RESEARCH LETTER

Domains involved in the *in vivo* function and oligomerization of apical growth determinant DivIVA in *Streptomyces coelicolor*

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Abstract

The coiled-coil protein DivIVA is a determinant of apical growth and hyphal branching in *Streptomyces coelicolor*. We have investigated the properties of this protein and the involvement of different domains in its essential function and subcellular targeting. In *S. coelicolor* cell extracts, DivIVA was present as large oligomeric complexes that were not strongly membrane associated. The purified protein could self-assemble into extensive protein filaments *in vitro*. Two large and conspicuous segments in the amino acid sequence of streptomycete DivIVAs not present in other homologs, an internal PQG-rich segment and a carboxy-terminal extension, are shown to be dispensable for the essential function in *S. coelicolor*. Instead, the highly conserved amino-terminal of 22 amino acids was required and affected establishment of new DivIVA foci and hyphal branches, and an essential coiled-coil domain affected oligomerization of the protein.

Introduction

Two distinct ways have been recognized in bacteria for directing elongation of the peptidoglycan cell wall and the acquisition of rod shape (Daniel & Errington, 2003). In most rod-shaped bacteria, including *Escherichia coli* and *Bacillus subtilis*, the actin-like MreB proteins form a helical cytoskeleton that organizes the insertion of new peptidoglycan into the lateral cell wall, thus leading to controlled elongation of the cell with maintained cell shape (reviewed in e.g. Carballido-Lopez, 2006; den Blaauwen *et al.*, 2008). An alternative mechanism is used by members of the phylum *Actinobacteria*, which do not use MreB proteins for cell elongation (Mazza *et al.*, 2006; Flärdh & Buttner, 2009) and that build their cell walls at the cell pole rather than inserting new material along the lateral wall (Flärdh, 2003b; Ramos *et al.*, 2003; Chauhan *et al.*, 2006). This apical growth appears to be directed by the coiled-coil protein DivIVA in organisms such as *Streptomyces*, *Corynebacterium*, and *Mycobacterium* (Flärdh, 2003b; Nguyen *et al.*, 2007; Hempel *et al.*, 2008; Kang *et al.*, 2008; Letek *et al.*, 2008).

Orthologs of DivIVA are ubiquitous among Gram-positive bacteria and have been associated with a range of processes. The *B. subtilis* DivIVA controls cell division by sequestering the division inhibitors MinC and MinD at the cell poles, effectively preventing polar divisions while allowing normal assembly of FtsZ rings at the cell midpoint (Cha & Stewart, 1997; Edwards & Errington, 1997; Marston *et al.*, 1998; Marston & Errington, 1999). The interaction between DivIVA and MinD is indirect and appears to be mediated by the MinJ protein (Bramkamp *et al.*, 2008; Patrick & Kearns, 2008). In addition, DivIVA is involved in attachment of chromosomes to the cell poles at the initiation of sporulation in *B. subtilis*, possibly via interactions with the RacA protein (Thomaides *et al.*, 2001; Ben-Yehuda *et al.*, 2003; Wu & Errington, 2003). Among other investigated members of the Gram-positive phylum *Firmicutes*, *divIVA* was implicated in cell division, cell shape, and chromosome segregation in *Enterococcus faecalis* (Ramirez-Arcos *et al.*, 2005; Rigden *et al.*, 2008), and affected cell shape, division, and maturation of cell poles in *Streptococcus pneumoniae* (Fadda *et al.*, 2003, 2007). In contrast, the investigated DivIVA orthologs from the other major type of

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Gram-positive bacteria, the *Actinobacteria*, have different functions and are involved in polar growth of the cell wall (Flärdh, 2003b; Nguyen *et al.*, 2007; Hempel *et al.*, 2008; Kang *et al.*, 2008; Letek *et al.*, 2008).

The most pronounced apical growth occurs in streptomycetes, which grow by tip extension and form branched hyphae and mycelia (Flärdh, 2003a; Flärdh & Buttner, 2009). The essential DivIVA protein accumulates at growing hyphal tips in *Streptomyces coelicolor* and strongly influences cell shape determination (Flärdh, 2003b). In addition, DivIVA appears to mark sites where new branches will appear and can recruit or activate the machinery for cell wall synthesis at new sites (Hempel *et al.*, 2008). Although *S. coelicolor* DivIVA can complement a DivIVA depletion strain of *Corynebacterium glutamicum* and restore apical growth and rod shape to this strain (Letek *et al.*, 2008), there are interesting differences between hyphal *Streptomyces* and rod-shaped cells such as the corynebacteria. In *Streptomyces*, new zones of cell wall growth are established *de novo* by creating new hyphal branches, and this is preceded by the appearance of foci of DivIVA at the new sites along the lateral wall (Hempel *et al.*, 2008). In contrast, cell wall growth occurs at cell poles generated by cell division in *Corynebacterium*, and in these organisms DivIVA is first targeted to sites of ongoing septation and after completed division remains at the new poles (Letek *et al.*, 2008). Thus, while recruitment of DivIVA to sites of cell division could explain the polar targeting in cells such as the corynebacteria, *Streptomyces* DivIVA is involved in creating entirely new cell poles from the hyphal side wall, independently of cell division sites and pre-existing poles (Hempel *et al.*, 2008).

