Analysis of the Bacillus cereus SpoIIS antitoxin-toxin system reveals its three-component nature

Jana Melničáková †, Zuzana Bečárová †, Jana Makroczyová and Imrich Barák *

Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovakia

Programmed cell death in bacteria is generally associated with two-component toxin-antitoxin systems. The SpoIIS toxin-antitoxin system, consisting of a membrane-bound SpoIISA toxin and a small, cytosolic antitoxin SpoIISB, was originally identified in Bacillus subtilis. In this work we describe the Bacillus cereus SpoIIS system which is a three-component system, harboring an additional gene spoIISC. Its protein product serves as an antitoxin, and similarly as SpoIISB, is able to bind SpoIISA and abolish its toxic effect. Our results indicate that SpoIISC seems to be present not only in B. cereus but also in other Bacilli containing a SpoIIS toxin-antitoxin system. In addition, we show that B. cereus SpoIISA can form higher oligomers and we discuss the possible role of this multimerization for the protein’s toxic function.

Keywords: Bacillus cereus, toxin-antitoxin system, SpoIIS, programmed cell death, Bacillus subtilis

Introduction

Programmed cell death (PCD) is a genetically regulated system in which a bacterial cell is able to commit suicide in response to a variety of different stresses. This response includes cell lysis or growth inhibition induced by harsh environmental conditions such as starvation or antibiotic treatment, active mother cell lysis during sporulation to release the spore, or altruistic suicide to release cell content to provide the nutrients required for the normal development of the remaining bacterial population (Engelberg-Kulka et al., 2006). PCD is usually mediated by a pair of toxin/antitoxin (TA) genes. Toxins are always highly stable proteins. Their antidotes, the antitoxins, are usually labile proteins or small RNAs. TA systems are classified according to the nature of the antitoxin. Type I and III are small RNAs which either inhibit the synthesis of the toxin or capture it. Examples include the type I hok-sok system (Pedersen and Gerdes, 1999) and the type III ToxIN system (Fineran et al., 2009). Types II, IV, and V, on the other hand, are all proteins. They include the type II mazEF TA system (Gerdes et al., 2005), the type IV yeeU-yeeV system (Masuda et al., 2012), and the type V ghoT-ghoS system (Wang et al., 2012). These three types are distinguished based on their mode of action. The type II antitoxin is a small protein with an N-terminal DNA-binding domain and a C-terminal toxin-binding domain, the type IV antitoxin is an antagonist of its cognate toxin and competes with it in binding to its target, and the type V antitoxin is an endoribonuclease that degrades the toxin-encoding mRNA (Goeders and Van Melderen, 2014).

Many bacteria harbor genes for TA systems on plasmids (Ruiz-Echevarria et al., 1995; Gerdes et al., 1997; Sayeed et al., 2000; Van Melderen, 2001; Camacho et al., 2002). These genes are part of a mechanism called post-segregational killing, which ensures that their host plasmids are retained in the daughter cells of a growing bacterial population. In this process, the stable,
The spoIIS locus was originally identified on the Bacillus subtilis chromosome during a study of the genetic mutants that block sporulation after the formation of the polar septum (Adler et al., 2001). Formerly, the locus was thought to consist of two genes, spoIISA coding for the toxin and spoIISB for proteic antitoxin (Adler et al., 2001), thus classifying as type II TA system. A condition-dependent analysis of the transcription of all B. subtilis genes indicated that a third transcriptionally active region, S458, might be present in the spoIS operon (Nicolas et al., 2012), which we name spoISC. Inactivation of the spoIISA toxin gene has no effect on sporulation, but inactivation of the spoIISB antitoxin gene decreases sporulation efficiency by four orders of magnitude. Furthermore, disruption of spoIISA in a spoIISB null mutant restores sporulation. Thus, spoIISA is required for sporulation only if SpoIISA is present in the cell (Adler et al., 2001). The morphological consequence of an artificially induced higher level of toxin expression is the formation of plasmolysis zones in the cytoplasmic membrane, leading to the death of the cell. The transcription of spoIISA, spoIISB, and spoISC is upregulated during sporulation from four to up to eight hours (Nicolas et al., 2012); however, the expression of SpoIISA is independent of the crucial sporulation initiation transcription factor, SpoOA (Rešetárová et al., 2010). Production of the SpoIISA toxin is also induced during ethanol stress and nutrient deprivation. During starvation, the production of SpoIISB was detected, which suggests that SpoIISB is able to diminish the toxic effect of SpoIISA. Moreover, SpoIISB is also produced during swarming and at times of high cell density. There is presently only a little information about spoIISB, but it is known that its transcription is activated during both sporulation and biofilm formation (Nicolas et al., 2012). The SpoIISB toxin is neutralized by the formation of a tight complex with the SpoIISB antitoxin. The crystal structure of this complex revealed that SpoIISB and the cytoplasmic domain of SpoIISA form a heterotetrameric complex with C-SpoIISA2:SpoIISB2 stoichiometry (Florek et al., 2011).

Homologs of SpoIISA and SpoIISB proteins have also been identified among other Bacillus species, but they display only a low level of homology. Both B. subtilis and B. cereus SpoIISA inhibit the growth of E. coli cells, and the SpoIISB antitoxin is able to neutralize SpoIISA toxicity in E. coli (Florek et al., 2008).

