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Molecular characterization of pathogenic Clostridium difficile strains

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Fundamental research

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Clostridium difficile 630Δ*erm* Spo0A regulates sporulation, but does not contribute to toxin production, by direct high-affinity binding to target DNA

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Abstract

Clostridium difficile is a Gram positive, anaerobic bacterium that can form highly resistant endospores. The bacterium is the causative agent of *C. difficile* infection (CDI), for which the symptoms can range from a mild diarrhea to potentially fatal pseudomembranous colitis and toxic megacolon.

Endospore formation in Firmicutes, including *C. difficile*, is governed by the key regulator for sporulation, Spo0A. In *Bacillus subtilis*, this transcription factor is also directly or indirectly involved in various other cellular processes.

Here, we report that *C. difficile* Spo0A shows a high degree of similarity to the well characterized *B. subtilis* protein and recognizes a similar binding sequence. We find that the laboratory strain *C. difficile* 630Δ*erm* contains an 18bp-duplication near the DNA-binding domain compared to its ancestral strain 630. *In vitro* binding assays using purified C-terminal DNA binding domain of the *C. difficile* Spo0A protein demonstrate direct binding to DNA upstream of *spo0A* and *sigH*, early sporulation genes and several other putative targets. *In vitro* binding assays suggest that the gene encoding the major clostridial toxin TcdB may be a direct target of Spo0A, but supernatant derived from a *spo0A* negative strain was no less toxic towards Vero cells than that obtained from a wild type strain, in contrast to previous reports.

These results identify for the first time direct (putative) targets of the Spo0A protein in *C. difficile* and make a positive effect of Spo0A on production of the large clostridial toxins unlikely.

Introduction

Sporulation is an adaptive strategy that enables bacteria to survive harsh environmental conditions for prolonged periods of time, and is an integral part of the transmission of sporulating pathogens and their tolerance and resistance towards antimicrobial compounds.

Spo0A is the key regulator for sporulation (1,2). Most of our knowledge about the protein is based on work in *Bacilli*. Spo0A is a response regulator that demonstrates phosphorylation dependent binding to DNA (3–5). Phosphorylation occurs through the concerted action of several proteins that together form a so called phosphorelay (6). The signaling cascade allows for the integration of environmental signals into the regulation of Spo0A dependent processes, including sporulation. The two functional domains, the N-terminal phosphorylation and dimerization domain (receiver domain), and the C-terminal DNA binding (effector) domain are separated by a hinge region that is relatively poorly conserved (7). Phosphorylation is believed to result in a structural rearrangement that facilitates dimerization (8,9), resulting in the disruption of transcription-inhibitory contacts between the receiver and effector domains. The isolated DNA binding domain can bind legitimate targets of the Spo0A protein due to the absence of the transcription inhibitory contacts, thereby bypassing the need for phosphorylation (10). Extensive characterization of Spo0A targets has revealed a motif that represents a high affinity Spo0A binding site, the 0A box (10,11). The crystal structure of the DNA binding domain confirms specific and non-specific contacts between the protein and the consensus sequence (12,13). It is noteworthy that Spo0A regulates many other processes than sporulation, such as competence for genetic transformation, DNA replication, and biofilm formation in *B. subtilis* (14–16), virulence factors and stress responses in for instance *B. anthracis* and *B. thuringiensis* (17–21), and solvent production in *Clostridium acetobutylicum* (22,23).

C. difficile is a Gram positive, anaerobic bacterium that is the causative agent of *C. difficile* infection (CDI) (for recent reviews see (24,25)). Though many people are asymptomatically colonized by *C. difficile*, the bacterium can cause serious health problems, such as pseudomembranous colitis and toxic megacolon, under the influence of risk factors such as age and antibiotic use. As a result, CDI was long regarded a nosocomial infection. Recently, however, an increase in the cases of community acquired CDI can be observed (26). Outbreaks of CDI have been linked to so called hypervirulent strains, such as PCR ribotypes (RT) 027 (BI/NAP1) and 078 (27,28). Its main virulence factors are the major clostridial Toxins A and B (29,30). In addition, certain strains of *C. difficile*, including RTs 027 and 078, additionally encode a binary toxin (31,32). *C. difficile* is transmitted via the

fecal-oral route. It is believed that spores are crucial to successfully infect new hosts, as they are able to withstand the harsh environment of the stomach, and survive antibiotic treatments that alter the endogenous flora, after which *C. difficile* can overgrow (24,25).

There is limited knowledge about the regulation of sporulation in *C. difficile*. It has been reported that *spo0A,* as expected, is required for the formation of spores (33) and the gene is required for persistence and transmission in mice (34). Though the pathways downstream of Spo0A seem to a large extent conserved between *B. subtilis* and *Clostridia*, this is less so for the pathways leading to activation of Spo0A (2). It has been suggested that the orphan histidine kinase CD2492 is involved in the activation of Spo0A (35). Similarly, it was reported that multiple orphan histidine kinases can phosphorylate Spo0A in *C. acetobutylicum* (36). Recently, it was reported that *spo0A* can be transcribed from a SigH-dependent promoter (37). It is unknown which genes are regulated by direct binding of Spo0A to their upstream regions.

Here, we establish an *in vitro* binding assay for *C. difficile* Spo0A and demonstrate for the first time direct binding of this transcription factor to DNA upstream of several putative target genes.

Material and Methods

Bacterial strains and media

Escherichia coli strains were routinely grown in Luria-Bertani broth or plates, supplemented with appropriate antibiotics. Chloramphenicol was used at a final concentration of 20 μg/mL for agar plates and 10 μg/mL for liquid cultures. Ampicillin was used at a final concentration of 100 μg/mL. Kanamycin was used at a final concentration of 20 μg/mL. Cloning was carried out using *E. coli* DH5*a*, overexpression was performed in E. coli Rosetta(DE3) pLysS (Novagen). C. difficile strains were grown in a glucose-free trypton-yeast based medium (TTY; 3% w/v bacto-trypton (BD), 2% yeast extract (Fluka), 0.1% w/v thioglycollate (Sigma) pH7.4), supplemented with 20 μg/mL of lincomycin when appropriate, or on CLO or TSS plates (Biomerieux).

Plasmid construction

All plasmids are listed in Table 1. Primers (obtained from Sigma Aldrich) are listed in Table 2 and specific cycling conditions are available on request. Unless noted otherwise, PCR reactions were carried out using Pfu polymerase (Fermentas) according to the instructions of the manufacturer.

Plasmid pWKS1251, for the overproduction of Spo0A-DNA Binding Somain (DBD) carrying a C-terminal 6xHis-tag, was constructed as follows. A sequence corresponding to the DBD of Spo0A was amplified using primers oWKS-1123a and oWKS-1124 using chromosomal DNA from *C. difficile* strain 630Δ*erm* as a template. The resulting fragment was cloned into pCR2.1-TOPO (Invitrogen), yielding pWKS1247. This plasmid was digested with NdeI and XhoI, separated on a 1% agarose/0.5x TAE (20 mM Tris Acetate, 0.5 mM EDTA) gel, the fragment corresponding to the DNA binding domain was recovered by gel-isolation (using a GeneJET Gel Extraction kit, Fermentas) and cloned into similarly digested pMF14 (10) that had been gel-isolated in the same manner. The construct was verified by PCR, restriction analyses and DNA sequencing using primers oWKS-135 and oWKS-136.

Plasmid pWKS1245, for the production of full length Spo0A carrying a C-terminal 6xHis-tag, was constructed in a similar manner using chromosomal DNA from *C. difficile* 630Δ*erm* as a template, but using the PCR product of primers oWKS-1122 and oWKS-1123a. Plasmids used as PCR templates for generating EMSA probes were constructed by cloning the PCR products into pCR2.1-TOPO. The inserts, and in the case of the mutated P*abrB* promoters the presence of the desired point mutations in the consensus 0A box, were verified by DNA sequencing using primers oWKS-24 and oWKS-25.