Could these differences in targeting and function be ascribed to any specific properties of the protein or regions in its amino acid sequence? In addition to the highly conserved amino-terminal domain of about 20 amino acids and the two extensively coiled-coil segments that are typical for all DivIVA-like proteins, two large domains are unique to *Streptomyces* DivIVA – a long domain rich in prolines, glutamines, and glycines that separates the two coiled-coil segments, and an unusual carboxy-terminal extension (Flärdh, 2003b). Here we report the analysis of a series of deletions of specific domains in *S. coelicolor* DivIVA, and how they affect the essential function, subcellular targeting, and oligomerization of the protein. In addition, we have investigated the properties of *S. coelicolor* DivIVA and its oligomerization into large oligomeric structures *in vitro* and *in vivo*.

Materials and methods

Bacterial strains, plasmids, and general methods

The bacterial strains and plasmids used in this study are described in Table 1. Culture conditions and genetic manipulations followed, in general, previously described procedures for *S. coelicolor* (Kieser *et al.*, 2000) and *E. coli* (Sambrook & Russel, 2001). DNA manipulations and cloning were carried out according to standard methods (Sambrook & Russel, 2001). Oligonucleotide primers are listed in Supporting Information, Table S1.

Recombinant protein purification

For purification of DivIVA, a derivative of the expression vector pTYB2 was used (pKF135), which gave rise to fusion of the DivIVA carboxy terminus to the intein-chitin-binding domain tag (intein-CBD) of the IMPACT-CN system (New England BioLabs Inc.). DivIVA-intein-CBD was produced in *E. coli* strain ER2566 and purified using chitin beads according to the instructions of the manufacturer (New England BioLabs Inc.). Self-cleavage of the intein tag released DivIVA with one added C-terminal glycine residue. The protein was eluted and dialyzed against appropriate buffer as described below, and the concentration was determined using the Bio-Rad *DC* protein assay kit using bovine serum albumin as the standard (Bio-Rad Laboratories Inc.).

Cell fractionation and immunoblotting

The *S. coelicolor* strain M145 was grown for 40 h in yeast extract–malt extract (YEME) medium at 30 °C (Kieser *et al.*, 2000), harvested by centrifugation, and washed twice in TBSME (25 mM Tris-HCl, pH 7.5; 150 mM NaCl; 3.6 mM β-mercaptoethanol; and 1 mM EDTA). The mycelium in TBSME supplemented with a protease inhibitor cocktail (Complete EDTA-free tablets, Roche) was lysed by two passages through a Constant Cell Disruptor (Constant Systems Ltd), cleared by centrifugation at 6000 ***g***, 4 °C, 25 min, and subjected to ultracentrifugation at 100 000 ***g***, 1 h. The supernatant was collected as the soluble fraction, and the pellet was resuspended in TBSME containing 2% Nonidet-P40 and diluted to the same volume as the original sample. The supernatants and pellets were analyzed by immunoblotting for detection of DivIVA.

For size exclusion chromatography, the 100 000 ***g*** cytoplasmic fractions of *S. coelicolor* M145 were obtained as described above, or purified recombinant DivIVA was dialyzed against TBS, and loaded onto a Superose 6 HR 10/30 column (Amersham Biosciences) that had been equilibrated with TBS. Fractions were collected and analyzed by immunoblotting.

Immunoblotting was carried out as described previously (Flärdh, 2003b). A rabbit antiserum was raised against *S. coelicolor* DivIVA (see Supporting Information), and this was used in immunoblotting at a dilution of 1 : 10 000. Donkey anti-rabbit horseradish peroxidase-linked secondary antibody (Amersham Biosciences) was used at 1 : 5000.

