Cloning and biochemical characterization of DivIVA protein from *Bacillus stearothermophilus*

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DivIVA is a bifunctional protein involved in division site selection, by directing the activity of MinCD complex during vegetative growth of *Bacillus subtilis* cell. Its second functional role is in segregation of the chromosome during early stages of sporulation process. The *divIVA*-like genes are present in many bacteria, however, the role of most of these DivIVA homologues is not well documented. We have studied DivIVA homologue from *Bacillus stearothermophilus* ATCC 12980 strain. The identified *divIVA* gene was cloned and sequenced. DivIVA protein was expressed in *Escherichia coli*, purified and characterized. Computer analysis of the secondary structure of *B. stearothermophilus* DivIVA homologue indicated the α-helical coiled-coil structure. Gel filtration experiments showed that *B. stearothermophilus* DivIVA oligomerizes similarly as DivIVA from *B. subtilis*.

**Key words:** bacterial cell division, DivIVA, *Bacillus subtilis*.

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mediated through DivIVA protein, which does not exhibit any sequence or structural similarity with MinE protein. It seems that DivIVA protein performs the same function as MinE but by a fully different mechanism (CHANG & STEWART, 1997; EDWARDS & ELLINGTON, 1997).

DivIVA is targeted to the new division sites in late step in their assembly, and it remains at the new cell pole after division is completed (MARSTON & ELLINGTON, 1999). Similar pattern of localization of B. subtilis DivIVA was observed when divIVA was cloned into E. coli and also into yeast Schizosaccharomyces pombe cells, in spite of a different mechanism of division (EDWARDS et al., 2000). Recently, it was shown that DivIVA accumulates at the cell poles of outgrowing spores and thus it seems that its localization is independent of all other division proteins (HAMPDEN & ELLINGTON, 2003).

DivIVA protein ensures that MinCD specifically inhibits division at the cell poles and allows division at the mid-cell during vegetative growth (CHANG & STEWART, 1997; EDWARDS & ELLINGTON, 1997). During sporulation, when the localization of the division septum must be switched from the mid-cell to polar site, the MinCD/DivIVA system must somehow allow the formation of a polar Z-ring. Although the mechanism of DivIVA action during asymmetric septum formation is not known, THOMAIDES et al. (2001) showed that DivIVA interacts with the chromosome segregation machinery and helps to localize the oriC region of the chromosome near the cell pole during sporulation. This interaction is probably interposed by Soj-Spo0J-RacA system. Soj protein is a protein associated with the chromosome, it specifically binds around the oriC region. Soj protein facilitates movement of the Spo0J/oriC complex towards the cell pole. RacA also binds to the chromosome around the oriC and attaches this region to the pole by possible interaction with DivIVA (WU & ELLINGTON, 2003). Switch of the division septum from the mid-cell to polar site is probably performed by dual function of DivIVA protein. It is responsible for sequestering MinCD at the poles during vegetative growth and RacA during sporulation (BEN-YEHUDA et al., 2003).

Role of DivIVA in cell division, nucleoid segregation and growth inhibition was observed in Gram-positive bacterium Streptococcus pneumoniae (FADDA et al., 2003). DivIVA homologue in Streptomyces coelicolor has a function related to asymmetrical growth and cell shape determination (FLAIRDH, 2003).

B. subtilis DivIVA protein displays a weak sequence similarity to eukaryotic tropomyosin (EDWARDS et al., 2000) forming dimers in solution. The predicted structure of B. subtilis DivIVA protein suggested an α-helical coiled-coil structure that is characterized by repeating heptad motif of amino acids being predominantly hydrophobic at the first and the fourth positions in each heptad and hydrophilic at the further positions (LUPAS et al., 1991; EDWARDS et al., 2000). These repeating motives are probably used for oligomerization as was shown for tropomyosin (LUPAS, 1996). Oligomerization of DivIVA in B. subtilis cell lysates was confirmed by Western blot analysis of non-denaturating polyacrylamide gels (MUCHOVA et al., 2002a). Similar results were obtained by electron microscopy of B. subtilis DivIVA. It has been shown that protein can form oligomers consisting of up to 6–8 monomers (STAHLBERG et al., 2004).