DNA sequencing

Sequence grade plasmids were isolated using a Nucleospin Plasmid QuickPure kit (Macherey Nagel) according to the manufacturer's instructions, except that two lysis reactions were combined onto a single filter and eluted with 65°C prewarmed AE buffer. All constructs were sequenced using BigDye Terminator chemistry (Invitrogen) on an ABI3130 sequencer (Perkin Elmer), according to the instructions of the manufacturers. In short, ~200 ng of plasmid was mixed with 3.2 pmol of primer, 1 μL Terminator Ready Reaction Mix (Invitrogen) in a final volume of 20 μL. After thermocycling, DNA was precipitated and washed with 65% isopropanol, and dissolved in 12 μL HiDi formamid (Invitrogen) at 96°C for 2 mins and stored in the dark at 4°C until the sequencing run. Sequence analyses were performed in CloneManager Professional Suite 7 (SciEd) and Geneious version 5.6.2 (Biomatters Ltd).

Table 1: Plasmids used in this study.

Name	Description *	Reference
pMF14	pET21b-*spo0A*-DBD-6xHis (Bsu)	(10)
pWKS1245	pET21b-*spo0A*-DBD-6xHis (Cdi)	This study
pWKS1251	pET21b-*spo0A*-6xHis (Cdi)	This study
pWKS1303	pCR2.1TOPO-P*abrB* (Bsu)	This study
pWKS1304	pCR2.1TOPO-P*abrB* (C4T)	This study
pWKS1305	pCR2.1TOPO-P*abrB* (C4T/G5T)	This study
pWKS1306	pCR2.1TOPO-P*abrB* (G2T)	This study
pWKS1307	pCR2.1TOPO-P*abrB* (G2T/C4T)	This study
pWKS1308	pCR2.1TOPO-P*abrB* (G5T)	This study
pWKS1309	pCR2.1TOPO-P*citG* (Bsu)	This study
pWKS1316	pCR2.1TOPO-P*spo0A* (Cdi)	This study
pWKS1317	pCR2.1TOPO-P*spoIIAA* (Cdi)	This study
pWKS1318	pCR2.1TOPO-P*spoVG* (Cdi)	This study
pWKS1322	pCR2.1TOPO-P*sigH* (Cdi)	This study
pWKS1323	pCR2.1TOPO-P*spoIIGA* (Cdi)	This study
pWKS1324	pCR2.1TOPO-P*spoIIE* (Cdi)	This study
pWKS1326	pCR2.1TOPO-P*tcdB* (Cdi)	This study
pWKS1329	pCR2.1TOPO-P*tcdA* (Cdi)	This study
pWKS1332	pCR2.1TOPO-P*fplA* (Cdi)	This study
pWKS1340	pCR2.1TOPO-P*tcd*R (Cdi)	This study
pWKS1341	pCR2.1TOPO-P*ssuA* (Cdi)	This study

* Bsu = DNA derived from *B. subtilis* JH642. Cdi = DNA derived from *C. difficile* 630Δ*erm*.

Protein purification

Plasmids pWKS1245 and pWKS1251 were transformed into *E. coli* Rosetta(DE3) pLysS (Novagen). Transformants were used to inoculate 25 mL of LB with appropriate antibiotics. After overnight incubation, the cells were 1:100 diluted in 500 mL fresh medium containing appropriate antibiotics. Protein production was induced with 1 mM IPTG at an OD_{600} of 0.7 and growth was continued for another three hours before harvesting. Cells were washed with ice cold PBS and stored at -80°C for later use. Purification of the proteins was essentially done as described (10). In short, cells were disrupted in 4 mL lysis buffer (2mM PMSF, 10 mM imidazole, 5 mM beta-mercaptoethanol, 300 mM NaCl, 50 mM NaH_2PO_4, pH7.9). Cleared cell lysates we incubated with 2 mL pre-equilibrated 50% TALON slurry (Clontech) in a final volume of 15 mL lysis buffer for 1hr. The resin was allowed to settle on a Poly-Prep column (BioRad) and washed with 2 mL wash

Table 2: Oligonucleotides used in this study.

Primer	Sequence (5'- 3')	Fragment
oWKS-24	GTGAGCGGATAACAATTTCACACAGG	pCR2.1TOPO insert
oWKS-25	GGTTTTCCCAGTCACGACGTTGTAA	pCR2.1TOPO insert
oWKS-135	GCGAAATTAATACGACTCACTATAGG	pET21b(+) insert
oWKS-136	CAGCCAACTCAGCTTCCTTTC	pET21b(+) insert
oWKS-1223a	TGCTCGAGTTTAACCATACTATGTTCTAGT	C. difficile spo0A-DBD
oWKS-1124	CTCATATGACTAGAAGTGATTTTGTTAAG	C. difficile spo0A and spo0A-DBD
oWKS-1122	TTTCATATGGGGGGATTTTTAGTGG	C. difficile spo0A
oWKS-1193	AGGATTTTTAGTAGGATAATAGC	*B. subtilis Pab*rB*
oWKS-1194	CTCCCAAGAGATACTTATTTG	*B. subtilis PabrB
oWKS-1223	CTCCCAAGAGATACTTATTTGTTTAAATTATATTT TTCTTCGTCATTATTCAACAAAATC	*B. subtilis PabrB -C4A
oWKS-1224	CTCCCAAGAGATACTTATTTGTTTAAATTATATTT TTCTTCGTCATTATTAAACAAAATC	*B. subtilis PabrB -C4A-G5A
oWKS-1225	CTCCCAAGAGATACTTATTTGTTTAAATTATATTT TTCTTCGTCATTATTCGAAAAAATC	*B. subtilis PabrB -G2A
oWKS-1226	CTCCCAAGAGATACTTATTTGTTTAAATTATATTT TTCTTCGTCATTATTCAAAAAAATC	*B. subtilis PabrB -G2A-C4A
oWKS-1230	CTCCCAAGAGATACTTATTTGTTTAAATTATATTT TTCTTCGTCATTATTAGACAAAATC	*B. subtilis PabrB -G5A
oWKS-1199	TGAAAGCGGAGGATACGAAG	*B. subtilis PcitG
oWKS-1200	CAATTCTGTATTCCATTTATGTATC	*B. subtilis PcitG
oWKS-1166	ACACAGGAGGTATCGTACAG	*C. difficile Pspo0A
oWKS-1167	CCCCCCATTAAAAAACATCTTC	*C. difficile Pspo0A
oWKS-1227	GGTGCTACTAGAATGTCTGC	*C. difficile PsigH
oWKS-1228	GACAATTGTCCACTAACTCATAAC	*C. difficile PsigH
oWKS-1231	GCCTCTACCCTTCTATTCAC	*C. difficile PplpA
oWKS-1232	TAACATTAAAGTAGTTCCTCCATAG	*C. difficile PplpA
oWKS-1235	GCTGATGGACGAACCATTTG	*C. difficile PssuA
oWKS-1236	CAATTAAAACTCCCCCAATTCCC	*C. difficile PssuA
oWKS-1166	ACACAGGAGGTATCGTACAG	*C. difficile Pspo0A
oWKS-1167	CCCCCCATTAAAAAACATCTTC	*C. difficile Pspo0A
oWKS-1277	GGTGCTACTAGAATGTCTGC	*C. difficile PsigH
oWKS-1228	GACAATTGTCCACTAACTCATAAC	*C. difficile PsigH
oWKS-1231	GCCTCTACCCTTCTATTCAC	*C. difficile PplpA
oWKS-1232	TAACATTAAAGTAGTTCCTCCATAG	*C. difficile PplpA
oWKS-1235	GCTGATGGACGAACCATTTG	*C. difficile PssuA
oWKS-1236	CAATTAAAACTCCCCCAATTCCC	*C. difficile PssuA
oWKS-1170	GGTTGGTGGAAGAAACATGG	*C. difficile PspoIIAA
oWKS-1171	AATCCCTCCTTCAATAGTTTTG	*C. difficile PspoIIAA
oWKS-1172	AAACGTGCGGATATTATAACTG	*C. difficile PspoIIE
oWKS-1173	ACAACTGCTACACTTCTTTG	*C. difficile PspoIIE
oWKS-1174	ACTTCTACCTAGATATATTGGATTC	*C. difficile PspoIIGA
oWKS-1175	ACTCAATATACACCATGTATCC	*C. difficile PspoIIGA
oWKS-1168	GGATATCCCTAGTTGTTCATAG	*C. difficile PspoVG

buffer (20 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH7.9). The protein was stepwise eluted in 1 mL fractions after applying 2 mL elution buffer to the column (identical to wash buffer but with 50, 100, 250 or 500 mM imidazole). The whole procedure was carried out at 4°C. Fractions were assayed for purity and yield and suitable fractions were dialysed against 2x 1L dialysis buffer (50 mM Tris-HCl pH8, 1 mM EDTA, 0.5 mM DTT) using Slide-A-Lyzer cassettes with a molecular weight cut-off of 3.5kDa (Pierce).