Table 1. Bacterial strains and plasmids

Strains	Relevant genotype	Sources/references
S. coelicolor A3(2)		
K114	*attB*$_{pSAM2}$::pKF58[*tipAp-divIVA*]	Flärdh (2003b)
K115	Δ*divIVA*::Ω*aacC4 attB*$_{pSAM2}$::pKF58[*tipAp-divIVA*]	Flärdh (2003b)
K116	*attB*$_{pSAM2}$::pPM927	Flärdh (2003b)
K120	*attB*$_{pSAM2}$::pKF67[*tipAp-FLAG-divIVA*]	This work
K129	*attB*$_{pSAM2}$::pKF129[*tipAp-divIVA*Δ*PQG*]	This work
K130	*attB*$_{pSAM2}$::pKF136[*tipAp-divIVA*Δ*Nterm*]	This work
K131	*attB*$_{pSAM2}$::pKF137[*tipAp-divIVA*Δ*Cterm*]	This work
K132	*attB*$_{pSAM2}$::pKF138[*tipAp-divIVA*Δ*CC2*]	This work
K133	*attB*$_{\Phi C31}$::pKF150[*tipAp-egfp*]	This work
K134	*attB*$_{\Phi C31}$::pKF151[*tipAp*-Φ(*divIVA-egfp*)Hyb]	This work
K135	*attB*$_{\Phi C31}$::pKF152[*tipAp*-Φ(*divIVA*Δ*PQG-egfp*)Hyb]	This work
K136	*attB*$_{\Phi C31}$::pKF153[*tipAp*-Φ(*divIVA*Δ*Cterm-egfp*)Hyb]	This work
K138	*attB*$_{\Phi C31}$::pKF155[*tipAp*-Φ(*divIVA*Δ*Nterm-egfp*)Hyb]	This work
K139	*attB*$_{\Phi C31}$::pKF156[*tipAp*-Φ(*divIVA*Δ*CC2-egfp*)Hyb]	This work
K141	Δ*divIVA*::Ω*aacC4 attB*$_{pSAM2}$::pKF129[*tipAp-divIVA*Δ*PQG*]	This work
K142	Δ*divIVA*::Ω*aacC4 attB*$_{pSAM2}$::pKF137[*tipAp-divIVA*Δ*Cterm*]	This work
M145	Prototrophic, SCP1$^-$SCP2$^-$ Pgl$^+$	Kieser *et al*. (2000)
E. coli		
BL21(DE3)	Expression strain	Lab stock
DH5α	Cloning strain	Lab stock
ER2566	*lacZ::T7 gene1*	NEB
ET12567/pUZ8002	*dam-13*::Tn*9 dcm-6 hsdM*, carries RK2 derivative with defective *oriT* for plasmid mobilization, Kana^R	Kieser *et al*. (2000)
GM2929	*dam-13*::Tn*9 dcm-6 hsdR2*	M. Marinus (University of Massachusetts Medical School)

Plasmids	Description*	Sources/references
C91	Cosmid carrying the *divIVA* region of the *S. coelicolor* chromosome	Flärdh (2003b)
pKF56	Cosmid C91 with *divIVA* replaced by Δ*divIVA*::Ω*aacC4*, Apra^R	Flärdh (2003b)
pKF58	*tipAp-divIVA* in integrating *S. coelicolor* vector pPM927, Spec^R	Flärdh (2003b)
pKF67	*tipAp-FLAG-divIVA* in integrating *S. coelicolor* vector pPM927, Spec^R	This work
pKF85	*divIVA* cloned in *E. coli* expression vector pET15b	This work
pKF129	*tipAp-divIVA*Δ*PQG* in integrating *S. coelicolor* vector pPM927, Spec^R	This work
pKF135	*divIVA* cloned in *E. coli* expression vector pTYB2	This work
pKF136	*tipAp-divIVA*Δ*Nterm* in integrating *S. coelicolor* vector pPM927, Spec^R	This work
pKF137	*tipAp-divIVA*Δ*Cterm* in integrating *S. coelicolor* vector pPM927, Spec^R	This work
pKF138	*tipAp-divIVA*Δ*CC2* in integrating *S. coelicolor* vector pPM927, Spec^R	This work
pKF150	*tipAp-egfp* in integrating *S. coelicolor* vector pIJ82, Hyg^R	This work
pKF151	*tipAp*-Φ(*divIVA-egfp*)Hyb in integrating *S. coelicolor* vector pIJ82, Hyg^R	This work
pKF152	*tipAp*-Φ(*divIVA*Δ*PQG -egfp*)Hyb in integrating *S. coelicolor* vector pIJ82, Hyg^R	This work
pKF153	*tipAp*-Φ(*divIVA*Δ*Cterm -egfp*)Hyb in integrating *S. coelicolor* vector pIJ82, Hyg^R	This work
pKF155	*tipAp*-Φ(*divIVA*Δ*Nterm -egfp*)Hyb in integrating *S. coelicolor* vector pIJ82, Hyg^R	This work
pKF156	*tipAp*-Φ(*divIVA*Δ*CC2 -egfp*)Hyb in integrating *S. coelicolor* vector pIJ82, Hyg^R	This work

*Sources of vectors and details on how plasmids were constructed are given in the Supporting Information.
NEB, New England BioLabs Inc.

Proteins were visualized by SuperSignal® West Pico Chemiluminescence (Pierce, Rockford, IL).

Immunoprecipitation

The appropriate *S. coelicolor* strains were grown in YEME medium for 40 h in the presence of 2 μg mL^{-1} of thiostrepton for the expression of DivIVA or FLAG-DivIVA from plasmids pKF58 and pKF67, respectively. The cytoplasmic fraction, obtained as described above, was used for immunoprecipitation. A final concentration of 1% Triton X-100 was added, and the samples were mixed with anti-FLAG M2 affinity gel (Sigma-Aldrich Inc.), pre-equilibrated in TBS. After overnight incubation at 4 °C and five washes in TBS

plus 1% Triton X-100, bound proteins were eluted by incubation in 3 × FLAG peptide solution (150 ng mL^{-1}) according to the instructions of the manufacturer (Sigma-Aldrich Inc.). The eluted material was used directly for blue native polyacrylamide gel electrophoresis (BN-PAGE) or boiled in sodium dodecyl sulfate (SDS) sample buffer for SDS-PAGE and immunoblotting.