In the present study we analyze the spoIS operon in B. cereus ATCC 14579. Even though a third transcriptionally active region in the spoIS operon of B. subtilis was identified, it is unclear whether its product is really part of this TA system. We have found that both B. subtilis and B. cereus spoISC encode an antitoxin that is able to diminish SpoIISA toxicity in E. coli. As in B. subtilis, the B. cereus spoIS operon consists of three genes: spoIISA, spoIISB, and spoISC. Using a bacterial two hybrid system we show that B. cereus C-SpoIISA interacts with other C-SpoIISA molecules, as well as with SpoIISB and SpoISC. These new positive interactions, identified in vivo, were also confirmed in vitro using a pull-down assay. In vitro analysis of the oligomeric states of B. cereus C-SpoIISA revealed that the soluble C-SpoIISA exists in monomeric, dimeric and trimeric forms.

Materials and Methods

Bacterial Strains, Growth Conditions, and Media

The bacterial strains E. coli XL1-BLUE, DH5a, and MM294 were used for routine DNA manipulations. The E. coli BTH101 reporter strain was employed in the bacterial adenylylate cyclase-based two-hybrid system. E. coli BL21 (DE3) cells were employed in expression of recombinant protein. E. coli cells were grown at 37°C, 28°C or room temperature in LB (Ausubel et al., 1987) or SOC medium (Hanahan, 1983) or on agar plates. When required, the medium was supplemented with appropriate antibiotics and other additives. E. coli transformation and DNA manipulations were performed using standard protocols (Sambrook et al., 1989).

The Kill/Rescue Assay Cultivation

To evaluate the effect of the expression of B. cereus spoIS genes on the growth of E. coli MM294, a single colony of bacterial cells was resuspended in 100 µl of LB and grown overnight on LB agar plates. The bacterial lawn was washed off with 1 ml LB and this primary culture was used to inoculate a second cell generation in LB containing 100 µg ml⁻¹ ampicillin and 0.5% glucose (w/v). The starting optical density (OD₆₀₀) of the cell cultures was 0.05–0.06. The cells were cultivated at 37°C in an orbital shaker at 150 rpm and growth was monitored by measuring the OD₆₀₀ in 1-h intervals. When the OD₆₀₀ reached 0.4, spoIS expression was induced by the addition of l-arabinose to a final concentration of 0.02% (w/v).

Recombinant Plasmid Construction

All bacterial strains and plasmids used in this study are listed in Table 1. All primers for cloning were designed for the PCR amplification of specific genes and regulatory regions and are listed in Table 2. Chromosomal DNA of B. subtilis PY79 (Youngman et al., 1984) and Bacillus cereus ATCC 14579 was used for amplification of spoIS genes.

Bacterial Two-hybrid System

Fragments T25 and T18 from the adenylate cyclase bacterial two-hybrid system (Karimova et al., 1998) were fused with the C-terminal domain of SpoIISA, full-length SpoIISB and SpoISC, all from both B. cereus and B. subtilis. Chromosomal DNA from B. subtilis PY79 and B. cereus ATCC 14579 were used as PCR templates. E. coli BTH101 was used as a host for testing protein-protein interactions. Cells were co-transformed with the relevant plasmid combinations and plated onto LB plates supplemented with 100 µg ml⁻¹ ampicillin, 30 µg ml⁻¹ kanamycin, 40 µg ml⁻¹ X-Gal and 0.1 mM IPTG and grown for 48 h at room temperature.
### TABLE 1 | Strains and plasmids used in this study.