We have previously performed crystallization trials with B. subtilis DivIVA protein (MUCHOVA et al., 2002b). However, the obtained crystals either did not diffract or diffracted weakly. Because of the experience that the thermophilic proteins are more suitable for crystallization trials than mesophilic proteins, we decided to use DivIVA homologue from B. stearothermophilus ATCC 12980 in order to increase the chance for successful crystallization. We have obtained B. subtilis DivIVA homologue from B. stearothermophilus ATCC 12980. Using alignment of B. subtilis divIVA and sequenced part of B. stearothermophilus DSM 13240 chromosome retrieved from the website http://www.genome.ou.edu/bstearo.html, we identified divIVA homologue in this thermophilic microorganism. Putative divIVA gene was cloned and DNA sequenced. Interestingly, DNA sequencing of this gene revealed high degree of differences between both B. stearothermophilus strains. The computer analysis of deduced amino acid sequence suggested coiled-coil structure. This DivIVA homologue was overproduced in E. coli and purified. Western blot analysis of non-denaturating polyacrylamide gel of B. stearothermophilus DivIVA protein and gel filtration experiment showed that it oligomerizes in a similar way as B. subtilis DivIVA (MUCHOVA et al., 2002a).

Material and methods

Bacterial strains and culture media
All bacterial strains and plasmids used in this study are listed in Table 1. E. coli strains were routinely grown in Luria-Bertani (LB) broth (AUSUBEL et al., 1987). E. coli competent cells were grown in SOB medium.
Table 1. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain and plasmid</th>
<th>Genotype or relevant characteristics</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td>E. coli MM294</td>
<td>endA1 hsdR17 supE44 thi/1 recA4</td>
<td>BACKMAN et al. (1976)</td>
</tr>
<tr>
<td>E. coli BL21(DE3)</td>
<td>F-ompT hsdSDE(r<del>m</del>)gal dcm</td>
<td>Novagen</td>
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<tr>
<td>B. stearothermophilus ATCC 12980</td>
<td></td>
<td>NAZINA et al. (2001)</td>
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<tr>
<td>IB 880</td>
<td>pETIVA1 in E. coli MM 294</td>
<td>This work</td>
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<td>IB 881</td>
<td>pETIVA2 in E. coli MM 294</td>
<td>This work</td>
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<tr>
<td>IB 882</td>
<td>pETIVA1 in E. coli BL 21 (DE3)</td>
<td>This work</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pET15b(+)</td>
<td>AmpR, lacI, T7 promoter</td>
<td>Novagen</td>
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<tr>
<td>pET26b(+)</td>
<td>KanR, lacI, T7 promoter</td>
<td>Novagen</td>
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<tr>
<td>pETIVA1</td>
<td>AmpR, lacI, T7 promoter, divIVA</td>
<td>This work</td>
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<tr>
<td>pETIVA2</td>
<td>AmpR, lacI, T7 promoter, divIVA</td>
<td>This work</td>
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(AUSUBEL et al., 1987). Selection of E. coli cells transformed with pETIVA1 and pETIVA2 was achieved by cultivation in the presence of ampicillin (100 µg/mL) or kanamycin (30 µg/mL) in agar or liquid medium, respectively.

**Cloning of divIVA into pET vectors**

B. stearothermophilus (ATCC 12980) chromosomal DNA was isolated according to the method by Curtiss & Vander-Horn (1990) and used as a template in PCR amplification. B. stearothermophilus divIVA was amplified by PCR using:

- Upstream primer: 5’-GAGGTGGCCCATATG-3’
- Downstream primer: 5’-GGCGGGCGGATCCGCGGATTTGCGGCAAT-3’

containing the underlined NdeI (upstream primer) and BamHI (downstream primer) restriction sites. Reaction mixture (50 µL) contained 100 pmol upstream and downstream primers, 1 µg template chromosomal DNA, 10 nmol dNTP and 0.5 µL DNA Taq polymerase (0.5 U) (Finenzyme). PCR was carried out as follows: 94°C for 1min, 39°C for 1 min and 72°C for 2 min in 30 cycles using an automatic thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer, Foster City, CA). PCR reaction product was analyzed on a 0.8% agarose gel. PCR fragment was isolated from gel using Promega Wizard™ PCR Prep DNA purification system. To prepare pETIVA1 and pETIVA2, the isolated PCR fragment coding for the B. stearothermophilus divIVA was digested with NdeI and BamHI and ligated into NdeI/BamHI cut pET15b(+) and pET26b(+) plasmids downstream of the IPTG inducible T7 promoter. Ligation mixture was used for transformation of E. coli MM294. Transformation of E. coli strain was performed by standard technique (AUSUBEL et al., 1987). Recombinant plasmids were verified by digestion with series of restriction endonucleases and subsequently DNA sequenced.