Proteins were stored at -80°C in storage buffer (identical to dialysis buffer but containing 20% glycerol). Protein concentrations were determined using Bradford reagent (BioRad), according to the manufacturer's instructions.

Electrophoretic mobility shift assays

DNA fragments for use in EMSA experiment were generated by PCR using GoTaq polymerase (Promega) and chromosomal DNA from *B. subtilis* JH642 (Bacillus Genetic Stock Center 1A96; http://www.bgsc.org), plasmids listed in Table 1, or chromosomal DNA from *C. difficile* 630Δ*erm* (38) as a template. Primers for generation of the EMSA probes are listed in Table 2. DNA fragments of the expected size were isolated from a 1xTAE / 8% native polyacrylamide gel using diffusion buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA pH 8, 0.1% SDS) and a QIAExII kit (Qiagen), according to the manufacturer's instructions. Recovered DNA was end-labeled with 32P-γ-ATP using FR buffer and T4 kinase (Invitrogen) according to the instructions of the manufacturer. Specific activity was determined on a LS6000 scintillation counter (Beckman).

EMSA conditions were based on previous studies (10). In short, binding reactions were carried out in binding buffer (10 mM Tris-HCl pH7.6, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, 5% glycerol) in the presence of 200 μg/mL bovine serum albumin (NEB) and 200 cpm/μL radiolabeled DNA fragment. Reactions were incubated for 20 minutes at 30 °C prior to loading on a 1xTAE / 8% non-denaturing polyacrylamide gel that was prerun for 20 minutes at 50V in 1x TAE buffer. Electrophoresis was carried out for 120 min at 85V. After vacuum drying the gels onto filter paper, they were imaged after overnight exposure on Phosphorimager screens on a Typhoon instrument (GE Healthcare).

Cytotoxicity assay

The toxic effects of *C. difficile* culture supernatants on Vero cells (39) were determined as follows. Supernatant from a bacterial culture was harvested by centrifuging cells for 3 minutes at 14000x g and filtered on a 0.45 μM cellulose acetate filter using a syringe. Supernatants were 2-fold serially diluted in cell culture

medium (Dulbecco modified Eagle medium (Lonza) supplemented with 100 μg/mL penicillin, 100 U/mL streptomycin, 10% fetal calf serum), before applying them to a monolayer of Vero cells, and incubation was continued for another hour. As a positive control, 50 μL 1:10 diluted purified toxin (Techlab) was added to the cells. To determine if observed cytotoxic effects were specific for the large clostridial toxins, commercially available anti-toxin against TcdA and TcdB (Techlab) was added to 10-fold diluted bacterial supernatant for 60 min prior to incubation on the Vero cells. Toxin end-point titres were defined as the lowest dilution at which no cytopathological effects (cell rounding) were observed. Statistical significance was evaluated with an independent sample t-test.

Generation of antibodies against Spo0A and immunoblot detection

Immunization of mice with full length *C. difficile* Spo0A-6xHis was kindly performed at the Welcome Trust Sanger Institute (Hinxton, UK). Cells from 1 mL of C. difficile culture were collected by centrifugation for 1 min at 14000 rpm in a table top centrifuge and resuspended in 200 μL resuspension buffer (10 mM Tris HCl pH8, 10 mM EDTA, 0.5 mg/mL lysozyme, 1 mM Pefabloc SC (Roche)). After incubation for 30 mins at 37 °C, 50 μL of 5x SDS sample buffer (0.1 M DTT, 2% SDS, 50 mM Tris HCl pH 6.8, 10% glycerol, 0.0025% BPB) was added, and samples were heated to 96°C for 5 mins. Total cell lysates (amounts corrected for OD_{600}) were separated on a 12% SDS-PAGE gel prior to semi-dry blotting for 1h at 10V to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked in PBST buffer (phosphate buffered saline with 0.1% v/v Tween-20) containing 5% membrane blocking reagent (Amersham Biosciences). To visualize Spo0A protein cleared polyclonal serum from a single mouse at a 1:3000 dilution was used, followed by either a goat-anti-mouse HRP-conjugated secondary antibody followed by ECL+ detection (Amersham Bioscience), or a goat-anti-mouse-biotin-conjugated secondary antibody (Dako) followed by a tertiary mouse-anti-biotin Cy3-conjugated antibody (Jackson). Detection was done using on a Typhoon instrument (GE Healthcare). Background corrected peak volumes were quantified using ImageQuant TL (Amersham Biosciences).

Bioinformatics and software

Alignments of *B. subtilis* and *C. difficile spo0A* were made using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) on the basis of the published genome sequences, Genbank accession numbers AL009126 and AM180355, respectively, and the 630Δ*erm spo0A* sequence as determined in this study. The sequence for *spo0A* of *C. difficile* strain 630Δ*erm* was deposited in Genbank (accession no *JX050222*). Consensus Spo0A boxes were identified using a Single string Search

command in Genome2D (40), allowing 0 mismatches. The box positions were linked to up- and downstream genes using the "Add nearest gene to List of DNA Motifs" feature and Microsoft Excel. The results were manually inspected for those boxes within 500 bp upstream of a gene on the same strand. Figures for publication were prepared using ImageQuant TL (Amersham Biosciences), Adobe Photoshop CS3 (Adobe Systems Inc) and Corel Graphics Suite X5 (Corel Corporation).

Results

Purification of Spo0A and its DNA binding domain

In order to characterize *C. difficile* Spo0A, the full length protein and its DNA binding domain (DBD) were expressed as a C-terminally 6xHis-tagged protein in the heterologous host *Escherichia coli* (Figure 1A) and purified to near homogeneity using metal affinity chromatography (Figure 1A; lanes P). Full length protein was used to raise antibodies to detect Spo0A in total lysates of *C. difficile* strains, and the purified DNA binding domain was used in subsequent *in vitro* binding assays (see below).

Spo0A is expressed throughout growth

We determined the expression of *C. difficile* Spo0A throughout growth. We found that the protein is present in lysates from exponential to stationary growth phase cells. We performed immunoblotting using polyclonal antibodies against *C. difficile* Spo0A on total lysates of wild type and spo0A mutant cells grown in a tryptone-yeast based medium (TTY). We found a clear signal of the size expected for full length Spo0A (~31 kDa) as early as 3 hours post inoculation (exponential growth phase), through transition phase (8h) as well as 24 and 48 hours post inoculation (stationary growth phase) (Figure 1B; 630Δ*erm*). The signals were specific for *C. difficile* Spo0A as they were absent from lysates from the *C. difficile spo0A* mutant (Figure 1B, CT::spo0A). We obtained similar results in other media, such as the commonly used supplemented brain heart infusion broth (BHIS; data not shown).

To determine relative levels of Spo0A throughout growth, we performed an immunoblot experiment using fluorescent antibodies, which gives more quantitative information compared to the use of horseradish peroxidase conjugated antibodies in our hands. We found that the levels of Spo0A increases approximately 20-fold from 6 hours post inoculation and remains at similar levels from 8 to 48 hours post inoculation (Figure 1C).