| Strain | Genotype or description | Reference or origin |
|--------|-------------------------|---------------------|
| **E. coli** | | |
| MM294 | F− endA1 hsdR17 (rK−, mk) supE44 thi-1 recA4 | Meselson and Yuan, 1968 |
| BTH101 | F− cya-99 araD139 galE15 galK16 rpsL1 (StrR) hsdR2 mcrA1 mcrB1 | Karimova et al., 1998 |
| DH5α | F− λq supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | Meselson and Yuan, 1968 |
| XL1-BLUE | Δ(mcrA1)Δ3 (mcrCB-hsdSMR-mrr173 endA1 supE44 thi-1 recA1 gyrA96 relA lac (F′ proAB lacR3 ΔM15Tn5 kanR1) | Stratagene |
| **IB890** | pBAD24 in MM294 | Florek et al., 2008 |
| **IB926** | pBAD24-BcIIISA in MM294 | Florek et al., 2008 |
| **PLASMIDS USED IN KILL/RESCUE ASSAY** | | |
| pBAD24 | AmpR, araC, PBAD promoter | Guzman et al., 1995 |
| pBADClIIISA | AmpR, araC, PBAD promoter, B. cereus spoIISA-like gene | Florek et al., 2008 |
| pBADISAB Bc | AmpR, araC, PBAD promoter, B. cereus spoIISAB-like genes | This study |
| pBADISC Bc | AmpR, araC, PBAD promoter, B. cereus spoIISC-like gene | This study |
| pBADISAC Bc | AmpR, araC, PBAD promoter, B. cereus spoIISAC-like genes | This study |
| pBADISA Bs | AmpR, araC, PBAD promoter, B. subtilis spoIISA | This study |
| pBADISAB Bs | AmpR, araC, PBAD promoter, B. subtilis spoIISAB | This study |
| pBADISC Bs | AmpR, araC, PBAD promoter, B. subtilis spoIISC-like gene | This study |
| pBADISAC Bs | AmpR, araC, PBAD promoter, B. subtilis spoIISAC | This study |
| **PLASMIDS FOR TESTING PROTEIN-PROTEIN INTERACTIONS IN VITRO** | | |
| pET15b | AmpR; T7lac promoter | Novagen |
| pETDuet-1 | AmpR; T7lac promoter | Novagen |
| pET15b-Bc-ClIIISA | AmpR; T7lac promoter, B. cereus C-spoIISA | Laboratory stock |
| pET15b-Bc-HClIIISA | AmpR; T7lac promoter, His6 tag fused with B. cereus C-spoIISA | This study |
| pETDuet-Bc-IsClIIAC | AmpR; T7lac promoter, B. cereus spoIISC | This study |
| pETDuet-Bc-HClIIAC | AmpR; T7lac promoter, His6 tag fused with B. cereus C-spoIISAC, T7lac promoter, B. cereus spoIISC | This study |
| pETDuetClIIISA Bc | AmpR; T7lac promoter, His6 tag fused with B. cereus C-spoIISA | This study |
| pETDuetClIIAS Bc | AmpR; T7lac promoter, B. cereus spoIISAC fused with S-tag | This study |
| pETDuetClIIAS Bc | AmpR; T7lac promoter, His6-tag fused with B. cereus C-spoIISA, T7lac promoter, B. cereus spoIISB fused with S-tag | This study |
| **PLASMIDS FOR THE BACTERIAL TWO-HYBRID SYSTEM** | | |
| pKT25 | KanR; Plac promoter, T25 | Karimova et al., 1998 |
| pKN25 | KanR; Plac promoter, T25 | Karimova et al., 1998 |
| pUT18 | AmpR; Plac promoter, T18 | Karimova et al., 1998 |
| pUT18C | AmpR; Plac promoter, T18 | Karimova et al., 1998 |
| pKT25-zip | KanR; Plac promoter, T25 fused with zip | Karimova et al., 1998 |
| pUT18-zip | AmpR; Plac promoter, T18 fused with zip | Karimova et al., 1998 |
| pKTCIIAS Bc | KanR; Plac promoter, T25 fused with B. cereus C-SpoIISA | This study |
| pKNTClIIAS Bc | KanR; Plac promoter, B. cereus C-spoIISA fused with T25 | This study |
| pUTClIIAS Bc | KanR; Plac promoter, B. cereus C-spoIISA fused with T25 | This study |
| pUTClIIAS Bc | KanR; Plac promoter, B. cereus C-spoIISA fused with T25 | This study |
| pUTClIIAS Bc | KanR; Plac promoter, B. cereus C-spoIISA fused with T25 | This study |
| pUTClIIAS Bc | KanR; Plac promoter, B. cereus C-spoIISA fused with T25 | This study |
| pKTIISAS Bc | KanR; Plac promoter, B. cereus C-spoIISA fused with T25 | This study |
| pKTIISAS Bc | KanR; Plac promoter, B. cereus C-spoIISA fused with T25 | This study |
| pKTIISAS Bc | KanR; Plac promoter, B. cereus C-spoIISA fused with T25 | This study |
| pKTIISAS Bc | KanR; Plac promoter, B. cereus C-spoIISA fused with T25 | This study |
TABLE 1 | Continued

| Strain       | Genotype or description | Reference or origin |
|--------------|-------------------------|---------------------|
| pUTClISB Bs  | Amp^{R}, P_{lac} promoter, 718 fused with B. subtilis spoIIB | This study          |
| pUTIISC Bs   | Amp^{R}, P_{lac} promoter, B. subtilis spoIISC fused with 718 | This study          |
| pUTClISC Bs  | Amp^{R}, P_{lac} promoter, 718 fused with B. subtilis spoIISC | This study          |
| pKTIISC Bs   | Kan^{R}, P_{lac} promoter, T25 fused with B. subtilis spoIISC | This study          |
| pKNTIISC Bs  | Kan^{R}, P_{lac} promoter, B. subtilis spoIISC fused with T25 | This study          |

TABLE 2 | Primers used in this study.

| Primer       | Sequence (5′–3′), restriction sites are in bold | Final construct |
|--------------|-----------------------------------------------|-----------------|
| SP/Bc-CIISA/Xhol | TCATCATCACTCGAGAATATGGGGTGCGAAATT              | pETDuet-Bc-HCIISA |
| ASP/Bc-CIISA/BamE | TCATCATCACTCGAGTTTACACATTAATCTGAT            | pETDuet-Bc-IISC |
| SP/BcIISB/Ndel | TCATCATCACTAGTTGATTGATGTAAGAAGGTGTA          | pBADIISAB Bc    |
| ASP/BcIISB/Xhol | TCATCATCACTCGAGTACACTTATGATTTCCTTTTTTT      | pBADIISAC (via pBADIISAB Bc) |
| SP/IISCBc/EcoRI | TCATCATCAATTCTAGCTTACTACATTCAAGAAAAGGAAGAGATG | pBADIISAC (via pBADIISAB Bc) |
| ASP/IISCBc/NcoI | TCATCATCACTCGAGTACACTTACTACATTCAAGAAAAGGAAGAGATG | pBADIISAC (via pBADIISAB Bc) |