BN-PAGE

For BN-PAGE (Schägger *et al.*, 1994), immunoprecipitated DivIVA was transferred to BN buffer (500 mM ε-amino caproic acid; 20 mM Bis–Tris, pH 7.0; 2 mM EDTA; 12 mM NaCl; 10% glycerol; and 1 mM PMSF) using Microcon YM10 centrifugal filters (Millipore). This was loaded onto a blue native 5–16.5% linear gradient polyacrylamide gel, which was run at 30 V overnight at 4 °C with 50 mM Bis–Tris, pH 7.0, as anode buffer and 50 mM tricine, 15 mM Bis–Tris, pH 7.0, containing 0.002% Coomassie Brilliant Blue G250 as cathode buffer. The proteins were detected by Coomassie Brilliant Blue R250 staining.

Transmission electron microscopy (TEM)

Purified DivIVA was dialyzed overnight against 10 mM Tris-HCl, pH 7.5, 150 mM NaCl. The proteins were attached to glow-discharged carbon films for 1 min, washed with water, and negatively stained with 2% uranyl acetate for 30 s. The solutions were blotted away and the grids were examined at 50 or 100 kV using a Philips CM 10 transmission electron microscope. Electron micrograph negatives were scanned and processed for publication by ADOBE PHOTOSHOP.

Disruption of the chromosomal *divIVA* locus

Disruption of the chromosomal *divIVA* locus was performed essentially as described previously (Flärdh, 2003b). Briefly, the cosmid pKF56 was passaged through *E. coli* strain GM2929, and nonmethylated and alkali-denatured cosmid DNA was used to transform *S. coelicolor* strains K129, K130, K131, and K132 to apramycin resistance on R2YE plates with 0.2 μg mL^{-1} thiostrepton. Transformants were tested for kanamycin resistance (Kana^R) to reveal the presence of the cosmid vector in single crossover recombinants. To allow segregation of double cross-over recombinants, apramycin-resistant (Apra^R) transformants were allowed to sporulate on mannitol soya flour agar (Kieser *et al.*, 2000) with thiostrepton, but no other antibiotic selection. Spores were plated to obtain single colonies, and 200 colonies of each strain were examined for antibiotic resistance in order to identify Apra^{R}Kana^S recombinants.

Fluorescence microscopy

Hyphae were prepared for fluorescence microscopy as described previously (Flärdh, 2003b). Microscopical preparations were viewed using a Zeiss Axio Imager.Z1 microscope equipped with X-Cite 120 Illumination (EXFO Photonic Solutions Inc.), a 9100-02 EM-CCD camera (Hamamatsu Photonics), or a Photometrics CoolSNAP$_{fx}$ CCD camera (Roper Scientific Inc.), and images were captured and processed using VOLOCITY 3DM software (Improvision).

Results and discussion

Streptomyces coelicolor DivIVA is cytoplasmic and exists as multimeric complexes in cell extracts

Given the crucial role of *S. coelicolor* DivIVA in tip extension and branching, it was important to investigate the basis for its assembly at the hyphal tip. Fractionation of cell extracts of *S. coelicolor* strain M145 left the bulk of the DivIVA protein in the soluble fraction (Fig. 1a), and a small amount of DivIVA that was found in the pelleted membrane fraction, disappeared almost completely after a single wash in the same buffer (data not shown). The soluble fraction was further separated by gel filtration chromatography, and DivIVA was eluted with an apparent molecular mass of 400–1000 kDa (Fig. 1d). Further, no DivIVA was detected in fractions that would have contained the monomeric form (41 kDa). It should be noted that the DivIVA complexes are likely to be elongated rather than globular like the size standards, and the estimations of molecular mass are therefore not very accurate.

To resolve the composition of these complexes, we constructed a strain (K120) producing DivIVA with the FLAG epitope fused to its N-terminus, and captured the complexes by immunoprecipitation. In agreement with the gel filtration analysis, DivIVA, which was pulled down by anti-FLAG antibodies, migrated as large complexes corresponding to around 900 kDa during BN-PAGE (Fig. 1b). This material is a mixture of FLAG-tagged and native DivIVA (data not shown). Upon denaturation and SDS-PAGE analysis, the complexes dissociated into monomeric DivIVA, and no other major constituents could be detected (Fig. 1c). We conclude that DivIVA forms homo-oligomeric high-molecular-weight complexes in *S. coelicolor*, and although they appear to line the hyphal apex, they are not strongly associated with the membrane.

