The *Bacillus subtilis* DivIVA Protein Has a Sporulation-Specific Proximity to Spo0J

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The *Bacillus subtilis* DivIVA protein controls the positioning of the division site and the relocation of the chromosome during sporulation. By performing coimmunoprecipitation experiments, we demonstrated that a myc-DivIVA protein is in proximity to FtsZ and MinD during vegetative growth and Spo0J during the first 120 min of sporulation.

The process by which *Bacillus subtilis* changes from vegetative growth into a distinct developmental pathway that results in spore formation has been studied extensively (5, 9). A key event in the initial stages of spore development is the movement of the two chromosomes away from the cell center and their eventual attachment to the cell poles (axial filament formation). This occurs in two distinct steps, beginning with the extraction of the chromosomal origin from its vegetative position and movement towards the cell pole (18). In a second step, the chromosome is decorated with the DNA binding protein RacA and is attached to the cell pole (1, 17). Interestingly, both of these events require the vegetative cell division positioning protein DivIVA (2, 4). DivIVA is a small, predominantly coiled-coil protein that is recruited to the vegetative cell division site after the assembly of FtsZ (12) and the incorporation of FtsW (data not shown). DivIVA remains associated with the division site as it matures and eventually splits to form the two new cell poles (4). The subcellular localization of DivIVA is crucial for the correct distribution of a bipartite cell division inhibitor complex consisting of the MinC and MinD proteins (MinCD) (12). By maintaining MinCD at the cell pole, DivIVA prevents the assembly of an FtsZ ring in the chromosome free space at the cell pole and promotes vegetative division at the midcell. Recently we identified a polar targeting mutant of DivIVA that functions in both vegetative growth and sporulation (14). DivIVA_{R18C} localizes to the chromosome in the presence of Spo0J/Soj and can be observed to occur transiently at the cell division site. It appears that the temporary association of this mutant protein with the division site is sufficient to partially localize MinCD. More intriguingly, the association of the mutant protein with the chromosome is sufficient to allow the relocation of the chromosome by MinD and Spo0J/Soj at the onset of sporulation. To identify proteins in proximity to DivIVA and DivIVA_{R18C}, we have developed a coimmunoprecipita-

TABLE 1. Complementation of a *divIVA* deletion by ectopic expression of myc-tagged versions of *divIVA*, *divIVA*_{R18C}, and *divIVA*_{W148A}

Strain (genotype)	Avg (±SD) cell length (μm) ^a	Frequency of minicells (%)	Sporulation efficiency (%) ^b
SE85 (<i>divIVA</i>)	4.9 ± 1.1	0.6	100
SE65 (<i>myc-divIVA</i>)	4.8 ± 1.2	1.4	100
SE35 (<i>divIVA</i> _{R18C})	5.6 ± 3.4	11.1	50
SE66 (<i>myc-divIVA</i> _{R18C})	6.2 ± 2.7	9.6	ND
SE47 (<i>divIVA</i> _{W148A})	18.3 ± 12.1	16.8	1
SE68 (<i>myc-divIVA</i> _{W148A})	14.7 ± 10.1	11.6	ND

^a More than 200 cells were measured (8).

^b Percentages are relative to levels for wild-type strain 168 and are the results of more than three experiments. ND, not determined.

tion (co-IP) protocol for the isolation of myc-DivIVA-containing complexes.

Immunoprecipitation of DivIVA interacting proteins. To precipitate a DivIVA complex from *B. subtilis*, we utilized a

TABLE 2. Bacterial strains used in this study

Strain	Genotype	Construction ^b	Reference or source
1751	<i>divIVA1</i>		4
1901	<i>trpC2 amyE ΔminD::ermC</i>		12
SE39	<i>trpC2 ΔminD::ermC</i>		14
SE65 ^a	<i>ΔdivIVA::tet</i>		This work
SE66 ^a	<i>ΔdivIVA::tet amyE::P_{spac}-myc-divIVA</i>		This work
SE68 ^a	<i>ΔdivIVA::tet amyE::P_{spac}-myc-divIVA_{R18C}</i>		This work
SE74	SE65 <i>ΔminD::erm</i>	1901(chr) → SE65	
SE75	SE66 <i>ΔminD::erm</i>	1901(chr) → SE66	
SE78	<i>trpC2 Δ(spo0J-soj)::spec</i>	AG1505(chr) → 168	10
SE79	SE78 <i>ΔdivIVA::tet</i>	pSP22 → SE78	
SE80	SE78 <i>ΔdivIVA::tet</i>	SE39(chr) → SE78	
SE81	SE39 <i>Δ(spo0J-soj)::kan</i>	JH21063(chr) → SE39	18
SE82	SE81 <i>amyE::P_{xyt} gfp-minD</i>	pMinD4 → SE81	
SE83	SE39 <i>amyE::P_{xyt} gfp-minD</i>	pMinD4 → SE39	
SE85 ^a	<i>trpC2 ΔdivIVA::tet amyE::P_{spac}-divIVA</i>		This work

^a Additional information on strain construction is available on request.

