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Characterization of the SigD Regulon of *C. difficile* and Its Positive Control of Toxin Production through the Regulation of *tcdR*

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**Abstract**

*Clostridium difficile* intestinal disease is mediated largely by the actions of toxins A (TcdA) and B (TcdB), whose production occurs after the initial steps of colonization involving different surface or flagellar proteins. In *B. subtilis*, the sigma factor SigD controls flagellar synthesis, motility, and vegetative autolysins. A homolog of SigD encoding gene is present in the *C. difficile* 630 genome. We constructed a sigD mutant in *C. difficile* 630 ∆erm to analyze the regulon of SigD using a global transcriptomic approach. A total of 103 genes were differentially expressed between the wild-type and the sigD mutant, including genes involved in motility, metabolism and regulation. In addition, the sigD mutant displayed decreased expression of genes involved in flagellar biosynthesis, and also of genes encoding TcdA and TcdB as well as TcdR, the positive regulator of the toxins. Genomic analysis and RACE-PCR experiments allowed us to characterize promoter sequences of direct target genes of SigD including *tcdR* and to identify the SigD consensus. We then established that SigD positively regulates toxin expression via direct control of *tcdR* transcription. Interestingly, the overexpression of FlgM, a putative anti-SigD factor, inhibited the positive regulation of motility and toxin synthesis by SigD. Thus, SigD appears to be the first positive regulator of the toxin synthesis in *C. difficile*.

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**Introduction**

*Clostridium difficile* is a Gram positive, anaerobic, spore-forming bacterium recognized as the major etiological agent of intestinal diseases associated with antibiotic therapy, with clinical manifestations ranging from diarrhea to pseudomembranous colitis [1]. The disruption of the commensal intestinal flora by antimicrobial therapy allows colonization of the intestinal tract by *C. difficile* [2]. Spores germinate, vegetative cells multiply and toxigenic strains produce two toxins, TcdA and TcdB, considered as major virulence factors, which are responsible for intestinal damage [3]. The epidemiology and severity of *C. difficile* infections has evolved over the past ten years, mainly due to the emergence and spread of a so-called hypervirulent strain belonging to PCR-ribotype 027 [4].

The mechanisms of regulation of genes encoding virulence factors are of major interest in *C. difficile*, since the spectrum of intestinal disease is highly variable. Beyond intestinal colonization, toxin synthesis is the critical event in *C. difficile* intestinal disease. The toxin encoding genes *tcdA* and *tcdB* are located in a 19.6 kb pathogenicity locus [5], with three accessory genes encoding TcdR, TcdC and TcdE. TcdR is an alternative sigma factor that directs transcription from the *tcdA* and *tcdB* promoters [6]. TcdC is an anti-sigma factor that negatively regulates TcdR-dependent transcription [7], although its role in toxin synthesis is still controversial [8,9]. TcdE is a holin-like protein required in the release of the toxins from the cells [10], although its role has also been discussed [11]. Several global regulators, such as CcpA, CodY, Spo0A and SigH regulate expression of toxin genes in response to diverse environmental stimuli. CcpA represses toxin expression in response to PTS sugar availability by binding to the
regulatory regions of the tcdA and tcdB genes [12], as well as regulatory regions of tcdR and tcdC genes [13]. CodY, which controls in B. subtilis many genes induced when cells make the transition from rapid exponential growth to stationary phase or sporulation, represses toxin gene expression by binding to the putative promoter region of the tcdR gene [14,15]. The role of SpoOA, the response regulator of sporulation initiation, in toxin production is still controversial [16,17]. Finally, the alternative sigma factor SigH, a key element in the control of the transition phase and of the initiation of sporulation, negatively modulates toxin and motility expression [18]. Most of these regulators control toxin genes expression in association with genes encoding major cell functions, suggesting a strong relationship between the physiology of C. difficile and the expression of the virulence factors of this bacterium.

Recently, Aubry et al. showed that regulation of the flagellar regulon differentially modulated toxin expression in C. difficile [19], according to a yet uncharacterized mechanism. The flagellar regulon of C. difficile includes a first region encoding late stage flagellar proteins such as FlIC ( filament protein) and FlI D (capping protein), a second region containing flagellar glycan biosynthetic genes and a third region encoding the hook basal body proteins and resembling the fla/che operon of B. subtilis [20,21] (Figure S1). In B. subtilis, the expression of genes of the fla/che operon depends on a promoter Pₐ recognized by SigA and a promoter Pₐ D recognizable by SigD [22]. Besides regulation of motility genes in B. subtilis, SigD plays also an important role in the control of peptidoglycan-remodeling autolysins (LytC, LytD and LytF) [23].