Purified DivIVA can assemble into filamentous cytoskeleton-like structures

To investigate whether DivIVA could form higher-order structures *in vitro*, we purified recombinant *S. coelicolor* DivIVA from an *E. coli* expression strain. The resulting

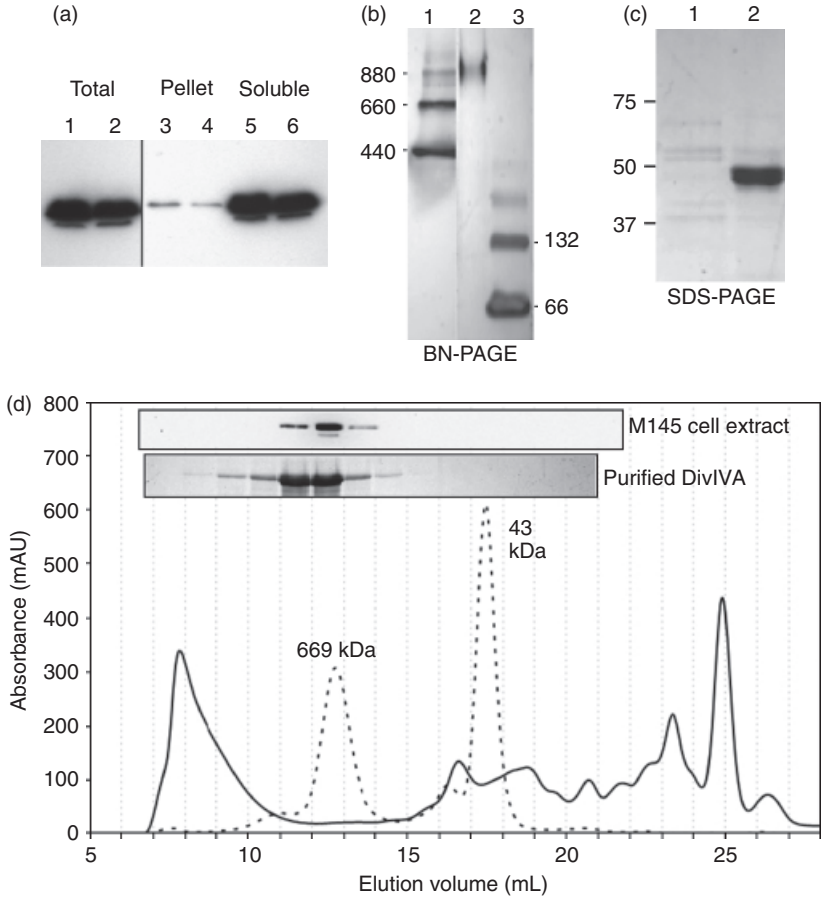

Fig. 1. DivIVA self-interacts and oligomerizes to form large cytoplasmic complexes. (a) Cell fractionation: total cell extracts of *Streptomyces coelicolor* strain M145 (lanes 1 and 2) were fractionated by ultracentrifugation at 100 000 ***g*** into insoluble membrane fractions (lanes 3 and 4) and soluble cytoplasmic fractions (lanes 5 and 6). The starting material was an intact mycelium (lanes 1, 3, and 5) or isolated protoplasts (lanes 2, 4, and 6). DivIVA was detected using immunoblotting with anti-DivIVA antiserum. (b, c) Analysis of immunoprecipitated FLAG-DivIVA from strain K120. Coomassie blue-stained gels are shown. (b) A blue native polyacrylamide gel, with molecular weight standard apoferritin (lane 1), isolated FLAG-DivIVA (lane 2), and molecular weight standard bovine serum albumin (lane 3). An irrelevant lane between lanes 1 and 2 has been excised from the image. (c) SDS-PAGE analysis of the isolated FLAG-DivIVA from the same experiment (lane 2) and a control immunoprecipitation from strain K114 expressing only nontagged DivIVA (lane 1). The positions of molecular weight standards are indicated. (d) Gel filtration analysis of DivIVA in *S. coelicolor* strain M145 cell extract or heterologously produced and purified DivIVA. Proteins were separated on a Superose 6 column. Absorbance detection of the total cell extract (solid line) and the two molecular weight standards ovalbumin (43 kDa) and thyroglobulin (669 kDa) (dotted line). The collected fractions, indicated by vertical dotted lines, were analyzed for the presence of DivIVA by SDS-PAGE and immunoblotting, as shown at the top of the panel, exactly aligned with the corresponding fractions.

protein was eluted after gel filtration chromatography with an apparent molecular mass of $>$ 700 kDa, which is consistent with the oligomeric complexes detected in *S. coelicolor* cell extracts (Fig. 1d). The purified DivIVA was examined by TEM after negative staining with uranyl acetate. This revealed the presence of striking filamentous structures of variable sizes, ranging from long filaments of sometimes several hundred nanometers to shorter structures (examples in Fig. 2). The apparent widths of the filaments were *c.* 2 nm, but thicker structures that appeared to be bundles of several filaments were also seen. In addition to the filaments, amorphous protein aggregates were also present in the preparations (examples can be seen surrounded by dense staining in Fig. 2a and c).