**Overproduction and purification of B. stearothermophilus DivIVA**

E. coli strain BL21 (DE3) was used for production of recombinant B. stearothermophilus DivIVA protein. This strain was transformed with recombinant plasmid pETIVA1 to generate strain IB 882. Cell culture was grown in LB medium supplemented with ampicillin (100 µg/mL) at 37°C. When cell culture reached OD600 of 0.6, expression of His6Tag-DivIVA was induced by the addition of IPTG to a final concentration of 1mM. After 5 hours of further growth at 37°C, cells were harvested by centrifugation and pellet was frozen at −70°C until use. Expression of DivIVA was confirmed by 12.5% SDS-PAGE analysis. Cells were resuspended on ice in binding buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM AEBSF), lyzed by sonication and centrifuged at 30 000 rpm for 30 min. To purify His6Tag-DivIVA, metal chelate affinity chromatography was used. The soluble fraction was applied onto a 2 mL Ni2+NTA column (Amersham Pharmacia) previously preequilibrated in binding buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl) and His6Tag-DivIVA was eluted with step gradient of a 0.1 M, 0.2 M, 0.3 M and 1 M imidazol, which bound to the Ni-NTA and displaced the tagged protein. Fraction of 0.2 M imidazol containing His6Tag-DivIVA was applied onto 1 mL Sephadex G-25 column preequilibrated in buffer B (10 mM Tris-HCl pH 8.0, 100 mM NaCl) and His6Tag-DivIVA protein was eluted with buffer B. The His6Tag-DivIVA was loaded onto Superose 12 gel filtration column and eluted with buffer A (50 mM Tris-Cl pH 8.0, 100 mM NACL, 1 mM EDTA).

**DNA sequencing**

The divIVA gene in pETIVA1 and pETIVA2 were sequenced by dideoxynucleotide chain termination procedure (SANGER et al., 1977) using T7 sequencing primers flanking divIVA. Nucleotide sequences were further analyzed using Vector NTI suite 6.

**SDS-PAGE and Western blot analysis**

SDS-PAGE electrophoresis was performed according LAEMLI et al. (1970). Western blot analysis of the re-
combinant protein was performed using the semi-dry method (Hirano & Watanabe, 1990). Proteins after electrophoresis through a blue native 5-18% linear gradient polyacrylamide gel (Schagger et al., 1994) were blotted onto a nitrocellulose membrane. The B. stea rothermophilus DivIVA was detected by incubation with the primary mouse polyclonal serum against B. subtilis DivIVA diluted 1:1000 followed by alkaline phosphatase-conjugated anti-mouse immunoglobulin G diluted 1:1000.

Computational analysis

Deduced amino acid sequence of B. stea rothermophilus DivIVA was analyzed using services on ExPASy molecular biology server (Gasteiger et al., 2003; http://ca.expasy.org). Secondary structure prediction of the protein was performed with nPredict (Kneller et al., 1990; http://www.cmpbio.unc.edu/~kneller/npredict.html) and PSA (Stultz et al., 1993; http://bmerc-www.bu.edu/psa/) programs. Putative coiled-coil structure was analyzed by COILS (Lupas et al., 1991; http://www.ch.embnet.org/software/COILS-form.html) and SMART (Letunic et al., 2002; http://smart.embl-heidelberg.de) programs. For determination of sequence similarity of predicted peptide sequence BLASTp (Altschul et al., 1990; http://www.ncbi.nlm.nih.gov/BLAST/) and MAXIOM (Sand er & Schneider, 1991; http://cubic.bioc.columbia.edu/) programs were used.

Nucleotide sequence accession number

The DNA sequence data reported here have been submitted to the GenBank (Benson et al., 2004) and assigned accession number AY533033.

Results and discussion

Cloning of the B. stea rothermophilus divIVA gene

PCR primers were designed according to the divIVA sequence of B. stea rothermophilus DSM 13240 (http://www.genome.ou.edu/bstearo.html) from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) (Fig. 1). The divIVA gene from B. stea rothermophilus ATCC 12980 was amplified by PCR. Obtained single fragment of expected 580 bp size was used for preparation of expression vectors pETIVA1 and pETIVA2. Amplified divIVA gene was cloned into pET15b(+) and pET26b(+) expression vectors using NdeI and BamHI restriction sites. Two recombinant clones, which were obtained after transformation of E. coli MM294 with ligation mixtures, were chosen to be DNA sequenced.