Though it should be noted that the Western blots do not provide information on the phosphorylation state of the protein, we conclude that the protein in active

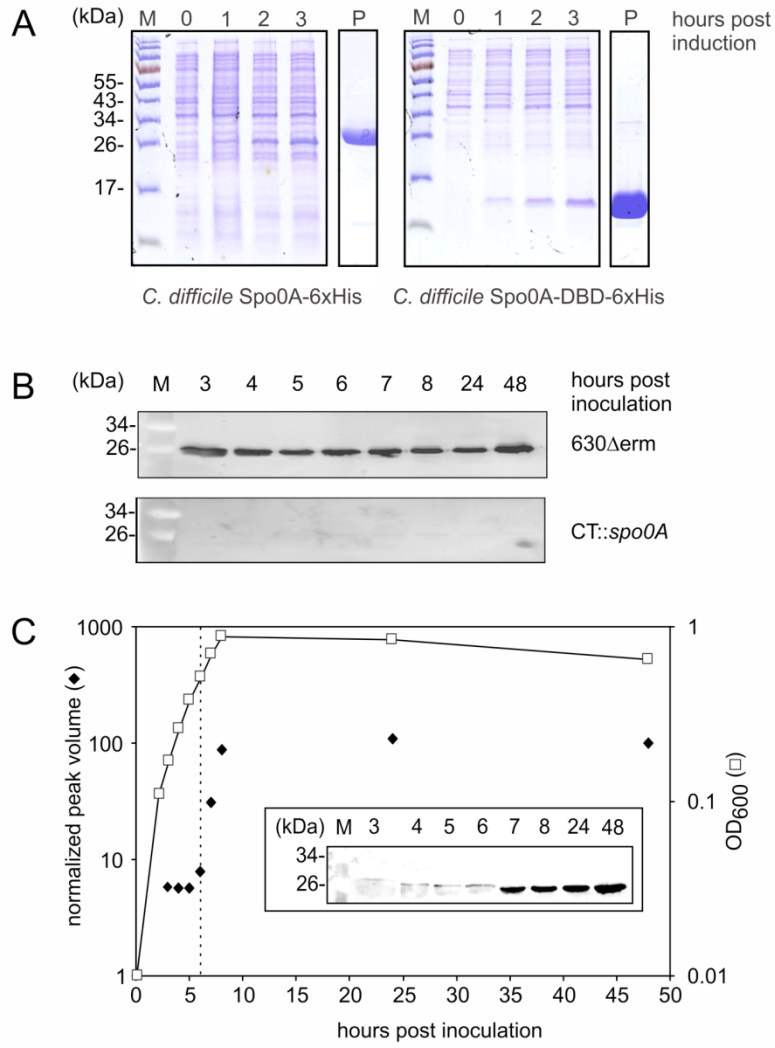

Figure 1: Purification and detection of *C. difficile* Spo0A. **(A)** Heterologous overproduction of Spo0A-6xHis and Spo0A-DBD-6xHis in *E. coli* Rosetta(DE3) pLysS. M = molecular weight marker, numbers indicate hours after induction with 1mM IPTG. P = metal affinity purified protein. Lysates were separated on a 12% SDS-PAGE. **(B)** *and* **(C).** Immunoblot detection of Spo0A in total cell lysates of a *C. difficile spo0A* mutant (CT::*spo0A*) and a wild type strain (630Δ*erm*). Times indicated range from early exponential (3h post inoculation) to late stationary growth phase (48h post inoculation). Sample volumes were corrected for OD_{600} to ensure loading of similar amounts of total cell lysate in each lane. For details see Materials and Methods. M = molecular weight marker. **(B)** ECL+ detection. **(C)** Fluorescent detection. Y-axes show peak volumes normalized to values at 48 hours post inoculation (closed diamonds; left axis) and optical density readings at 600nM (open squares; right axis). Inset shows the blot on which the curve is based. Vertical dashed line indicates the moment Spo0A levels increase sharply (6 hours post inoculation).

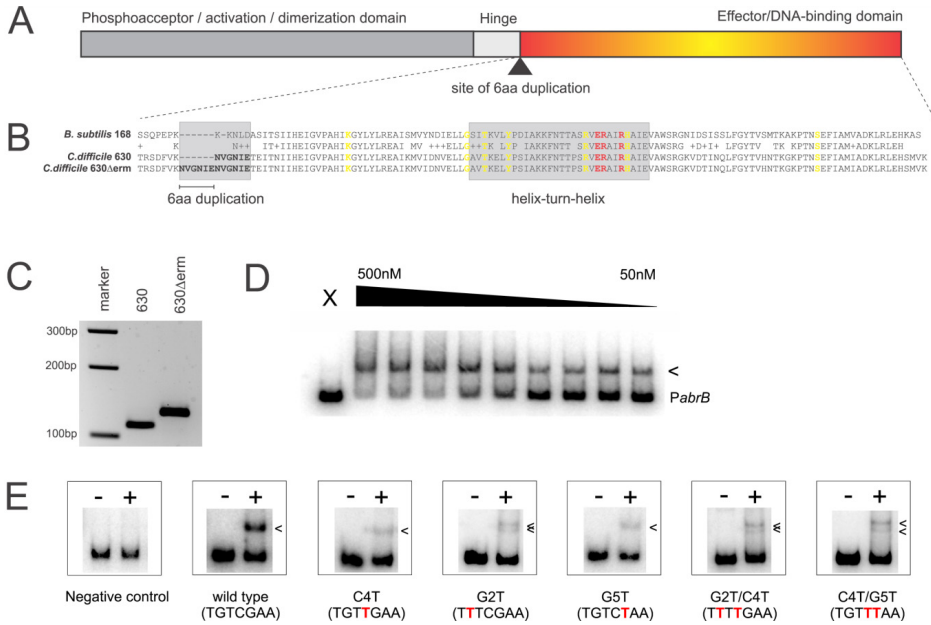

Figure 2: (A) Spo0A box is important for high affinity binding by *C. difficile* Spo0A. **(A)** Domain organization of Spo0A. The site of the duplication in strain 630Δ*erm* identified in this study is indicated by an arrow. **(B)** Sequence alignment of the C-terminal regions of the Spo0A proteins of *B. subtilis* 168 and *C. difficile* strains 630 and 630Δ*erm*. Residues identified in structural studies using Bacillus Spo0A as involved in backbone interactions are indicated in yellow, residues forming base-specific contacts are indicated in red (13). The region of the 6aa duplication and the helix-turn-helix motifs are boxed in gray and the duplication in strain 630Δ*erm* is underlined. **(C)** PCR showing the presence of the duplication near the DNA binding domain in *C difficile* 630Δ*erm* compared to 630. **(D)** Electrophoretic mobility shift assay using purified *C. difficile* Spo0A-DBD-6xHis and a radiolabeled P*abrB* DNA fragment. X = no protein control, the triangle indicates 1.3-fold serial dilutions of protein to the indicated concentrations. The arrow indicates a DNA:protein complex. **(E)** Electrophoretic mobility shift assays without (-) or with (+) 150 nM Spo0A-DBD-6xHis added to radiolabeled P*abrB* fragments carrying mutations in the consensus Spo0A box (in red). Arrows indicate DNA:protein complexes. The negative control is P*citG* from *B. subtilis*.

or inactive form is present throughout growth and is more abundant in stationary growth phase.

Spo0A of *C. difficile* strain 630Δ*erm* contains a 6-aminoacid duplication

BLAST homology searches readily identify a homolog of the well-characterized *B. subtilis* Spo0A protein in *C. difficile* 630 (CD1214) and previous work demonstrated that a spo0A mutant (an insertional inactivation of cd1214) – as expected – no longer forms spores (41). In silico analyses suggest a similar secondary structure for both proteins (Figure 2A), with a conserved dimerization and DNA binding domain, separated by a poorly conserved hinge region (7,12).We compared the sequence of CD1214 obtained from our lab strain 630⊿*erm* (38) to that of the published *C. difficile*630 genome (42). Strain 630⊿*erm* is a spontaneous erythromycin sensitive strain, which is commonly used in mutagenesis studies and was obtained by serial passaging of strain 630 (33,38). The 630⊿*erm spo0A* sequence (Genbank accession no *JX050222*) was derived from the expression plasmids constructed for this study, and confirmed in a whole genome sequence of strain 630⊿*erm* generated in our lab (data not shown). We found that 630⊿*erm* spo0A contains an 18 base pair direct repeat,resulting in a 6 amino acid (NVGNIE) duplication compared to the published reference sequence. The duplication maps to a region of the protein with relatively low sequence conservation (hinge), flanking the highly conserved DNA binding domain (Figure 2A and B). We verified the absence of this duplication in strain 630 by PCR (Figure. 2C) as well as sequencing from the chromosomal DNA of *C. difficile* 630 (data not shown), to rule out an error in the original genome sequence and to demonstrate that the difference in size of the PCR product was specific to the 18bp insertion. In addition, we checked several other strains of PCR ribotypes 12 (to which 630 and 630⊿*erm* belong) by PCR, but the duplication was found to be unique to 630⊿*erm* among the isolates tested (data not shown).