^b Plasmid or chromosomal (chr) DNA from the strain to the left of the arrow was used to transform the strain to the right of the arrow.

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FIG. 1. Identification of candidate proteins from co-IP experiments performed with myc-DivIVA variants during vegetative growth. Proteins were eluted after co-IP experiments using strains expressing DivIVA, myc-DivIVA, myc-DivIVA_{R18C}, or myc-DivIVA_{W148A}. Samples were separated by SDS-PAGE and Western blotting. Western blots were probed with (A) anti-FtsZ, (B) anti-MinD, (C) anti-Spo0J, or (D) anti-YpsB. Lanes C, proteins precipitated from strain SE85 (*divIVA*); lanes L, loading control consisting of whole-cell extract from strain SE65 (myc-*divIVA*); lanes 1, proteins precipitated from strain SE65 (myc-*divIVA*); lanes 2, proteins precipitated from strain SE66 (myc-*divIVA*_{R18C}); lanes 3 (for panels A and B), proteins precipitated from strain SE68 (myc-*divIVA*_{W148A}); lane 4 (for panel C), proteins precipitated from strain SE75 (Δ *minD* myc-*divIVA*_{R18C}). Bars show the sizes and positions of molecular size markers. nd, not determined.

myc-DivIVA protein that could support both normal vegetative growth and wild-type levels of sporulation (Table 1). For our preliminary co-IP experiments, we used strain SE65, which contains a *divIVA* deletion (Δ *divIVA*) and an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible myc-*divIVA* (*P*_{spac} myc-*divIVA*::*amyE*) (for descriptions of strains and plasmids used in this study, see Tables 2 and 3, respectively). This strain is viable and when grown in the presence of 0.5 mM IPTG has a phenotype similar to that of the isogenic strain SE85 (Δ *divIVA* *P*_{spac} *divIVA*::*amyE*) (Table 1). In order to validate our protocol, we also chose to work with two partially active mutant proteins. The first, DivIVA_{R18C}, is unable to target the cell poles and localizes to the chromosome in a Spo0J/Soj-dependent manner (14). The second, DivIVA_{W148A}, has not been described previously and contains a mutation in a highly conserved tryptophan that is located close to the C terminus of the protein. In complementation experiments, this mutant was able to support vegetative growth but produced a phenotype similar to that of the original *divIVA1* mutant (16) (Table 1). The two alleles were cloned to enable the controllable expres-

sion of the epitope-tagged derivatives myc-*divIVA*_{R18C} and myc-*divIVA*_{W148A} in a *divIVA* deletion background (Table 1).

In preliminary studies, we performed co-IP experiments with mid-exponential-phase cultures of test strain SE65 (myc-*divIVA*::*amyE*) and control strain SE85 (*divIVA*::*amyE*). All cells were grown in LB supplemented with 0.5 mM IPTG to an optical density at 600 nm of 0.1. A 20-ml aliquot of the culture was harvested by centrifugation at 4,000 rpm for 5 min and protoplasts produced by incubation for 20 min at 37°C in 1× SMM (0.5 M sucrose, 20 mM maleic acid, 20 mM MgCl₂, pH 6.5) with 1 mg/ml lysozyme and 1 U/ml DNase I. The protein concentration of each sample was determined using the Bradford assay (1a). Dithiobis(succinimidyl propionate) (11) (20 μ mol per 100 μ g protein) was added to each aliquot to cross-link proteins within the protoplasts. The cross-linking reactions were allowed to proceed for 5 min at 37°C. Excess dithiobis(succinimidyl propionate) was inactivated by the addition of 20 mM Tris-HCl, pH 7.5 (final concentration), and the protoplasts lysed by the addition of 0.02% to 0.05% Triton X-100 (final concentration). Dyna-

FIG. 2. myc-DivIVA is in proximity to Spo0J during the early stages of sporulation. (A and B) Western blots of SDS-PAGE gels probed with anti-Spo0J. For panel B, proteins were isolated from strain SE75 (Δ *minD* myc-*divIVA*_{R18C}). Lanes C, whole-cell extract from strain SE65 (myc-*divIVA*), lanes 10 to 180, proteins precipitated from strain SE65 (myc-*divIVA*) 10, 90, 120, or 180 min after inducing sporulation. (C and D) Western blots of SE65 whole-cell extracts separated by SDS-PAGE and probed with (C) anti-Spo0J or (D) anti-DivIVA. Lanes from left to right represent samples taken immediately prior to resuspension (0 min) and then at 10, 90, 120, and 180 min after inducing sporulation, respectively.