The C. difficile 630 genome carries a gene (CD0266) encoding a putative SigD factor homologous to SigD of B. subtilis. In the present study, we first analyzed the gene expression profile of C. difficile wild-type compared to the sigD mutant and identified the consensus sequence of the SigD-controlled promoters. Then, we demonstrate the role of SigD as a direct and positive regulator of tcdR expression and consequently of toxin synthesis in C. difficile. Thus, we identified a SigD dependent consensus sequence upstream of tcdR gene and we showed that SigD positively acts on the tcdR transcription as an alternative sigma factor of the RNA polymerase. In support of this result we showed that the putative anti-SigD factor FlgM represses motility and toxin genes expression via the inhibition of SigD activity.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are presented in Table 1. C. difficile strains were cultured on blood agar (Oxoid), BHI agar (Difco), BHI broth (Difco) and TY medium in an anaerobic environment (H₂ 10%, CO₂ 10%, N₂ 80%) at 37°C. When necessary, cycloserine (250 µg/ml), thiamphenicol (15 µg/ml), erythromycin (5 µg/ml) and anhydrotetracycline (ATc) (20 ng/ml) were added to C. difficile cultures. Escherichia coli strains were cultured aerobically at 37°C in LB broth or LB agar (MP Biomedicals) containing chloramphenicol (25 µg/ml) or ampicillin (100 µg/ml) when required.

General DNA techniques

Chromosomal DNA extraction from C. difficile colonies was performed using the InstaGene Matrix kit (Bio-Rad). PCRs were carried out in a reaction volume of 25 µl using GoTaq Green Master (Promega) or FastStart High Fidelity PCR System (Roche). The primers used (Eurofins MWG Operon, Eurogentec) are listed in Table S1. PCR products and plasmids were purified using a Nucleospin Extract II kit and a Nucleospin plasmid kit (Macherey-Nagel), respectively.

RNA isolation and quantitative real time PCR

Total RNA of C. difficile was extracted with the RNeasy Mini kit (Qiagen). Samples were treated with two different DNases, DNase I (Sigma) and Turbo DNA-free kit (Ambion) according to the respective manufacturer’s instructions. The total RNA quantity and purity were spectrophotometrically measured (Nanovue, GE Healthcare) and two micrograms of total RNA was reverse transcribed using the Omniscript enzyme (Qiagen) and random 15-mer primers (Eurofins MWG Operon). A total of six nanograms of cDNA were used for subsequent PCR amplification with the IQ SYBR green Supermix (Bio-Rad) and the appropriate primers (0.5 µM final concentration). Specific primers used for PCR amplification were designed with Beacon Designer software (PREMIER Biosoft International) (Table S1). Quantification of 16S rRNA was used as an internal control. Amplification, detection (with automatic calculation of the threshold value), and real-time analysis were performed in duplicate and with three different RNA samples for each condition, by using the CFX96 real time PCR detection system (Bio-Rad). The value used for the comparison of gene expression levels was the number of PCR cycles required to reach the threshold cycle (Cₜ). Expression of an mRNA species was calculated as fold changes using the formula: Fold changes = 2^ΔΔCₜ , with ΔΔCₜ = (Cₜ gene X – Cₜ 16S rRNA wild-type) – (Cₜ gene X – Cₜ 16S rRNA mutant). Statistical analysis was performed with Student’s t test and a P value of ≤ 0.05 was considered significant.

Construction of a C. difficile sigD mutant

The Clostron system was used as described previously [24] to inactivate the sigD gene. Briefly, primers were designed (http://www.sigmaaldrich.com) to retarget the group II intron of pMTL007 to sigD (Table S2), and used to generate a 353 pb DNA fragment by overlap PCR according to the manufacturer’s instructions. These PCR products were cloned into the HindIII and BsrGI restriction sites of pMTL007 and sequenced to verify plasmid constructions with primers pMTL007seqF and pMTL007seqR. pMTL007::Cdi-sigD-228s was transformed into the conjugative E. coli HB101 (RP4) and then transferred via conjugation into C. difficile 630Δerm. C. difficile transconjugants were selected by subculturing on BHI agar containing cycloserine and thiamphenicol. Then, the integration of the group II intron RNA into the sigD gene was induced and selected by plating onto BHI agar containing erythromycin. PCR using the primers ErmRAM-F and ErmRAM-R confirmed the erythromycin resistant phenotype due to the splicing of the group I intron from the group II intron following integration. To verify the insertion of group II intron in the sigD gene, we


performed PCRs using (i) two primers flanking sigD (sigD-F-
sigD-R), (ii) a primer in sigD, sigD-F and the intron primer
EBSu and (iii) ErmRAM-F and ErmRAM-R (Table S1, Figure
S2).

Southern hybridization
For Southern blot analysis, 5 µg of genomic DNA from C.
difficile strain 630::erm and the sigD mutant strain were
digested to completion with HindIII, subjected to agarose gel
electrophoresis (0.8%) and then transferred from the gel onto
Hybond-N+ filter (Amersham). The Southern blot probe was
generated by PCR using pMTL007 plasmid as a template and
primer pair OBD522 and OBD523 (Table S1), yielding a 374 bp
PCR product that hybridizes within the group II intron. Southern
blot analyses were performed using Amersham ECL Direct
Nucleic Acid labeling and detection reagents, according to the
manufacturer’s guidelines. The hybridization signal was
detected using Super Signal West Femto Maximum Sensitivity
Substrate (Thermo Scientific).