C. difficile Spo0A-DBD shows similar specificity as _B. subtilis_ Spo0A-DBD

Next, we examined the conservation of the Spo0A-DBD between *B. subtilis* and *C. difficile*. In *B. subtilis* amino acid residues contacting the backbone of the DNA and interacting with specific residues of the Spo0A binding sequence have been defined (13). We found that all these residues were conserved in the *C. difficile* protein sequence (Figure 2B), indicating that the protein likely recognizes a similar motif.

C. difficile Spo0A-DBD shows similar specificity as _B. subtilis_ Spo0A-DBD

Next, we examined the conservation of the DNA binding domain of Spo0A (Spo0A-DBD) between *B. subtilis* and *C. difficile*. In *B. subtilis* amino acid residues

contacting the backbone of the DNA and interacting with specific residues of the Spo0A binding sequence have been defined (13). We found that all these residues were conserved in the *C. difficile* protein sequence (Figure 2B), indicating that the protein likely recognizes a similar motif.

DNA binding by full length Spo0A in *B. subtilis* requires phosphorylation dependent dimerization (8,9). However, it was shown that the isolated DBD is capable of binding to legitimate targets of the full length protein (10). Analogously, we purified the *C. difficile* Spo0A-DBD for use in *in vitro* binding assays. As no direct targets for the *C. difficile* protein have been reported so far, we used the upstream region of the *abrB* gene (P*abrB*) of *B. subtilis*. P*abrB* is commonly used as a high-affinity control in binding assays with the *B. subtilis* Spo0A or Spo0A-DBD protein (43,44). It is noteworthy that we failed to identify a homolog of *abrB* in *C. difficile* using BLAST, indicating that potential indirect regulation by Spo0A cannot occur through *abrB* in *C. difficile* as it does in *B. subtilis*. We found that *C. difficile* Spo0A-DBD bound with high affinity to P*abrB* (Figure 2D and E). We performed electrophoretic mobility shift assays (EMSAs) using radiolabeled P*abrB* and increasing amounts of purified *C. difficile* Spo0A-DBD that was purified using a C-terminal 6xHis-tag. The addition of protein leads to a dose-dependent retardation of the DNA fragment with an apparent K_D of <50nM. In the same range of protein concentrations, no binding was observed for a negative control (a DNA fragment of *B. subtilis citG* (*45*)) (Figure 2E), suggesting that binding was specific for the *abrB* promoter region. *B. subtilis* Spo0A recognizes a distinct sequence (0A box), that is characterized by a 7bp core motif (TGTCGAA) (10,11). Structural studies have revealed that the protein makes specific contacts with the G at position 2 (G2), and the C at position 4 (C4) and 5 (G5) of this motif (13). We introduced G2A, C4A, G5A, G2A/C4A and C4A/G5A mutations in the perfect consensus core 0A-box present in P*abrB*. We found that the affinity of *C. difficile* Spo0A for these mutated P*abrB* fragments was highly reduced (Figure 2E). We performed EMSAs using radiolabeled P*abrB* containing the mutated core sequence. For the single point mutations in the DNA, the affinity decreased ~10-fold. There did not seem to be an additive effect of a second point mutation for the two combinations tested. None of the mutations abolished binding of *C. difficile* Spo0A completely, most likely as the result of binding of Spo0A to other (non-consensus) 0A boxes in the *abrB* promoter (44).

Taken together, we conclude that the guanine and cytosine residues in the core TGTCGAA motif of P*abrB* are important for specific binding of this fragment by *C. difficile* Spo0A-DBD.

The presence of a consensus Spo0A box has predictive value for binding by *C. difficile* Spo0A-DBD

Above, we have established that the Spo0A-DBD of *C. difficile* is highly homologous to that of the *B. subtilis* Spo0A protein, and that the proteins recognize a similar consensus sequence (Figure. 2). Based on this information, we identified the several genes as putative direct targets of *C. difficile* Spo0A. We queried the *C. difficile* 630 genome sequence for perfect matches to the core 0A box using Genome2D (40). Such an analysis revealed the presence of 102 matching motifs, of which 45 were located within 500bp of the initiating ATG of an open reading frame on the same strand (data not shown). Our attention was drawn to *spo0A* and *sigH*, as these two genes were previously found to be regulated by Spo0A in *B. subtilis* and/or play important roles in sporulation (3,46–48). We found that *C. difficile* Spo0A bound to DNA sequences upstream of *spo0A* and *sigH*.

We performed EMSAs with DNA encompassing 220-281bp upstream of the initiating ATG codon of the *spo0A*, *sigH* and *spoVG* open reading frames. We found that the addition of Spo0A-DBD to the reactions caused retardation of the *spo0A* and *sigH* DNA fragments (Figure 3A), but not of a *spoVG* fragment which did not contain a consensus 0A box (Figure 3B). It should be noted that the affinity of Spo0A-DBD for the region upstream of *spo0A* was the highest we have

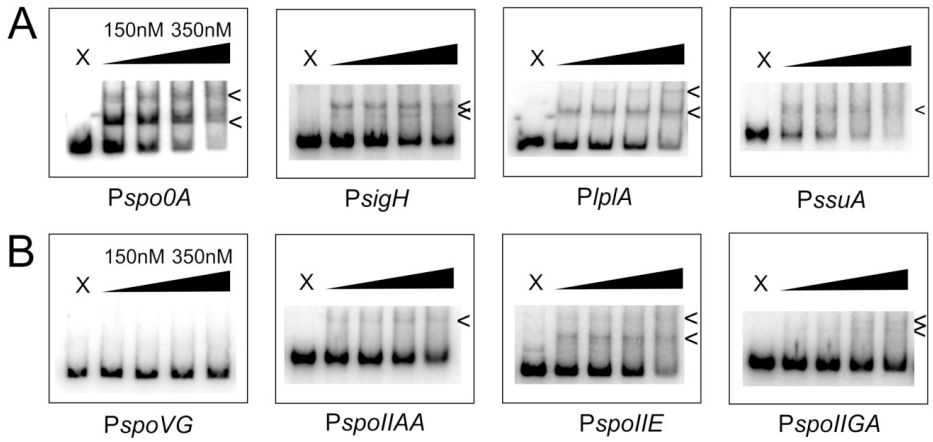

Figure 3: *C. difficile* Spo0A binds to predicted and expected target sequences. Electrophoretic mobility shift assay using purified *C. difficile* Spo0A-DBD-6xHis and a radiolabeled DNA fragments. X = no protein control, the triangle indicates 1.3-fold serial dilutions of protein to the indicated concentrations. The arrows indicate DNA:protein complexes. **(A)** *In silico* predicted target sequences upstream of the genes encoding Spo0A (*spo0A*), σH (*sigH*), a lipoate ligase (*lplA*) and an aliphatic sulphonates ABC transporter (*ssuA*). **(B)** Target sequences predicted on the basis of findings in other organisms: *spoIIAA*, *spoIIE* and *spoIIGA*. The DNA upstream of *spoVG* serves as a negative control.