Analysis of the B. stea rothermophilus divIVA gene

The two fragments, 627 bp of pETIVA1 and 632 bp of pETIVA2, were DNA sequenced. pETIVA1 plasmid contained B. stea rothermophilus divIVA gene encoding 190 amino acid residues of His6-tagged DivIVA. This DivIVA protein was used in gel filtration experiment and Western blot analysis. The divIVA gene in pETIVA2 plasmid encoded 170 amino acid residues of DivIVA. The DNA sequences of open reading frames of divIVA from pETIVA1 and pETIVA2 were compared. Their comparison confirmed 100% identity of these sequences. Thus, we propose that the clones, which we obtained after transformation of E. coli strain MM294, had no mutations in the divIVA gene. In contrast, when the similar experiments were carried out with divIVA gene from B. subtilis, the authors obtained mutations and deletions in the divIVA gene in each clone (Muchova et al., 2002a). It is very likely that DivIVA from B. subtilis can interact with the E. coli division apparatus in a way that inhibits growth because B. subtilis DivIVA protein is targeted to the division sites in E. coli (Edwards et al., 2000). It is also probable that point mutations and deletions in divIVA gene are selected allowing the E. coli host strain to escape the negative effect of B. subtilis DivIVA on cell growth. However, this effect was not observed for B. stea rothermophilus DivIVA. When we compared DivIVA from B. stea rothermophilus with DivIVA from B. subtilis, alignment revealed relatively low degree of homology: 66.8% identity in nucleotide sequences (Fig. 1) and 66.5% identity in amino acid sequences (Fig. 2). We suggest that B. stea rothermophilus DivIVA interacts with E. coli division apparatus differently than B. subtilis DivIVA does.

Interestingly, DNA sequence identity between the divIVA from B. stea rothermophilus ATCC 12980 and from B. stea rothermophilus DSM 13240 is only 86.4% (Fig. 1). The proteins predicted from the open reading frames share, however, 94.7% identity (Fig. 2).

Expression and purification of recombinant DivIVA

To overexpress His6-Tag-DivIVA, E. coli BL21(DE3) strain transformed with pETIVA1 plasmid was used. The highest expression of DivIVA after induction with IPTG was obtained after five hours. Analysis of expression of DivIVA in E. coli cells showed that the protein was present both in the soluble fraction and cell pellet (Fig. 3A). The amount of protein in soluble fraction of cell lysate

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Fig. 1. Comparison of DNA of a segment of contig 619 from sequenced *B. stearothermophilus* DSM 13240 (BstDSM), *divIVA* gene from *B. subtilis* KSS1168 (Bsu) and *divIVA* gene from *B. stearothermophilus* ATCC 12980 (BstATCC). Sequence identity between the *divIVA* from *B. stearothermophilus* ATCC 12980 and those from *B. stearothermophilus* DSM 13240 and *B. subtilis* is 86.4% and 66.8%, respectively. The start and stop codons are underlined. The oligonucleotide primers used for PCR are marked.

Fig. 2. Comparison of amino acid sequences of DivIVA: *B. stearothermophilus* DSM 13240 (BstDSM), *B. stearothermophilus* ATCC 12980 (BstATCC) and *B. subtilis* (Bsu). H1, H2 and H3 denote three predicted α-helices. Letters a and d denote predominantly hydrophobic amino acids at first and fourth position in predicted heptad repeats. Sequence identity between the DivIVA from *B. stearothermophilus* ATCC 12980 and those from *B. stearothermophilus* DSM 13240 and *B. subtilis* is 94.7% and 66.5%, respectively.

was sufficient, therefore the recombinant protein was purified from soluble fraction of cell lysate. Most efficient elution of the His-tagged protein from the Ni-column was achieved by 0.2 M and 0.3 M imidazole (Fig. 3B). Sephadex G-25 column was used for buffer exchange. The protein yield from the soluble fraction of the cell lysate was about 3 mg purified protein per 50 mL of cell culture.

