diamonds). The smallest observable species is a dimer, whereas the largest is at least a 10 mer. (b) Two species plot of $1/M_w$ vs $1/M_z$ of two loading concentrations of DivIVA9 at 15000 r.p.m. (closed circles). The open circles represent the calculated molecular masses of each of the oligomeric species as a comparison. Here it can be seen that the molecular mass averages extend from values associated with a dimer to that associated with at least a 10 mer.

previously at mid-cell and polar septal sites (Edwards & Errington, 1997). It is not known whether DivIVA 10–12 mers observed here are intermediates for the assembly of these rings.

Western blot analysis of *B. subtilis* cell lysates revealed the presence of the same 10–12 mer in the *divIVA1* mutant strain (Fig. 1b; lane 3). This allele contains a G to A transition, resulting in an alanine to threonine substitution at amino acid residue 78. It is predicted that this amino acid is located in the hydrophobic core of the proposed coiled-coil structure. This suggests that the loss of DivIVA function in this mutant strain is not due to altered oligomerization and that oligomerization *per se* is not sufficient for correct DivIVA function. An

alternative interpretation is that this mutation affects the interaction of DivIVA with other components of the division machinery. The single DivIVA band corresponding to the 10–12 mer with no trace of smaller or higher order oligomeric forms is in contrast to the analytical ultracentrifugation observations, which suggest oligomers ranging from dimers to 10–12 mers. We assume that in native blue electrophoresis experiments the 10–12 mer of DivIVA is stabilized by the glycerol present in the sample buffer in these experiments.

In contrast to the above, the protein DivIVA2 behaves differently, with 5–6 mer complexes prominent in addition to the 10–12 mers (Fig. 1b; lane 5). The *B. subtilis* IB704 strain harbouring the *divIVA2* mutation grows so poorly that we were unable to load enough cell lysate to clearly detect the DivIVA2 protein. Fig. 1(b) shows recombinant DivIVA2 in an *E. coli* cell lysate (lane 5). The *divIVA2* mutation causes a Leu to Pro substitution at amino acid residue 120, which is also predicted to be located in the hydrophobic core, but at the end of the proposed coiled-coil structure. This mutation is probably not compatible with the proposed coiled-coil structure in this region (Edwards *et al.*, 2000).

Biological effect of *divIVA* mutations

The *divIVA1* mutation causes a misplacement of the septum during vegetative growth, resulting in the formation of mini-cells and filaments (Cha & Stewart, 1997; Edwards & Errington, 1997). To check the phenotype of the *divIVA9* and *divIVA2* mutants, we transformed derivatives of plasmid pUK19 containing the cloned mutant genes into the wild-type *B. subtilis* strain PY79 selecting for kanamycin resistance. Correct recombination by a single crossover event to replace the wild-type copy of *divIVA* with the two mutant genes was checked by Southern blot hybridization analysis (not shown). Light microscopy revealed that the *divIVA9* mutation has no significant effect on cell length or morphology, and no mini-cells were observed (Fig. 3a, c). In contrast, the *divIVA2* mutant has a similar phenotype to *divIVA1* mutant cells, characterized by longer cells and easily detectable mini-cells (Fig. 3b, d). The formation of septa in the wild-type and mutant *divIVA* strains was observed by fluorescence microscopy using the vital stain FM 4-64 (Fig. 3a*–d*). This stain specifically labels the lipid bilayer of the plasma membrane and is useful for the detection of septa (Pogliano *et al.*, 1999). Normal septa are formed in wild-type and *divIVA9* mutant strains, in contrast to the *divIVA1* and *divIVA2* mutant strains in which many partially formed septa are visible (Fig. 3a*–d*).

DivIVA is a key protein in septum positioning during binary fission in *B. subtilis* and the elucidation of its function is crucial for our understanding of bacterial cell division. In addition to its role in mid-cell division, DivIVA may also be involved in the regulation of polar division, a process that occurs at the onset of spore formation. Multimerization of DivIVA appears to be an important element in its biological function. The

Fig. 3. Light and fluorescence micrographs of *B. subtilis* late-vegetative growth cultures of the following strains: a and a*, wild-type PY79; b and b*, KSS1168 (*divIVA1*); c and c*, IB703 (*divIVA9*); d and d*, IB704 (*divIVA2*). Panels a, b, c and d are light micrographs, where the formation of mini-cells is marked by arrows. Panels marked with an asterisk are fluorescence micrograph counterparts of panels a, b, c and d, showing cell membranes by detection of the fluorescent stain FM 4-64. The scale bar (4 μ m) is at the bottom-right of the panel.

smallest observable species corresponds to a dimer, and the largest to either a 10, 11 or 12 mer. Macromolecules that exhibit a mixture of five- and twofold symmetry occur frequently in biology, a well-known example being viruses. Similarly, RecA displays six- and twofold symmetry (Brenner *et al.*, 1990), and the functional unit of the RNA-binding protein from *B. subtilis*, TRAP, with which our laboratory is familiar is an 11 mer (Antson *et al.*, 1999). Clearly, the oligomerization of DivIVA raises new questions requiring additional experimentation.

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