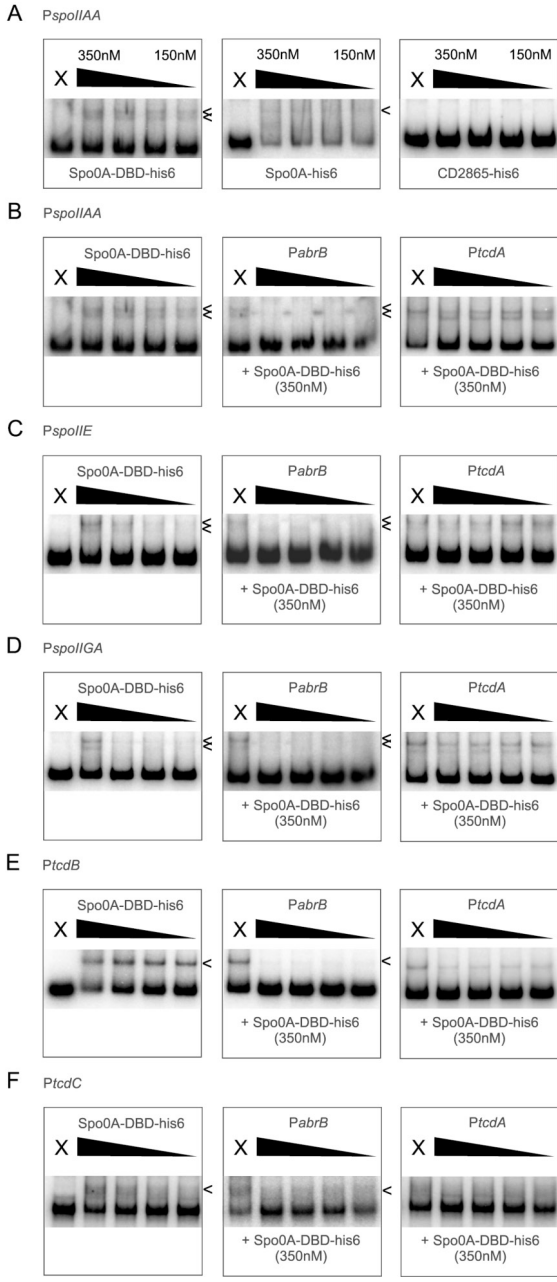

Figure 4: Specificity controls for binding by Spo0A-DBD-his6. Arrows indicate the position of shifted species (DNA:protein complexes). Titrations with PCR fragments of P*abrB* (containing a high affinity binding site) and P*tcdA* (lacking such a site) correspond to approximately 0.1nM/µL - 0.03 nM/µL

(A) Comparison of binding of Spo0A-DBD-his6, Spo0A-his6 and CD2195-his6 binding to the upstream region of *spoIIAA*.

(B) Binding of Spo0A-DBD-his6 to the upstream region of *spoIIAA* is reversed by the addition of P*abrB*, but not by the addition of P*tcdA*).

(C) Binding of Spo0A-DBD-his6 to the upstream region of *spoIIE* is reversed by the addition of P*abrB*, but not by the addition of P*tcdA*.

(D) Binding of Spo0A-DBD-his6 to the upstream region of *spoIIGA* is reversed by the addition of P*abrB*, but not by the addition of P*tcdA*.

(E) Binding of Spo0A-DBD-his6 to the upstream region of *tcdB* is reversed by the addition of P*abrB*, but not by the addition of P*tcdA*.

(F) Binding of Spo0A-DBD-his6 to the upstream region of *tcdC* is not or moderately affected by the addition of P*abrB* and/or P*tcdA*.

observed so far for any *C. difficile* DNA. Moreover, the presence of multiple shifted species could indicate the presence of more than one strong binding site. These results establish that *spo0A* and *sigH* are likely legitimate targets of Spo0A in *C. difficile*, and confirm that *spoVG* is not, in line with results obtained in *B. subtilis* (10).

We were interested to see if Spo0A in *C. difficile* could potentially regulate genes that have no documented function in sporulation. Our *in silico* analysis identified several genes with no obvious link to sporulation that had a consensus 0A box within 100bp upstream of their start codon. This positioning is similar to that observed for *spo0A* (-75) and *sigH* (-78). We confirmed *in vitro* binding of the *C. difficile* Spo0A-DBD to the promoter regions of *lplA* and *ssuA*.

We carried out EMSA experiments using probes that included the perfect consensus site and purified Spo0A-DBD protein. We observed binding of the protein to fragments upstream of the *lplA* gene (CD1654; box at -67) and the *ssuA* gene (CD1484; box at -82) (Figure 3A). The *lplA* gene encodes a predicted lipoate-protein ligase, and *ssuA* is annotated as an aliphatic sulfonates ABC transporter; to our knowledge, neither of these have been directly implicated in sporulation or have found to be targets for Spo0A in other organisms.

Together our results establish the potential for binding of Spo0A to DNA upstream of *spo0A* and *sigH*, two genes that are important for sporulation, and indicate that Spo0A may have functions that go beyond the regulation of sporulation in *C. difficile*.

C. difficile Spo0A-DBD binds to early sporulation promoters

It has been established that a *spo0A* mutant of *C. difficile* does not produce any spores, consistent with a crucial role in the sporulation pathway (33). However, the *in silico* identification of upstream regions with a consensus Spo0A binding site did not point to any of the early sporulation genes (downstream of *spo0A* itself) as direct targets of Spo0A. This is likely the result of variations in the 0A-box in these promoters that were disregarded in the box search. In support of this, many well-characterized legitimate direct targets of *B. subtilis* Spo0A (such as *spoIIAA* and *spoIIE*) do a 100% match to the core motif, but rather one or more near-consensus boxes (5,49). We found that Spo0A-DBD of *C. difficile* is capable of binding the DNA upstream of the open reading frames of *spoIIAA, spoIIE* and *spoIIGA* with low affinity.

We performed EMSA experiments using increasing amounts of purified Spo0A-DBD from *C. difficile* 630Δ*erm* and the DNA fragments indicated above (Figure 3B). For *spoIIAA* (encoding an anti-anti sigma-factor) and *spoIIE* (encoding a serine phosphatase), we observed a low intensity shifted species at concentrations as low as 150 nM. For *spoIIGA* (encoding a sporulation specific protease) we

observed the shifted species only at higher concentrations of protein (>200nM). The negative control (*spoVG*) did not demonstrate binding of Spo0A-DBD at these concentrations. Moreover, the. shift we observed was reversible using unlabeled DNA containing a high affinity binding site, but not using unlabeled DNA that lacked such a site not contain (Figure 4 B-D). Therefore, we consider the binding to *spoIIAA*, *spoIIE* and *spoIIGA* genes to be specific, despite the fact that increasing the amount of protein did not seem to cause a significant increase in the amount of DNA in the complex. Together, these results suggest that Spo0A in *C. difficile* might regulate the transcription of at least a subset of early sporulation genes by direct binding to their promoter regions.

C. difficile Spo0A-DBD binds to DNA upstream of *tcdB*

It has previously been reported that the deletion of Spo0A in *C. difficile* results in a significantly lower toxin production and a ~1000-fold reduction in the toxicity of culture supernatant derived from *spo0A* negative cells towards Vero cells (35). Considering the absence of a homolog of the *abrB* repressor, direct binding of Spo0A and concomitant activation of toxin gene transcription is a likely mechanism through which this could occur. We found evidence for direct binding of Spo0A-DBD to the region upstream of *tcdB*, encoding one of the major clostridial toxin genes, and possibly *tcdC*, but this did not seem to result in lower toxin levels in our hands.

We performed EMSAs using DNA upstream of *tcd*R (encoding a sigma factor responsible for the activation of toxin gene transcription), *tcdB* (encoding toxin B), *tcdA* (encoding toxin A). In order to test regions upstream of all open reading frames in the PaLoc, we also tested binding of Spo0A to DNA upstream of *tcdE* (encoding a holin-like protein (50,51)) and *tcdC* (encoding a putative negative regulator of toxin production (52–54)), even though this regulator does not have a significant effect on toxin levels under the conditions we used (55,56). Of the regions tested, we only observed a clear shifted species, indicative of Spo0A binding, for *tcdB* (Figure 5A); the shifted species in our EMSA assay was reversed by the addition of unlabeled DNA containing a high affinity binding site, but not by DNA lacking such a site (Figure 4E).