FIG. 3. Phenotype of strains SE79 and SE80 [$\Delta divIVA \Delta (spo0J-soj)$] and localization of GFP-MinD in the absence of DivIVA and Spo0J/Soj. (A) Phase-contrast image of mid-exponential-growth-phase SE79 cells fixed in 70% ethanol for measurement (8). (B) Corresponding fluorescent image. Chromosomes were stained with DAPI (4',6'-diamidino-2-phenylindole) to reveal the positions of the nucleoids. (C) Minicells of SE79 and SE80 [$\Delta divIVA \Delta (spo0J-soj)$] strains. All differential interference contrast (DIC) images were obtained using a 100 $\times$ objective, 1.6 $\times$ Optivar, Hamamatsu Orca ERII charge-coupled-device camera and Openlab 3.0.7 (Improvision). (i) Oblique minicell observed in SE79. The arrow indicates the production of an oblique minicell, a characteristic shared with the original *divIVA1* mutant (4, 16). (ii) Different-sized minicells observed in SE80. The arrow indicates a larger-than-usual minicell that has arisen from a second cell division close to the cell pole. (iii) Pairs of SE80 minicells arising from the cell pole. The arrow indicates a small minicell that is the result of an oblique cell division. (D and E) Expression of GFP-MinD in (D) SE83 ($\Delta divIVA \Delta minD$) and (E) SE82 [$\Delta divIVA \Delta minD \Delta (spo0J-soj)$]. (i) Phase-contrast images of living cells induced to express GFP-MinD by the addition of 0.1% xylose for 30 min. (ii) Corresponding fluorescent images, revealing (for panel D) localization of GFP-MinD as transverse bands and (for panel E) punctate localization of GFP-MinD. Arrows indicate the positions of obvious cell division sites. Bar, 10 μ m.

beads protein G coated magnetic beads (DynaL Biotech Ltd.), which had been cross-linked to mouse monoclonal anti-c-myc antibody 9E10, were then added to each sample. The magnetic bead-cell extract mix was then incubated at 25°C on a rotary shaker for 1 h. The beads were washed four

times with 1 ml phosphate-buffered saline. Next, the myc-DivIVA-containing complexes were eluted in 1 $\times$ Laemmli sample buffer supplemented with dithiothreitol, and the cross-links holding the complexes together were reduced. Eluted proteins were separated by sodium dodecyl sulfate-

TABLE 3. Plasmids used in this study

Plasmid	Genotype	Description ^a	Reference
pSP22	<i>bla cat 3'ylmH tet ΔdivIVA ileS5'</i>		14
pMinD4	<i>bla amyE3' spc P_{yl} gfp-minD' amyE5'</i>	<i>'minD</i> (PCR BamHI) into pSG1729 (BamHI-EcoRV)	
pPYrbs	<i>bla amyE3' cat P_{spac} rbs lacI 'spoVG-lacZ amyE5'</i>	Annealed primers RBS1 and RBS2 into pPY18 (HindIII-XbaI)	3
pPYmyc	<i>bla amyE3' cat P_{spac} rbs myc lacI 'spoVG-lacZ amyE5'</i>	Annealed primers MYC1 and MYC2 into pPYrbs (XbaI-BglIII)	
pDH3	<i>bla amyE3' cat P_{spac} divIVA lacI amyE5'</i>		14
pSP4	<i>bla amyE3' cat P_{spac} divIVA_{R18C} lacI amyE5'</i>		14
pSP12	<i>bla amyE3' cat P_{spac} divIVA_{W148A} lacI amyE5'</i>		This work
pSP26	<i>bla amyE3' cat P_{spac} myc-divIVA lacI 'spoVG-lacZ amyE5'</i>	<i>'divIVA</i> (PCR BglII-ClaI) into pPYmyc	
pSP27	<i>bla amyE3' cat P_{spac} myc-divIVA_{R18C} lacI 'spoVG-lacZ amyE5'</i>	<i>'divIVA_{R18C}</i> (PCR BglII-ClaI) into pPYmyc	
pSP30	<i>bla amyE3' cat P_{spac} myc-divIVA_{W148A} lacI 'spoVG-lacZ amyE5'</i>	<i>'divIVA_{W148A}</i> (PCR BglII-ClaI) into pPYmyc	