Our findings confirm that *S. coelicolor* DivIVA shares the ability to oligomerize with orthologs from both *Actinobacteria* and *Firmicutes* (Muchová *et al.*, 2002; Fadda *et al.*, 2007; Nguyen *et al.*, 2007; Rigden *et al.*, 2008). The *Streptomyces* oligomers could assemble further into extensive filamentous structures, reminiscent of cytoskeletal proteins (Fig. 2), and the appearance of these smooth filaments differed from those reported to be formed by end-to-end association of the 'doggy-bone'-like structures consisting of six to eight monomers of *B. subtilis* DivIVA (Stahlberg *et al.*, 2004). The find-

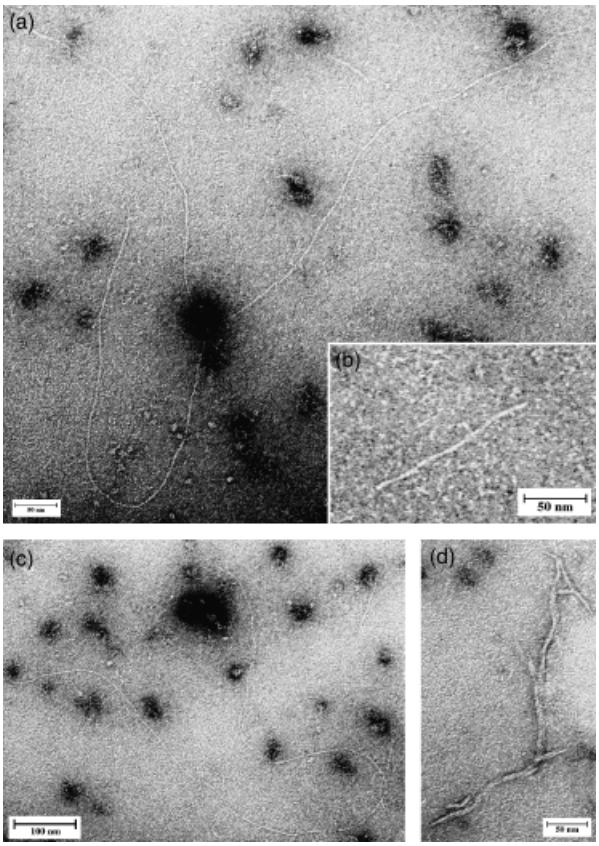

Fig. 2. Examples of filamentous structures formed by purified recombinant *Streptomyces coelicolor* DivIVA and detected by uranyl acetate negative staining and electron microscopy. Examples of long thin filaments can be seen in (a)–(c), and bundles of filaments can be seen in (d). (c) shows several examples of more amorphous aggregates surrounded by dark staining, as mentioned in the text. Scale bars = 50 nm in (a), (b), (d), and 100 nm in (c).

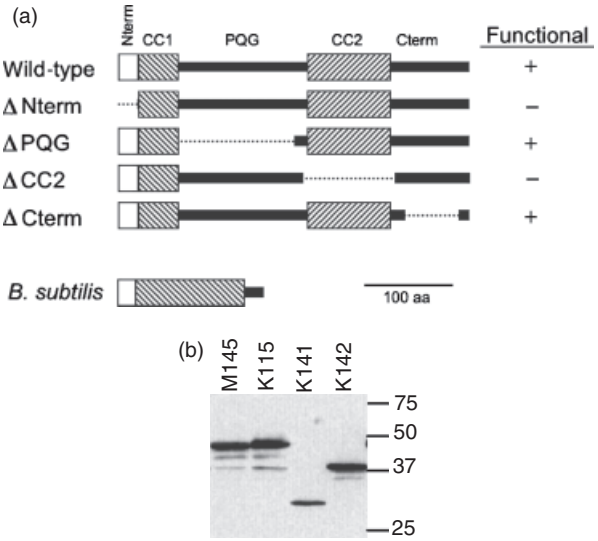

Fig. 3. Identification of two large dispensable segments of DivIVA. (a) Schematic map of *Streptomyces coelicolor* DivIVA and the tested deletion derivatives. An open box indicates the conserved 22 N-terminal amino acids. Striped boxes denote the segments predicted to adopt a coiled-coil conformation. Black lines indicate poorly conserved segments of DivIVA. *Bacillus subtilis* DivIVA is drawn in the same way for comparison. The deleted segments, indicated by dotted lines, are for DivIVAΔNterm amino acid residues 2–22; for DivIVAΔPQG residues 69–201; for DivIVAΔCC2 residues 210–314; and for DivIVAΔCterm residues 326–388. The functionalities of the deletion derivatives was tested by determining whether they could replace the normal DivIVA in *S. coelicolor*. (b) Western blot analysis of the cellular DivIVA contents in the parent M145 and strains in which the chromosomal *divIVA* locus had been deleted (Δ*divIVA*::Ω*aacC4*) and instead full-length *divIVA* (strain K115), *divIVAΔPQG* (strain K141), and *divIVAΔCterm* (strain K142) were expressed from the *tipAp* promoter on an integrating plasmid. This demonstrates the absence of normal DivIVA from the deletion strains K141 and K142. Numbers indicate the positions of molecular weight marker bands.

ings reinforce the view that DivIVA oligomers can assemble into higher-order structures. Microscopy studies of enhanced green fluorescent protein (EGFP)-tagged DivIVA in *Streptomyces* have not revealed any long filaments *in vivo*. However, a dense network of short DivIVA filaments could make up the large foci observed at hyphal tips.