SDS-PAGE Analysis

One dimensional SDS-PAGE was performed according to Laemmli (1970). Samples of whole cell lysates of recombinant-protein expressing E. coli  BL21 (DE3) cells, protein complexes, or purified protein samples were resuspended in sample buffer [4% SDS (w/v); 10% β-mercaptoethanol (v/v); 20% glycerol (v/v); 0.25 M Tris-Cl, pH 8] and boiled for 10 min. Denatured proteins were separated in 12% polyacrylamide gels. Due to the low
molecular weight of *B. cereus* SpoIISC (6.6 kDa), this protein was analyzed using 16.5% Tricine–SDS-PAGE (Schägger and von Jagow, 1987), which better resolves such small proteins. As for the SDS-PAGE, samples of whole cell lysates of *E. coli* BL21 (λDE3) cells expressing recombinant SpoIISC and purified protein samples were resuspended in Novex sample buffer (Invitrogen, USA), then heated for 5 min in a boiling water bath and briefly spun down. The gels were run at 25 mA and stained with Coomassie brilliant blue R-250.

**Pull-down Assay**

Pull-down assays were used to confirm *in vitro* the interactions between *B. cereus* C-SpoIISA and SpoIISB, SpoIISC and *B. subtilis* C-SpoIISA. In order to investigate the interaction of His$_6$-tagged *B. cereus* C-SpoIISA with S-tagged SpoIISB, the following proteins were isolated: His$_6$-tagged C-SpoIISA, S-tagged SpoIISB and His$_6$-tagged C-SpoIISA expressed together with S-tagged SpoIISB. *E. coli* BL21 (λDE3) competent cells were transformed with the pETDuet-CIISA Bc and pETDuetIISB Bc plasmids (Table 1) for the overexpression of His$_6$-tagged C-SpoIISA and S-tagged SpoIISB, respectively. Transformation with pETDuetCISAB C was performed to obtain co-expression of His$_6$-tagged C-SpoIISA with S-tagged SpoIISB. The resulting cell cultures were grown at 28°C in LB medium supplemented with 100 μg ml$^{-1}$ ampicillin and 0.5% glucose. Recombinant protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM, when the culture reached an OD$_{600}$ of ~0.6. Cells were harvested 5 h after induction, centrifuged, and resuspended in solubilization buffer [20 mM Tris–Cl, pH 8; 150 mM NaCl; 10% glycerol (v/v); 10 mM MgCl$_2$; 1 mM AEBSF]. Proteins were solubilized by overnight incubation at 14°C in the presence of 10 mM CHAPS (Sigma Aldrich). Samples were centrifuged for 30 min at 60 000 × g and 4°C. Soluble fractions were loaded onto a Ni Sepharose HP column (Amersham Bioscience) and washed; bound proteins were eluted with an imidazole step gradient from 0.2 M, to 0.4 M, 0.6 M and 1 M. The most concentrated fraction of the His$_6$-tagged C-SpoIISA, that with 1 M imidazole, was used in further experiments. The S-tagged *B. cereus* SpoIISB 0.2 M imidazole fraction was used as a control for non-specific binding to the Ni column. Finally, the 0.4 M imidazole fraction of SpoIISB was used in the assay to confirm that His$_6$-tagged C-SpoIISA interacts with S-tagged SpoIISB. These proteins and the C-SpoIISA–SpoIISB protein complex were fractionated by 16.5% Tricine–SDS–PAGE. The fractioned proteins were transferred onto a nitrocellulose membrane and subsequently Western blotted.

The pull-down assay of His$_6$-tagged *B. cereus* C-SpoIISA with untagged SpoIISC was performed similarly as described above. In this case, *E. coli* BL21 (λDE3) cells were transformed with pETDuet-Bc-HCIISAC for the interaction study and pETDuet-Bc-IISC (Table 1) to control for the non-specific binding of *B. cereus* SpoIISC to the Ni column.

**Glutaraldehyde Crosslinking**

The oligomeric state of recombinant *B. cereus* His$_6$-C-SpoIISA was assessed by glutaraldehyde crosslinking. *E. coli* BL21 (λDE3) competent cells were transformed with pETDuetCISAB Bc, and protein expression was induced with 0.5 mM IPTG for 5 h at 28°C. Cells were then harvested and resuspended in a buffer containing 20 mM HEPES pH 7.5 and 150 mM NaCl and sonicated. The soluble fractions were centrifuged for 30 min at 60 000 × g and 4°C and then loaded onto a Ni Sepharose HP column (Amersham Bioscience). Proteins were eluted with an imidazole step gradient from 0.1 M to 0.2 M, 0.3 M and 1 M. For the crosslinking, 80 μg of protein was mixed with 5 μl of a freshly prepared solution of 2.3% glutaraldehyde to make a total volume of 100 μl. This reaction mixture was incubated for 30 min at 37°C and the reaction was then stopped by the addition of 10 μl of 1 M Tris–HCl, pH 8.0. The crosslinked molecules of *B. cereus* C-SpoIISA were loaded onto a 12% SDS-PAGE gel and detected by Western blotting.