^a PCR fragments were amplified from purified chromosomal or plasmid DNA with specific primers incorporating suitable restriction sites. The sequences of primers and additional information on plasmid and mutant construction are available on request.

polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting. This approach confirmed that myc-DivIVA could be precipitated from strain SE65 (*myc-divIVA::amyE*) but not from strain SE85 (*divIVA::amyE*) (data not shown). We repeated the procedure with several strains and screened for the precipitation of four candidate proteins, MinD, FtsZ, Spo0J, and YpsB. MinD is known to require the presence of DivIVA for its correct localization, and FtsZ is required for the recruitment of DivIVA to the cell division site (12). Spo0J is required for the proper organization and trapping of the chromosome origin at the onset of sporulation (18) and is essential for the localization of a DivIVA_{R18C} mutant to the chromosome (14). The fourth protein, YpsB, localizes to the cell division site in an FtsZ-dependent manner but does not interact with either DivIVA or MinD (data not shown) and was therefore intended to act as a negative control.

The FtsZ and MinD proteins were precipitated with myc-*divIVA*, but Spo0J and YpsB were not (Fig. 1, lane 1). The precipitation of MinD was expected, and the identification of FtsZ is consistent with the subcellular location of DivIVA at the cell division site. The myc-DivIVA_{R18C} and myc-DivIVA proteins precipitated similar levels of FtsZ (Fig. 1, lane 2), but myc-DivIVA_{R18C} precipitated lower levels of MinD (Fig. 1, compare lane 1 and lane 2). myc-DivIVA_{R18C} does not localize to the cell pole (14) and therefore would be expected to precipitate less MinD. However, since myc-DivIVA_{R18C} is rarely observed to occur at the cell division site, the amount of FtsZ precipitated by this mutant protein was surprising. A possible explanation may be provided by the observation that a ring of DivIVA molecules is visualized only rarely in close proximity to FtsZ and the majority

of DivIVA at the midcell is actually incorporated into a coating that lines the invaginating septum (7). One hypothesis would be that the rarely observed coincidence of DivIVA and FtsZ represents the initial recruitment of the protein and that this is the same point in division when DivIVA_{R18C} is in proximity to the division apparatus. We then envisage that, once recruited, DivIVA goes on to form the coating that is ultimately responsible for the polar cap, a process that DivIVA_{R18C} cannot follow. While this hypothesis would explain why both proteins precipitate similar amounts of FtsZ, we cannot exclude the possibility that myc-DivIVA_{R18C} is locked in a conformation that remains associated with FtsZ molecules after it has left the division site or that the mutant protein may interact with FtsZ molecules that are not part of the cell division ring. More importantly, myc-DivIVA_{R18C} also precipitated Spo0J, and the isolation of this protein was not dependent on MinD (Fig. 1, lane 2 and lane 4). This set of biochemical data is consistent with genetic and cell biology data which indicate that DivIVA_{R18C} and Spo0J are in close proximity during vegetative growth (14). To understand the biological significance of this result, we turned our attention to sporulation, specifically, the initial 90 min when DivIVA, MinD, and Spo0J/Soj are all required for the relocation of the origin (14).

Strains expressing myc-DivIVA in the presence (SE65) or absence (SE74) of MinD were harvested at time points from t_{10} (10 min after the initiation of sporulation) to t_{180} (13). Spo0J was found to precipitate with myc-DivIVA in a MinD-independent manner during the first 120 min of sporulation (Fig. 2A and B). Notably, the amount of Spo0J precipitated remained constant from t_{10} to t_{120} ; however, by t_{180} the amount of Spo0J pulled down with myc-DivIVA decreased dramatically. We confirmed by Western blotting that this reduction was not caused by a change in the levels of either Spo0J or myc-DivIVA (Fig. 2C and D). We also determined the amount of myc-DivIVA precipitated at t_{180} and noted that the efficiency of our co-IP experiments did not alter (data not shown). Therefore, we propose that an association between myc-DivIVA and Spo0J occurs at a specific point during development and that myc-DivIVA_{R18C} is trapped in a conformation that mimics this developmental change.