The Streptomyces-specific PQG-rich and carboxy-terminal domains are dispensable for the essential function of DivIVA

The amino acid sequence of *S. coelicolor* DivIVA contains a highly conserved amino-terminal part of 22 amino acids (hereafter designated Nterm), which is followed by a segment predicted to adopt a coiled-coil structure (Flärdh, 2003b) (Fig. 3a). This coiled-coil region is split into two parts, which we designate CC1 (amino acid 23–65) and CC2 (amino acid 202–309). Between these regions is an extended uncharged segment of low complexity, rich in proline, glutamine, and glycine residues, which we term PQG (amino acids 66–201). This PQG segment and the carboxy-terminal part (amino acids 310–398, designated as Cterm) are only similar to other *Streptomyces* DivIVAs. A series of deletion alleles of *divIVA* were constructed (Fig. 3a) and placed under the control of the thiostrepton-inducible *tipAp* promoter in integrating plasmids in *S. coelicolor* (see Table 1 and Supporting Information).

The functionality of the new deletion alleles was tested by asking whether their expression *in trans* would allow the native chromosomal *divIVA* allele to be deleted. After having been transformed with the cosmid pKF56, which carries the relevant chromosomal region from *S. coelicolor* with a Δ*divIVA*::Ω*aacC4* deletion/insertion allele conferring $Apra^R$ and a $Kana^R$ gene in the cosmid vector sequence, both strains K129 (*tipAp-divIVAΔPQG*) and K131 (*tipAp-divIVAΔCterm*) readily gave rise to $Apra^R Kana^S$ progeny. The allelic exchanges were

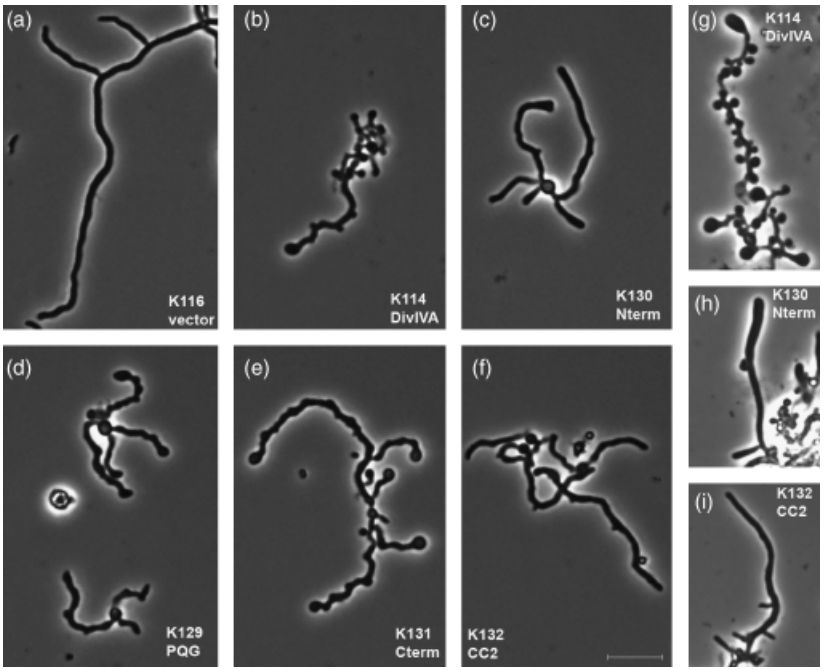

Fig. 4. Ectopic overexpression of *divIVA* deletion alleles. The deletion alleles were expressed from the induced *tipAp* promoter for 3 h (a–f) or 8 h (g–i) in *Streptomyces coelicolor* strain M145. The panels show examples of representative morphologies for strain K116 carrying the empty vector pPM927 (a); K114 expressing the full-length *divIVA* (b, g); K130 expressing *divIVA*Δ*Nterm* (c, h); K129 expressing *divIVA*Δ*PQG* (d); K131 expressing *divIVA*Δ*Cterm* (e); and K132 expressing *divIVA*Δ*CC2* (f, i). Scale bar = 8 μm.

confirmed by PCR (data not shown) and, as shown in Fig. 3b, only the deletion protein could be detected by anti-$DivIVA_{SC}$ antiserum in the resulting strains K141 (Δ*divIVA/tipAp-divIVA*Δ*PQG*) and K142 (Δ*divIVA/tipAp-divIVA*Δ*Cterm*). The viability of these strains showed that the *Streptomyces*-specific internal PQG-rich segment and carboxy-terminal extension were not essential for growth.

The conserved coiled-coil and amino-terminal domains of *S. coelicolor* DivIVA are required for function and affect oligomerization and targeting new sites

When *S. coelicolor* strains K130 (*tipAp-divIVA*Δ*Nterm*) and K132 (*tipAp-divIVA*Δ*CC2*), expressing Nterm and CC2 deletion alleles, were transformed with the pKF56 cosmid, only $Apra^{R}Kana^{R}$ progeny were obtained. Thus, only single recombinational crossovers were recovered leading to integration of pKF56. This strongly suggests that the amino-terminal and CC2 coiled-coil domains are essential for DivIVA function.