For *tcdC,* some smearing was observed at all concentrations of proteins tested (Figure 5A), and there did not seem to be a clear effect of the addition of unlabeled DNA fragments (Figure 4F). The probes for *tcdA*, *tcdE* and *tcd*R were indistinguishable from those obtained with our negative control, *spoVG*.

We wanted to determine if toxin levels in culture supernatants were directly or indirectly affected by Spo0A, as was previously suggested. We found no lower toxicity towards Vero cells of culture supernatants derived from spo0A mutant cells compared to wild type.

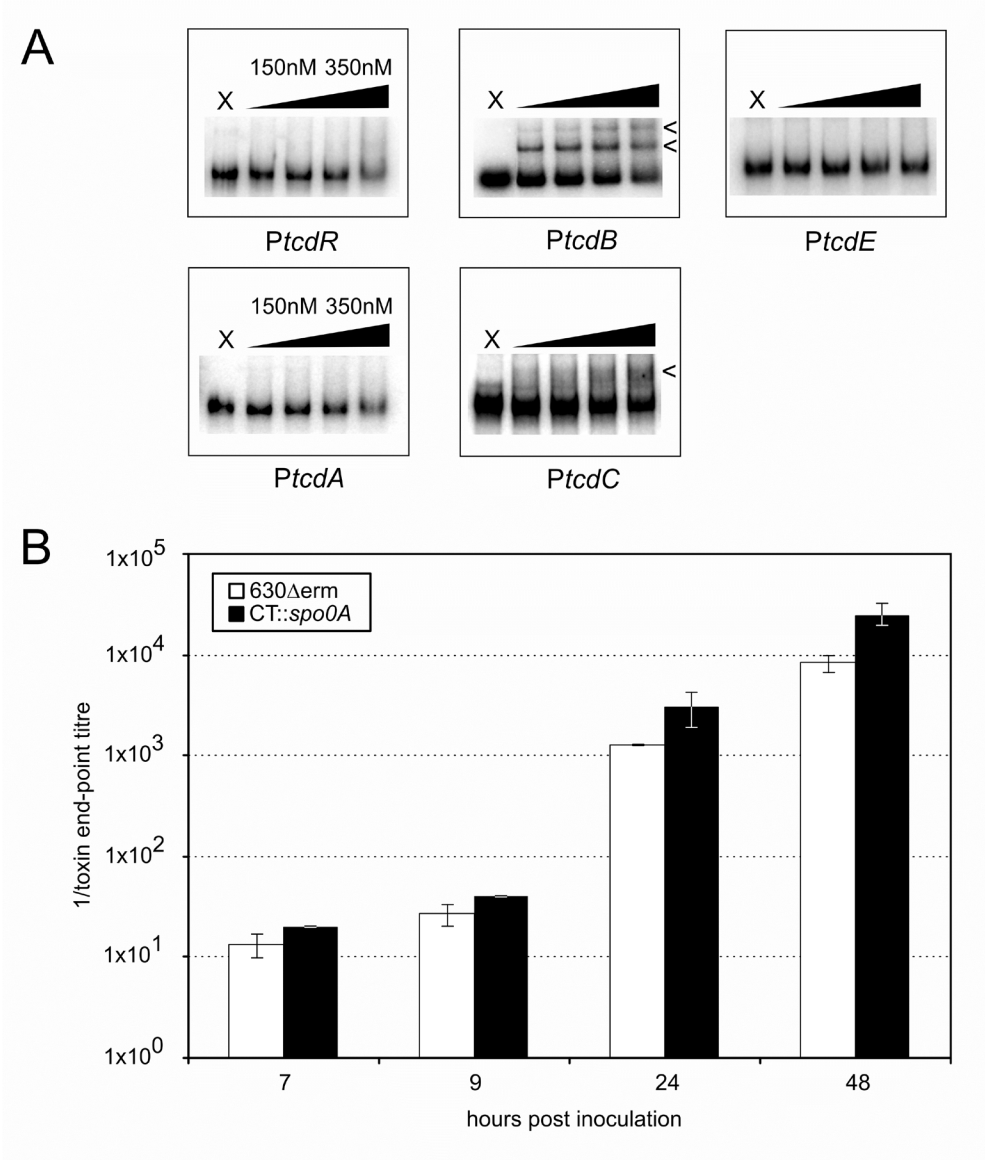

Figure 5: *C. difficile* Spo0A does not contribute to toxin production directly. **(A)** Electrophoretic mobility shift assays using purified *C. difficile* Spo0A-DBD-his6 and radiolabeled DNA fragments corresponding to the upstream regions of the indicated toxin-related genes. X = no protein control, the triangle indicates 1.3-fold serial dilutions of protein to the indicated concentrations. Arrows indicate (possible) protein:DNA complexes. **(B)** Toxin end-point titre of culture supernatant from wild type (630Δ*erm*; white bars) or *spo0A* mutant (CT::*spo0A*; black bars) *C. difficile* cells. For details see Materials and Methods. Error bars represent standard error of the mean (n=3). None of the observed differences were significant in an independent sample t-test.

We grew three independent biological replicates of a wild type (630⊿*erm*) or Clostron-generated *spo0A* mutant (CT::*spo0A* - a kind gift of the Minton lab) in glucose-free TTY medium. We harvested culture supernatant at late-exponential phase (approximately 7 hours post inoculation), the transition phase between exponential and stationary growth phase (approximately 9 hours post inoculation), as well as two time points in stationary phase (24 and 48 hours post inoculation) and determined the toxin endpoint titres (see Materials and Methods). In contrast to previous findings, we observed a small (≤4-fold) increase in the toxicity of supernatants derived from spo0A mutant cells compared to wild type, but in all cases this difference was not statistically significant (p>0.05, independent sample t-test). In other medium (BHIS), we observed no differences at all (data not shown). We conclude that Spo0A does not positively affect toxin production in *C. difficile* 630⊿*erm* and the in vivo relevance of the binding to regions upstream of *tcdB* and/or *tcdC* is therefore limited under our experimental conditions.

5

Discussion

The Spo0A-box of *C. difficile*

In *B. subtilis*, the binding site of Spo0A on target DNA has been well-characterized, through a combination of in vitro binding assays, determination of in vivo binding profiles and mutagenesis of regulated promoter sequences. This work has led to the identification of a conserved core motif, TGTCGAA, or Spo0A box (5,10,11,45). Depending on the analysis, this motif is flanked by one or more adenine or thymine residues (10,11). Interestingly, many target genes do not harbor a perfect match to this consensus sequence, but rather contain one or more degenerate motifs. The differences in these motifs may reflect different promoter architectures (e.g. AT content), modes of action (e.g. activation or repression) or levels of regulation. Spo0A genes in *B. subtilis* can be divided in different classes that respond to different levels of phosphorylated Spo0A (43,57).

For *C. difficile*, we conclude that the Spo0A protein likely recognizes a motif that is similar to the *B. subtilis* Spo0A box on the basis of four lines of evidence; 1. All DNA binding/contacting residues are conserved (Figure 2B), 2. *C. difficile* Spo0A can bind with high affinity to a target of *B. subtilis* Spo0A (Figure 2D), 3. Mutagenesis of key residues in the *B. subtilis* Spo0A box reduces affinity of *C. difficile* Spo0A for DNA (Figure 2E) and 4. A *B. subtilis* Spo0A box has predictive value for DNA binding by *C. difficile* Spo0A (Figure 3A). It is conceivable that our model system, using the purified DNA binding domain, does not accurately reflect binding to all target sites, if target site selectivity is determined in part by other parts by of

the full length protein. It is likely that differences do exist between the preferred binding sites for both proteins that will be evident when a comprehensive analysis is performed of in vivo DNA binding of *C. difficile* Spo0A; based on the limited data set of this study, a MEME analysis (58) already suggests possible differences in the extended Spo0A motif (W.K. Smits, unpublished observations). These differences may relate to the much higher AT content of C. difficile compared to B. subtilis (71 vs. 56.5 %, respectively), or phosphorylation dependent dimerization, for instance.