**Western Blotting**

To visualize the interaction of *B. cereus* C-SpoIISA with the heterologous *B. subtilis* C-SpoIISA as well as the interaction of *B. cereus* C-SpoIISA with SpoIISB, we performed Western bloting using the general protocol of Ausbel et al. (1987). Briefly, proteins were fractionated by either 12% SDS-PAGE or 16.5% Tricine-SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond ECL; Amersham Bioscience). To prevent non-specific binding, the membrane was treated using 5% non-fat milk in Tris-buffered saline with 0.05% Tween 20 (v/v). His$_6$-tagged *B. cereus* C-SpoIISA was plotted with an anti His$_6$-tag monoclonal antibody (Novagen; catalog no. 70796-3) while S-tagged *B. subtilis* C-SpoIISA and S-tagged *B. cereus* SpoIISB were probed with an anti S-tag monoclonal antibody (Novagen; catalog no. 71549-3). Protein interactions were detected using antimouse horseradish peroxidase-conjugated secondary antibodies (Promega; catalog no. W402B).

**Gel Filtration**

To analyze the oligomerization of *B. cereus* C-SpoIISA using gel filtration, we developed a procedure for purifying untagged *B. cereus* C-SpoIISA. First, *E. coli* BL21 (λDE3) cells were transformed with the plasmid pET15b-Bc-CIISA. Next, the cell culture was grown at 28°C in LB medium supplemented with 100 μg ml$^{-1}$ ampicillin. When the culture reached an OD$_{600}$ of 0.6, the expression of untagged C-SpoIISA was induced with 0.5 mM IPTG. The cells were harvested 5 h after induction, centrifuged and resuspended in a resuspension buffer containing 50 mM glycine, pH 10; 50 mM NaCl; 10 mM MgCl$_2$; 10% glycerol (v/v); and 1 mM AEBSF. The protein was solubilized by incubating at 14°C overnight in the presence of 10 mM CHAPS (Sigma Aldrich). The soluble fractions were cleared by centrifugation for 30 min at 60 000 × g and 4°C and loaded onto a HiPrep DEAE Sepharose FF 16/10 column (GE Healthcare Life Sciences), which had previously been equilibrated with a resuspension buffer containing 10 mM CHAPS. The protein eluted in the flow-through fraction and was loaded onto a HiPrep Q Sepharose HP 16/10 column (GE Healthcare Life Sciences), previously equilibrated with the same solution. The protein was eluted from this column with a continuous salt gradient ranging from 0.2 to 1 M NaCl. The purified protein was applied to a Superose 6 10/300 GL column (GE Healthcare Life Sciences).
connected to an FPLC (GE Healthcare Life Sciences) instrument controlled by UNICORN 5.11 software, at a flow rate of 0.4 ml min⁻¹. The elution was followed using UV absorbance at 280 nm.

**Dynamic Light Scattering Measurements**

DLS experiments were performed at 20°C on a Zetasizer Nano ZS instrument (Malvern Instrument) controlled by DTS software (version 5.1, Malvern Instruments Ltd). The instrument has a 90° scattering angle. The purified protein, at a concentration of 100 μM in a resuspension buffer at pH 8 containing 10 mM CHAPS, was filtered through 20 nm filters into a 40 μl cuvette. A single measurement consisted of 20 runs of 12 s each. All measurements were done in triplicate. The samples gave a clear signal (the y-intercept was 0.95) and required only moderate attenuation (set at 7).

**Bioinformatics Analysis**

Promoter analysis was performed using BPROM (Solovyev and Salamov, 2011). Identification of Rho-independent bacterial terminators was done using was done using ARNold web tool (Naville et al., 2011; http://rna.igmors.u-psud.fr/toolbox/arnold/). B. cereus SpoIISA membrane topology prediction was done using the MEMSAT3 and MEMSAT-SVM algorithms (http://bioinf.cs.ucl.ac.uk/psipred/; Nugent and Jones, 2009).

**Results and Discussion**

**The SpoIIABC Toxin-antitoxin System**

The SpoIIS toxin-antitoxin system in *Bacillus subtilis* consists of a SpoIISA toxin that is neutralized by a SpoIISB antitoxin (Adler et al., 2001; Florek et al., 2008). However, profiling of the condition-dependent transcription of *B. subtilis* revealed the presence of a third transcriptionally active region, denoted as S458 (Nicolas et al., 2012), located 55 bp downstream of spoIISB in the spoIS operon, which we named spoIISC. Adler et al. (2001) identified two promoters in the *B. subtilis* spoIIS operon. The first promoter (P₁) is located upstream of spoIISA and is important for regulating the expression of both spoIISA and spoIISB. The second promoter (P₂) is located within the spoIISA gene and serves to regulate the expression of spoIISB. A promoter search using BPROM (see Materials and Methods) revealed a possible additional promoter (P₃) downstream of spoIISB which could be used to regulate the expression of spoIISB. Its -35 sequence is 5'-TTTCTTT-3' and its -10 sequence is 5'-ACATATAAT-3'. In addition, a search for Rho-independent bacterial terminators using the ARNold tool identified the terminator (5'- AAAGAAAATGCAATAGACATTTTTTTTGTTTTTTTTA-3'; letters in bold indicate bases contributing to the loop structure, underlined letters are bases forming the stem of terminator hairpin) immediately after the spoIISC STOP codon.