Overexpression of the functional PQG and Cterm deletion alleles resulted in similar swelling of the tips and induction of multiple branch-like outgrowths from the hyphal side walls as were seen with full-length *divIVA* (Fig. 4b, d, e, and g) (Flärdh, 2003b; Hempel *et al*., 2008). After 8 h of overproduction of the Nterm deletion protein, most hyphae had developed a club-like shape, indicating an effect on pre-existing hyphal tips (Fig. 4h). However, overexpression of the Nterm deletion allele did not lead to multiple swellings along the lateral wall (Fig. 4c and h). In support of this, the EGFP-tagged DivIVAΔNterm protein formed much fewer lateral foci of fluorescence upon overexpression than did the full-length, ΔPQG, and ΔCterm EGFP fusion proteins (Fig. S1). This suggests that the amino-terminal part is important in establishing new foci of DivIVA at sites along the lateral wall. Such initial assembly of DivIVA foci at new sites represents the first recognized stage in the formation of hyphal branches and establishment of new sites of cell wall synthesis (Hempel *et al*., 2008).

Interestingly, all tested deletion derivatives fused to EGFP, except ΔCC2, accumulated clearly at the hyphal tips when produced at levels corresponding to normal DivIVA (Fig. S2). We conclude that newly produced molecules/oligomers of DivIVA–EGFP were efficiently recruited to the resident apical DivIVA assembly, and that none of the tested domains were alone required for this self-interaction, except for CC2. However, the DivIVA ΔCC2–EGFP protein appeared unstable and accumulated at very low levels in *S. coelicolor* (data not shown). When the deletion alleles were expressed in *E. coli* and the products were purified and separated by BN-PAGE, the normal DivIVA, Nterm-, PQG-, and Cterm-deletion mutant proteins formed similar large aggregates, while deletion of the CC2 coiled-coil domain disturbed oligomerization and resulted in resolution of a series of differently sized complexes (Fig. 5). The aberrant oligomerization is a likely reason for the lack of functionality of the ΔCC2 protein (Figs 3a and 4f).

In conclusion, broadly conserved features such as the amino-terminal stretch and the CC2 coiled-coil segment are essential for the functioning of *S. coelicolor* DivIVA, which is in

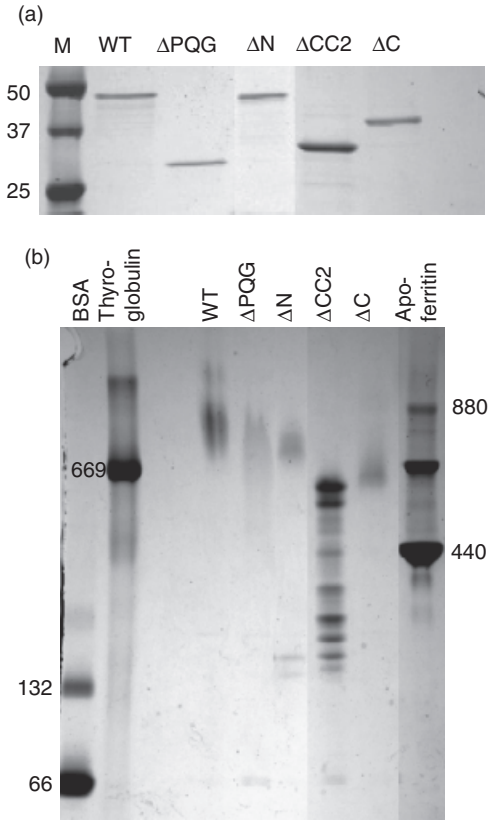

Fig. 5. Purification and oligomerization of deletion derivatives of DivIVA. (a) SDS-PAGE analysis of *Streptomyces coelicolor* DivIVA deletion derivatives that had been expressed and purified from *Escherichia coli* using the IMPACT system. A Coomassie blue-stained gel is shown. (b) BN-PAGE of purified derivatives of DivIVA showing their oligomerization properties. The molecular weight standards are indicated. Irrelevant lanes have been excised from the images between ΔN and ΔCC2, and on the left side of the apoferritin marker.

agreement with recent reports on the essential nature of the corresponding parts of DivIVA from *E. faecalis* and *C. glutamicum*, and the involvement of the CC2-like domains in oligomerization (Rigden *et al.*, 2008; Letek *et al.*, 2009). More surprisingly, the two conspicuous *Streptomyces*-specific segments of DivIVA are dispensable and do not contain critical determinants for subcellular targeting and oligomerization. However, these PQG and Cterm domains appear to influence the morphology of hyphae to some extent (unpublished data) and may be important under certain circumstances. Presumably, they may affect, for example, the architecture of the apical DivIVA assemblies, the regulation of their formation, or the interaction with specific partners.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplementary materials and methods.
Fig. S1. Subcellular localization of ectopically overproduced DivIVA deletion derivatives.
Fig. S2. Subcellular localization of DivIVA deletion derivatives expressed at normal levels.
Table S1. Oligonucleotide primers used in this study.

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