Regulation of sporulation; feedback regulation of *spo0A*

The initiation of sporulation in *B. subtilis* is subject to complex regulation (for review see ref (1,59)). The activation of Spo0A is controlled by a multi-component phosphorelay that can integrate environmental cues (60) and ensures a gradual increase in the level of phosphorylated Spo0A in the cell (57). In addition, the transcription of the spo0A gene is controlled by multiple feedback loops. For instance, Spo0A regulates its own transcription by binding to the spo0A promoter (46), as well as by indirectly stimulating the transcription of *sigH*, encoding a sigma factor that recognizes the *spo0A* promoter (48). In *C. difficile*, there are some interesting differences and similarities in the regulatory pathways. Most notably, there seems to be no phosphorelay (2) and the phosphorylation state of Spo0A is supposedly controlled by orphan histidine kinases (35). The transcription of *spo0A* in *C. difficile* is under control of the transition state sigma factor Sigma H (37), as it is in *B. subtilis* (61). Our data indicate that both spo0A and sigH could be targets for direct regulation by Spo0A in *C. difficile* (Figure 3A), raising the possibility of auto-regulation of *spo0A*. The putative direct regulation of *sigH* by Spo0A may reflect that the *C. difficile* genome does not harbor a homolog of the pleiotropic regulator AbrB, which is responsible for the Spo0A-dependent regulation of *sigH* in *B. subtilis* (48). Consistent with a model in which *spo0A* is positively auto-regulated, we noted a sharp increase in the levels of Spo0A as cells approach the stationary growth phase (Figure 1C).

Downstream of Spo0A, we found binding of Spo0A to DNA upstream of several early sporulation genes, such as *spoIIAA*, *spoIIE*, and *spoIIGA* (Figure 3B). All these observations are consistent with direct regulation of these genes by Spo0A in other organisms (5,45,49,62), and the conservation of the sporulation pathway (2).

Regulation of processes outside sporulation

Though Spo0A is the key regulator for sporulation in Firmicutes, it regulates numerous other processes in various bacteria. In the non-pathogenic *B. subtilis*, for instance, the protein also affects competence development, biofilm formation, the

production of and resistance to antimicrobial compounds, chromosome dynamics and aspects of phage biology (10,14–16). Importantly, several of these processes are indirectly regulated, through the Spo0A-dependent repression of *abrB*. Additionally, transcription of *abrB* responds already to low levels of Spo0A~P (43). As a result these effects are detectable in late-exponential and early stationary phase, as some Spo0A is present throughout growth in *B. subtilis* cells.

Though *abrB* is absent from *C. difficile*, this does not exclude the possibility of indirect transcriptional regulation through Spo0A-dependent effects on other regulators. Alternatively, Spo0A may exert a direct effect. In *Clostridium acetobutylicum* and *C. beijerinckii*, Spo0A is a direct regulator of solvent formation, as well as sporulation (22,23). It seems therefore conceivable that Spo0A in *C. difficile* also affects aspects of metabolism. In this respect, it is important to note that also in *C. difficile* Spo0A is detectable from early exponential growth phase on (Figure 1B).

We observed direct binding of *C. difficile* Spo0A to the promoter region of *sigH* (Figure 3A). This gene encodes the key sigma factor for the transition phase, and regulates processes outside sporulation as well (37). Moreover, we found significant levels of Spo0A from early stationary phase on (Figure 1B and unpublished observations), indicating the regulatory actions of Spo0A need not be limited to stationary phase in *C. difficile*. In line with this idea, we found a potential regulatory link between Spo0A and two genes that to our knowledge are not related to the sporulation process, the lipoate ligase *lplA* and the aliphatic sulfonates transporter *ssuA* (Figure 3A). The presence of a putative Spo0A binding site upstream of these genes, as well as the spacing compared to the start codon, is conserved in the problematic Stoke-Mandeville strain (R20291), a member of RT 027. This could indicate that these aspects of regulation by Spo0A are conserved in multiple strains of *C. difficile*.

It should be noted that our work so far has been limited to an in vitro analysis of Spo0A binding, and therefore does not indicate whether activation or repression of the putative target genes occurs in vivo. To answer this question, detailed transcriptome and/or proteome studies have to be performed. In order to distinguish direct from indirect effects, in vivo binding profiles of Spo0A should be performed. The antibodies generated for this study should prove to be useful for this type of experiments.

Regulation of toxin production

Amongst the pathogenic Firmicutes, Spo0A has been reported to affect toxin production in multiple species. In *B. anthracis* a *spo0A* mutation results in elevated levels of AbrB, and concomitantly lower levels of the toxin genes *pagA*, *cya* and *lef* that are under AbrB control (17). Similarly, the production of the emetic toxin

cereulide in *B. cereus* is greatly repressed in a spo0A mutant, in an AbrB-dependent manner (63). In contrast, Spo0A directly represses the expression of the *cry* toxin genes in *B. thuringiensis* and a *spo0A* mutant is therefore a hyper-producer of the insecticidal crystal protein (18,21). In *Clostridium perfringens* TpeL, a member of the large clostridial toxins just like TcdA and TcdB, is directly dependent on Spo0A (64) and also the production of enterotoxin in this organism seems to be (indirectly) dependent on sporulation (65,66).

In *C. difficile* an insertional *spo0A* mutant generated using Clostron technology was reported to have ~10-fold reduced levels of Toxin A (TcdA), both intracellularly and extracellularly as well as ~1000-fold reduced toxicity towards Vero cells, which are primarily sensitive towards Toxin B (TcdB) (35). Our in vitro binding data indicate a potential binding site for Spo0A upstream of *tcdB* and possibly *tcdC* (Figure 5A). However, the in vivo relevance of this binding seems limited as in our hands an independently derived but otherwise identical mutant (a kind gift of the Minton lab; (33)) did not demonstrate a reduced toxicity towards Vero cells. In contrast, we found that in TTY medium toxin levels were slightly elevated in *spo0A* mutant cells compared to wild type (≤2-fold in exponential phase cells up to 4-fold in late-stationary phase cells). The small, and not significant, differences in toxin levels in our experiments might be attributed to differences in the susceptibility of cells for lysis rather than the production of toxin, but could also indicate a negative regulatory effect of Spo0A on toxin production. In support of the latter hypothesis, it was recently reported that a spo0A mutant of *C. difficile* strain R20291 (a RT 027/BI/NAP1 epidemic strain) demonstrates ~10-fold higher toxin levels than its isogenic wild type 30h post inoculation, and is significantly more virulent in a mouse model of disease (34).

The differences between Underwood *et al* (35) on the one hand and our study as well as the study of Deakin and coworkers (34) on the other hand may be explained by differences in experimental conditions, such as the medium used. However, we observed no difference in cytotoxicity between supernatant derived from wild type or *spo0A* mutant cells when they were grown in BHIS, a medium nearly identical to that used previously (data not shown). Alternatively, the differences could indicate integration of the group II intron at more than one location in the chromosome in the strain used in Underwood *et al* (35). In the absence of a complementation experiment and/or Southern blot data, this remains to be established.

In summary, our data are consistent with a model in which the regulation of the major clostridial toxins in *C. difficile* is not positively affected by Spo0A, in contrast to previous findings and other pathogenic Clostridia. Whether Spo0A is truly a negative regulator of toxin production remains to be confirmed using in vitro and in vivo transcription assays.

Concluding remarks

In the present study we have for the first time demonstrated direct binding of the DNA binding domain of *C. difficile* Spo0A to putative target DNA. This work has revealed that aspects of Spo0A binding are conserved between *Bacillus* and *C. difficile* (0A box, possible auto-regulation and binding to early sporulation promoters), whereas others are not (the absence of *abrB* as a direct target in *C. difficile*, binding to DNA upstream of *lplA*, *ssuA*). The effects of Spo0A on toxin production may be similar to those observed for *B. thuringiensis* (18,21). Future work will be aimed at determining the effect of Spo0A on the transcription of the putative target genes, and carry out a comprehensive analysis of Spo0A binding in vivo. The identification of genes affected by Spo0A in *C. difficile* may shed light on the role of the protein in virulence and pathogenesis of this organism.

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