We previously found that a two-component SpoIIS system also exists in *B. cereus* (Florek et al., 2008). The position of its locus on the chromosome is completely different from that of the spoIS operon in *B. subtilis*. While the *B. subtilis* spoIIS operon is 115° away from the origin of replication, the *B. cereus* spoIIS locus is 158° away. The *B. cereus* spoIIS operon consists of spoIISA (BC_2436), which encodes a 245-residue SpoIISA-like protein, and BC_2437, which encodes a hypothetical protein with 58 residues. As shown in Florek et al. (2008), BC_2437 is found 316 bp downstream of the spoIISA-like gene and was named spoISB since its SpoIISB-like product was able to neutralize the toxicity of the SpoIISA-like protein in *E. coli*. Prompted by the identification of a putative third transcript in the *B. subtilis* spoIS operon (Nicolas et al., 2012), we revisited the bioinformatics analysis of the *B. cereus* spoIS operon and found that the *B. cereus* spoIS operon also likely contains three genes: the BC_2436 ORF encoding a 245-residue SpoIISA-like protein; a 138-bp ORF 103 bp downstream of this gene, which encodes a 45-residue, putative SpoISB; and a further 72 bp downstream of that, the BC_2437 ORF, which encodes the 58-residue protein we had previously called SpoIISB, but which we now call SpoISC (Figure 1; Florek et al., 2008). As in the *B. subtilis* analysis, BPROM identified putative promoters in this operon. *B. cereus* spoIISA appears to be driven by the putative promoter P₁, the putative P₂ promoter for controlling spoIISB gene expression is found within the spoIISA gene, and the putative P₃ promoter that likely regulates the expression of spoIISC is located downstream of the spoIISB gene. ARNold tool predicts that a Rho-independent bacterial transcription terminator, with the sequence 5'-AAAGAAAATGCAATAGACATTTTTTTTGTTTTTTTTA-3' (letters in bold indicate bases contributing to the loop structure, underlined letters are bases forming the stem of terminator hairpin). This sequence overlaps with the end of *B. cereus* spoIISC gene (Figure 1A).

The presence of three promoters in the spoIS locus may be due to the different conditions under which the expression of individual genes is induced. The transcription of all three *B. subtilis* spoIS genes is clearly induced during sporulation, but during nutrient deprivation only the spoIISA and spoIISB genes are transcribed (Nicolas et al., 2012). Moreover, there are conditions which induce transcription of only one of these genes: spoIISA is transcribed during ethanol stress, spoISB during swarming and at high cell density, and spoIISC during biofilm formation (Nicolas et al., 2012).

Both *B. subtilis* and *B. cereus* SpoIISB-SpoIISB systems are clear examples of type II TA systems (Adler et al., 2001; Florek et al., 2011). The spoIS operon has been identified only in Bacilli, and only a low level of homology can be detected between the SpoIIS proteins of *B. subtilis* and *B. cereus* (Florek et al., 2008). SpoIISB proteins display 17.3% identity and 30.2% similarity, while the SpoISB proteins have only 12.5% identity and 17.9% similarity. The SpoIISC proteins have the lowest homology, with only 8.6% identity and 15.5% similarity. On the other hand, the SpoIISB and SpoIISC proteins from one of these organisms exhibit a higher level of homology with each other than with their counterparts in the other organism. Thus the *B. subtilis* SpoIISB and SpoIISC proteins show 37.5% homology and 12.5% identity while *B. cereus* SpoIISB and SpoIISC have 35.6% similarity and 27.1% identity (Figure 1B).

Bacterial type II TA systems are normally organized so that the first gene in the operon codes for the antitoxin and the toxin is positioned farther downstream; both genes are usually preceded by their own promoters. This arrangement ensures an abundance of antitoxin is produced to prevent toxin activity when it is
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FIGURE 1 | Comparison of the spoIIS loci of Bacillus cereus and Bacillus subtilis. (A) Genomic organization of the spoIIS locus in B. cereus and B. subtilis. (B) Alignment of the SpoIIS proteins of B. cereus (Bc) and B. subtilis (Bs). Amino acids printed in normal weight on a gray background indicate similar amino acids, while bold weight on a gray background indicates identical amino acids.

undesirable. One exception to this arrangement is the higBA TA module in pathogenic Proteus species (Hurley and Woychik, 2009). As noted above, the spoIIS system is another, with the toxin preceding its two putative antitoxin genes. The SpoIIS TA system is unusual in another way as well. The typical type II TA system is a two-component system, but the SpoIIS TA system consists of three components: the SpoIISA toxin, the SpoIISB antitoxin and the third component SpoIISC (a likely antitoxin). Other three-component type II TA systems have previously been reported, including the ω-ε-ζ TA module encoded by the Streptococcus pyogenes plasmid pSM19035, the paaR-paaA-parE TA module encoded by E. coli O157:H7, and the pasA/pasB/pasC module of the Thiobacillus ferrooxidans plasmid pTF-FC2 (reviewed in Unterholzner et al., 2013). In all of these systems, at least one of the three components is involved in autoregulating the operon.

There is presently no information about whether the expression of the spoIIS operon in Bacilli is autoregulated.