**Oligomerization of *B. stearothermophilus* DivIVA**

The purified His6-Tag-DivIVA protein (molecular weight of monomer is 22.04 kDa) was loaded
Fig. 3. Expression, purification and oligomer formation analysis of DivIVA protein. a – His$_6$-Tag-DivIVA from B. stearothermophilus, b – B. stearothermophilus His$_6$-Tag-DivIVA oligomer, c – B. subtilis DivIVA oligomer. (A) 15% SDS-PAGE analysis of expression of DivIVA. Lane 1, molecular mass marker; lane 2, total cell protein from uninduced E. coli BL21 (DE3) with pETIVA1; lane 3, soluble fraction of cell lysate from induced E. coli BL21 (DE3) harboring pETIVA1; lane 4, cell pellet from induced E. coli BL21 (DE3) harboring pETIVA1. (B) 12.5% SDS-PAGE analysis of elution of His$_6$-tagged DivIVA from Ni-NTA-column. Lanes 1-4, fractions eluted by 0.1 M, 0.2 M, 0.3 M, 1.0 M imidazol, respectively. (C) Western blot analysis of blue native gel of B. stearothermophilus DivIVA protein. Lane 1, B. subtilis DivIVA; lane 2, B. stearothermophilus ATCC 12980 DivIVA.

Fig. 4. Superose 12 gel filtration of B. subtilis DivIVA and His$_6$-tagged B. stearothermophilus DivIVA. 0.5 mL of B. subtilis DivIVA after FastQ and MonoQ ion exchange columns was loaded. 0.5 mL of B. stearothermophilus DivIVA after Ni-NTA-column and Sephadex G-25 column was loaded. Chromatogram of molecular weight standards: Blue dextran (2,000 kDa), aldolase (158 kDa) and cytochrome c (12.5 kDa).

onto Superose 12 gel filtration column. The retention volume of His$_6$-Tag-DivIVA (B. stearothermophilus DivIVA) protein was between the molecular weight standards of the 158 kDa and 2,000 kDa (Fig. 4). The same result was obtained for B. subtilis DivIVA (molecular weight of monomer is 19.5 kDa) and B. subtilis His$_6$-Tag-DivIVA (molecular weight of monomer is 21.5 kDa). Based on this result and assuming that B. stearothermophilus DivIVA forms a similarly shaped oligomers, we can suggest that thermophilic DivIVA forms oligomers with a similar molecular weight as B. subtilis DivIVA oligomers. Oligomerization of B. subtilis DivIVA was confirmed by analytical ultracentrifugation (MUCHOVA et al., 2002a). These observations showed existence of various oligomeric species with the molecular mass averages extended from values associated with a dimer to that associated with at least a decamer. Recently, the ability of B. subtilis DivIVA to form “doggy bone” shaped particles was observed. They were interpreted as DivIVA 6-8mers (STAHLEBERG et al., 2004). Thus, we would like to propose that B. stearothermophilus DivIVA forms 6-8mers similarly as B. subtilis DivIVA.

Oligomerization of B. stearothermophilus DivIVA was also observed by Western blot analysis of non-denaturating polyacrylamide gel (Fig. 3C). The positions of signals for B. stearothermophilus and B. subtilis DivIVA oligomers were slightly different. However, it does not mean that they differ in quaternary structure. This observation can be caused by different interaction of Coomassie Brilliant Blue (CBB) with both proteins. CBB interacts with all positively charged amino acids on the surface of a protein. The total charge of the protein is then characterized by its initial negative charge and by charge obtained from CBB.
Electrophoretic mobility of protein in blue native gel is dependent on the shape of molecule and on the charge of the protein complex. His6-tagged *B. stearothermophilus* DivIVA has more positively charged amino acid residues than its *B. subtilis* counterpart and thus it has higher mobility in blue native gel.

**Computational analysis of DivIVA sequence**

Based on the results of nnPREDICT and PSA programs, secondary structure elements of *B. stearothermophilus* DivIVA were predicted. It is expected that the protein contains three α-helices Glu22-Ile143, Arg147-Leu151 and Leu159-Glu165, marked as H1, H2 and H3 (Fig. 2). Analyses by the COILS program and the web-service SMART confirmed heptad repeat in the DivIVA sequence (Fig. 2) and predicted the start and the end of the coiled-coil region at Phe28 and Ile103, respectively. These analyses show that the coiled-coil region is localized inside of H1. The similar localization of coiled-coil region with start at the N-terminus of protein sequence was determined in the *B. subtilis* DivIVA sequence (EDWARDS et al., 2000). Breaking of the α-helical structure by Leu120Pro (DivIVA2) mutation in the coiled-coil region leads to the change of ability to oligomerize, while Ala78Thr (DivIVA1) mutation had no effect on the DivIVA oligomerization (MUCHOVA et al., 2002a), although the latter mutation disrupts functional abilities of DivIVA.

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**References**


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