***divIVA* can be deleted in the absence of *spo0J-soj*.** We were unable to inactivate *divIVA* in a wild-type background and could obtain a viable deletion strain only in the absence of either *minD* or *minC* (4, 14; also data not shown). Since myc-DivIVA could be demonstrated to precipitate with both MinD

TABLE 4. Summary of phenotypes of strains SE79, SE78, and 1751

Strain (genotype)	Avg ($\pm$ SD) cell length (μ m) ^a	Frequency of minicells (%)	Sporulation efficiency (%) ^b
SE79 ^c [Δ <i>divIVA</i> Δ (<i>spo0J-soj</i>)]	12.5 $\pm$ 10	26	0.03
SE78 [Δ (<i>spo0J-soj</i>)]	4.7 $\pm$ 1.9	0	120 ^d
1751 (<i>divIVA1</i>)	15.8 $\pm$ 11.1	12.2	1

^a More than 200 cells were measured (8).

^b Percentages are relative to levels for wild-type strain 168 and are the results of more than three experiments.

^c Isogenic strain SE80 produced almost-identical figures, which are available upon request.

^d See reference 18.

and Spo0J, we determined whether *divIVA* could be deleted in the absence of *spo0J-soj*. Strain SE78 [$\Delta(spo0J-soj)$] was transformed with the *divIVA* deletion plasmid pSP22 (14), and 28 Tet^r Cam^s colonies were identified. For one transformant (SE79), we confirmed the deletion of *divIVA* by PCR and the genotype [$\Delta divIVA \Delta(spo0J-soj) minD^+$] by Western blotting (data not shown). We considered it necessary to repeat this result by transforming the *spo0J-soj* deletion strain (SE78) with chromosomal DNA isolated from SE39 ($\Delta minD::erm \Delta divIVA::tet$). Once again we confirmed that a deletion of *divIVA* could be tolerated in the presence of *minC* and *minD* (SE80). Analysis of strains SE79 and SE80 revealed an identical phenotype that was characterized by a mixture of filamentous cells and minicells (Table 4 and Fig. 3A and B). Although their phenotype was similar to the original *divIVA1* phenotype (4, 16), SE79 and SE80 produced a significantly higher percentage of minicells. Unusually, these minicells often occurred in pairs or short chains that suggested successive rounds of polar cell division (Fig. 3C).

GFP-MinD does not recognize the septum in the absence of DivIVA and Spo0J/Soj. The lethality of a *divIVA* deletion is proposed to result from the uncontrolled activity of the bipartite cell division inhibitor complex MinCD (2, 4). Therefore, to understand the viability of SE79 and SE80 we determined the localization of MinCD in the absence of *divIVA* and *spo0J-soj*. For this work, we constructed a strain (SE82) that combined the *spo0J-soj*, *divIVA*, and *minD* deletions with a xylose-inducible green fluorescent protein (GFP)-MinD fusion protein. In the absence of xylose, SE82 exhibited the minicell phenotype characteristic of a *minD* deletion strain. In the presence of 0.1% xylose, GFP-MinD was expressed and after 30 min the cells began to filament. When we examined these cells by epifluorescence microscopy, we observed discrete patches of fluorescence but no bands of GFP-MinD at potential cell division sites (Fig. 3E, panel ii). This pattern contrasted with that of our control strain SE83 ($\Delta divIVA \Delta minD P_{xy} gfp-minD$), which under identical conditions produced a diffuse GFP-MinD signal that appeared to coat the cytoplasmic membrane and form a series of transverse bands that corresponded to the location of septa or presumed cell division sites (Fig. 3D, panel ii). Therefore, we conclude that in the absence of both *spo0J-soj* and *divIVA* MinD assembles into patches on the cell membrane and that this is sufficient to titrate the level of MinCD inhibitor and allow a small number of FtsZ rings to assemble.

In conclusion, by using a fully functional epitope-tagged version of DivIVA, we demonstrated in co-IP experiments that myc-DivIVA is in proximity to FtsZ and MinD during vegetative growth and Spo0J during sporulation. This proximity to Spo0J does not exist prior to resuspension but occurs rapidly upon resuspension and is independent of MinD. Therefore, the key question of how DivIVA switches from controlling the bipartite cell division inhibitor MinCD during vegetative growth to controlling the positioning of the chromosome during the initial stages of sporulation appears to involve

an increased proximity to Spo0J. This association between DivIVA and Spo0J may explain the chromosome partition function assigned to other members of the DivIVA protein family (6, 15).

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