The spoIISB and spoIISC Genes Encode Antitoxins in Bacillus Cereus

B. subtilis transcription analysis by Nicolas et al. (2012) and in this study have revealed that the spoIIS operon is formed by the spoIISA, spoIISB, and spoIISC genes. In E. coli, B. subtilis SpoIISA inhibited bacterial growth and SpoIISB was able to neutralize SpoIISA toxicity (Florek et al., 2008). Previously, we observed that B. cereus SpoIISA, like B. subtilis SpoIISA, has a toxic effect on E. coli growth (Florek et al., 2008), but at that time, we had incorrectly designated ORF BC_2437 as spoIISB. A new bioinformatics analysis, prompted by the likely existence of a third gene in the B. subtilis spoIIS operon by Nicolas et al.
(2012), shows that BC_2437 indeed contains spoIISC and that SpoIISB is a 45-residue protein of unknown function encoded by a small ORF (only 138 bp) located between the spoIISA and spoIISC genes.

To determine if B. cereus SpoIISB and SpoIISC are both able to neutralize the toxicity of B. cereus SpoIISA in E. coli, the corresponding genes spoIISAB and spoIISAC were cloned into pBAD24 vectors under the control of arabinoose-inducible pBAD promoters to generate pBADIISAB Bc and pBADIISAC Bc. These plasmids were subsequently introduced into E. coli MM294 cells. The growth of these transformed cells, together with the control strains IB890 (E. coli MM294 / pBAD24) and IB926 (E. coli MM294/pBAD-BCIISA) (Florek et al., 2008), was monitored after the induction of protein expression. As found previously (Florek et al., 2008), the growth of E. coli cells expressing only B. cereus SpoIISA was inhibited. On the other hand, both SpoIISB and SpoIISC were able to neutralize the toxicity of SpoIISA: the growth curves of those strains which expressed both SpoIISA and either the SpoIISB antitoxin or SpoIISC were similar to that of the wild-type IB890 E. coli cells (Figure 2A). Because B. cereus SpoIISB and SpoIISC disturb SpoIISA toxicity when expressed in E. coli cells, it can be concluded that both spoIISB and spoIISC encode antitoxins and that they are likely to have similar functions as the antitoxins in B. subtilis.

Indeed, an identical set of experiments using the B. subtilis genes rather than the B. cereus ones gives very similar results (Figure 2B).

The Interactions of SpoIIS Proteins in a Bacterial Two Hybrid System

The clearest evidence that B. subtilis SpoIISA and SpoIISB directly interact can be found in the crystal structure of the C-terminal domain of SpoIISA in complex with SpoIISB (Florek et al., 2011). To analyze the protein–protein interactions of the B. cereus SpoIIS proteins in vivo, we made use of the bacterial adenylate cyclase two hybrid system (Karimova et al., 1998). Like B. subtilis SpoIISA, B. cereus SpoIISA is predicted to be a membrane protein with three membrane-spanning segments. However, we decided to use only the cytoplasmic domains in this protein–protein interaction study, since the whole SpoIISA protein is toxic for E. coli as we have shown previously. We prepared fusions of the C-terminal domain of B. cereus SpoIISA, SpoIISB, and SpoIISC with the adenylate cyclase fragments T25 and T18. All possible interactions were tested and compared with those of similar SpoIIS fusion proteins from B. subtilis (Figure 3).

Our results confirmed the dimerization of B. subtilis C-SpoIISA as well as the interaction of B. subtilis C-SpoIISA with SpoIISB described in Florek et al. (2011). A positive interaction was also observed for B. subtilis C-SpoIISA with SpoIISC (Figure 3). Finally, we found that the B. cereus C-terminal domain of SpoIISA can interact with another C-SpoIISA protomer, with SpoIISB and with SpoIISC (Figure 3).

B. cereus SpoIISB and SpoIISC are Able to Bind the C-terminal Domain of SpoIISA in vitro

To analyze these protein–protein interactions in vitro, we prepared three derivatives of the pETDuet recombinant expression plasmid, each containing one of the following genes, all under the control of an IPTG-inducible T7 promoter: a gene coding for a His6-tagged B. cereus C-SpoIISA, an S-tagged SpoIISB and an untagged SpoIISC (Table 1). We found that His6-tagged C-SpoIISA binds the Ni column and that S-tagged SpoIISB and untagged SpoIISC create a tight complex with C-SpoIISA which can be eluted by a solubilization buffer step gradient containing 0.1–1 mM imidazole (Figure 4).
Our bacterial two-hybrid experiments showed that they may also act. Earlier studies also suggested that whole SpoIISA bound C-SpoIISA, the two are pulled down together as a complex during elution with 0.4 M imidazole (Figure 4A). This complex could be detected by Western blotting using an anti-His6-tag monoclonal antibody to identify His6-tagged C-SpoIISA (Figure 4A, lane 3) and an anti-S-tag monoclonal antibody to identify the S-tagged SpoIISB (Figure 4A, lane 6).

A similar approach was used to test the interaction of untagged B. cereus SpoIISC with His6-tagged C-SpoIISA in vitro (Figure 4B). B. cereus SpoIISC expressed in E. coli BL21 (DE3) appears in the insoluble fraction of the cell lysate according to 16.5% Tricine/SDS–PAGE (data not shown). However, when co-expressed with B. cereus His6-tagged C-SpoIISA in the same cells, they form a complex which is able to pull SpoIISB out of the insoluble fraction. The whole complex can then be solubilized and purified from the soluble fraction by affinity chromatography.

**B. cereus C-terminal Domain of SpoIISA forms an Oligomer**

The crystal structure of the B. subtilis SpoIISA C-terminal domain shows that the protein dimerizes by forming a four-helix bundle using the first and last α-helices of each molecule (Florek et al., 2011). Our bacterial two-hybrid experiments showed that B. cereus C-SpoIISA interacts with other B. cereus C-SpoIISA molecules (Figure 3), suggesting that this molecule also forms oligomers. The oligomeric form of C-SpoIISA was examined by measuring the hydrodynamic radius of dissolved particles using dynamic light scattering. A cumulant analysis showed that the sample was monomodal (i.e., had only one peak, Figure 5A), and was polydisperse, with a polydispersity index of 0.255 and an overall polydispersity of 50.32%. The polydispersity indicates broader particle size distribution, and thus the hydrodynamic radius and corresponding molecular mass cannot be reliably calculated.

The SpoIISA oligomerisation was examined further by size-exclusion chromatography of C-SpoIISA using a Superose 6 10/300 GL column. In this analysis, most of the protein appeared in the void volume fraction of the column, which was determined from the elution of Blue dextran 2000 (∼2000 kDa, Pharmacia) (Figure 5B). Three small peaks were detected, however, and likely correspond to the 59.1 kDa trimer, the 39.4 kDa dimer and the 19.7 kDa monomer of C-SpoIISA. The existence of monomeric, dimeric and trimeric states was confirmed by glutaraldehyde crosslinking (Figure 5C), but the existence of higher oligomeric forms could not be confirmed because such large species would not have been able to enter the crosslinking gel.

Taken together, the above results indicate that B. cereus C-SpoIISA is able to form higher multimers, even if their nature is unclear. In this respect, its behavior differs from that of B. subtilis C-SpoIISA, which formed only dimers (Florek et al., 2011). Whole B. subtilis SpoIISA does seem to form higher oligomers, but this seems to require its N-terminal transmembrane domain rather than just its C-terminal cytosolic domain (Makroczynová et al., 2014). Earlier studies also suggested that whole SpoIISA oligomerizes, and moreover suggested that it forms holin-like pores (Adler et al., 2001). Whether either the B. subtilis or B. cereus proteins actually do form such pores remains unknown, however.

Finally, this study describes the SpoIISC protein, a third component of the spoIIS locus. This protein serves as an antitoxin and shows similarity to SpoIISB. The presence of two antitoxin genes in the spoIIS locus of both B. subtilis and B. cereus naturally poses the question of the role of such duplication. One possibility is that the different proteins are linked to different conditions under which they might be expressed, as was shown for B. subtilis SpoIIS system (Nicolas et al., 2012). They may also act as transcription regulators, as some other antitoxins are known to. It is also possible that their different amino-acid compositions could affect their affinity for SpoIISA, leading to different degrees of inhibition. In any case, our results show that the SpoIIS TA system is much more complex than had previously been thought.

**FIGURE 3 | Interaction study of the SpoIIS proteins using the BACTH system.** Since fusions with SpoIIS proteins in both orientations were positive in some cases, only representative ones were selected. A strain expressing a pair of leucine zipper proteins, T25-Zip and T18-Zip, served as the positive control; the negative control was a strain expressing the pair T25-CIISA Bs and T18-Zip. Abbreviations: Bc, B. cereus; Bs, B. subtilis; CIISA, C-terminal domain of SpoIISA; IISB, SpoIISB; IISC, SpoIISC.
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FIGURE 4 | Pull-down assays of B. cereus SpoIISB and SpoIISC with C-SpoIISA. The soluble fractions of lysed bacterial cells were applied to a Ni Sepharose HP column. The eluted proteins were identified by Western blotting (A) and Coomassie brilliant blue R-250 staining (B). (A) In the Western blot, the eluted proteins were probed with an anti-His\textsubscript{6} monoclonal antibody (lanes 1–3) or with an anti-S monoclonal antibody (lanes 4–6). Lanes 1 and 4 contain purified His\textsubscript{6}-tagged C-SpoIISA, lanes 2 and 5, purified S-tagged SpoIISB. S-tagged SpoIISB does not bind a Ni Sepharose HP column. Lanes 3 and 6 show that His\textsubscript{6}-tagged C-SpoIISA can pull down S-tagged SpoIISB and therefore that there is an interaction between them. (B) A pull-down assay showing an interaction between His\textsubscript{6}-tagged C-SpoIISA and SpoIISC when both proteins are co-expressed. TF, total fraction; SF, soluble fraction; IF, insoluble fraction; FT, flow-through fraction; 0.04; 0.2; 0.4, 0.6, and 1.0—molarity of imidazole used in washing and elution. The arrows mark the following positions on the protein ladder from top to bottom: 116, 66.2, 45, 35, 25, 18.4, and 14.4 kDa.

FIGURE 5 | Analysis of the multimeric state of B. cereus C-SpoIISA. (A) Dynamic light scattering analysis of C-SpoIISA oligomer. Size distribution (by intensity) of B. cereus C-SpoIISA, at 20°C, average hydrodynamic radius = 55 nm. (B) Gel filtration of C-SpoIISA. The stars indicate the positions at which the following protein standards eluted from the column (left to right): 2000, 450, 66, 45, and 29 kDa. (C) Western blot analysis of glutaraldehyde-crosslinked His\textsubscript{6}-tagged C-SpoIISA.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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