Table 1. Strains and plasmids used in this study.

| Strains/plasmids | Relevant features | Reference or source |
|------------------|-------------------|---------------------|
| C. difficile      |                   |                     |
| 630              | wild type ErmR    | [65]                |
| 630Δerm           |                   |                     |
| 630Δerm sigD::intron-erm | ErmR | [66]                |
| 630Δerm + pMTL::PCD2767-flgM | TrmR | This study          |
| 630Δerm + pMTL007 | TrmR              | This study          |
| 630Δerm + pMTL84121 | TrmR | This study          |
| sigD mutant + pMTL84121 | TrmR | This study          |
| 630Δerm + pRPF185 | TrmR ATEC R       | This study          |
| sigD::erm + pRPF185 | TrmR ATEC R     | This study          |
| sigD::erm + pRPF-sigD | TrmR ATEC R     | This study          |
| sigD::erm + pRPF-sigD to CD0272 | TrmR ATEC R | This study          |
| sigD mutant + pDIA5941 | TrmR | This study          |
| 630Δerm + pDIA5941 | TrmR              | This study          |
| E. coli          |                   |                     |
| TOP10            |                   | Invitrogen          |
| HB101 (RP4)      |                   | Laboratory stock    |
| M15              |                   | Qiagen              |
| Plasmids         |                   |                     |
| RP4              |                   |                     |
| pMTL007          |                   |                     |
| pMTL007::sigD-228s | TrmR         | This study          |
| pQE30            |                   |                     |
| pMTL84121        |                   |                     |
| pDIA5941         |                   |                     |
| pDIA5941 derivative carrying tcOR with its promoter region | This study |
| pRPF185          |                   |                     |
| pRPF-sigD        |                   | This study          |
| pRPF-sigD to CD0272 | TrmR ATEC R    | This study          |
| pMTL::PCD2767-flgM | pMTL007 derivative containing flgM gene with PCD2767promoter | This study |

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Construction of complemented strains
DNA fragments containing the sigD gene alone or with genes
downstream of sigD (from CD0267 to CD0272) were generated
by PCR from genomic DNA of C. difficile strain 630::erm using
sigDcomptetF-sigDcomptetR and sigDcomptetF-
CD0272comptetR primers, respectively (Table S1). The PCR
products were then cloned into pRPF185 digested by ScaI and
BamHI placing genes under control of a tetracycline inducible
promoter [25]. Using the E. coli HB101 (RP4) as donor,
plasmids were transferred by conjugation into the C. difficile
630Δerm sigD mutant, giving the sigD::erm + pRPF-sigD and
sigD::erm + pRPF-sigD to CD0272 strains.

Microarray design for the C. difficile 630genome, DNA-
array hybridization and data analysis
The C. difficile 630 genome was obtained from EMBL
database. Probe design for the microarray was performed by
using the OligoArray 2.0 software[26]. One or 2
oligonucleotides were designed for each 3785 genes (we were
unable to design oligonucleotides for 28 genes) and the
microarrays were produced by Agilent. Probes were replicated
Overexpression of \textit{nm}) laser scanner (GenePix). All data were analyzed with R with ethanol. The cDNAs were then mixed with Cy3 or Cy5 presented in Table S1.

A mixture of 5\mu{}g of RNA and 1\mu{}g hexanucleotide primers (pd(N)6 Roche) was heated to 70{}°C for 5 min and quicky chilled on ice. We then sequentially added: 1X first-strand buffer, dithiothreitol (20mM), dNTP mix, Rnase OUT and 1600 units of Superscript III reverse transcriptase in a total volume of 24\mu{l}. The reaction was incubated 3h at 42{}°C to generate cDNAs. After alkaline hydrolysis and neutralization, cDNAs were purified on SNAP columns (Invitrogen) and precipitated with ethanol. The cDNAs were then mixed with Cy3 or Cy5 dyes (GE healthcare), incubated 1 h at room temperature in the dark, and purified on SNAP columns. 200 pmol of Cy3 and Cy5-labeled cDNAs was mixed and concentrated with microcon (Millipore). Hybridization was performed in microchambers for 17 h at 65{}°C according to the manufacturer’s recommendations. 8 differential hybridizations were performed and each RNA preparation was hybridized with a dye switch. The array was then washed successively with Gene Expression Wash Buffer 1 and 2 (Agilent). We realized arrays scanning with a GenePix Pro 6 dual-channel (635 nm and 532 nm) laser scanner (GenePix). All data were analyzed with R and Limma (Linear Model for Microarray Data) software from the Bioconductor project (www.bioconductor.org). The background was corrected with the “Normexp” method [28], resulting in strictly positive values and reducing variability in the log ratios for genes with low levels of hybridization signal. Then, we normalized each slide with the “Loess” method [29]. In order to identify genes differentially expressed, we used the bayesian adjusted \textit{-statistics and performed a multiple testing correction of Benjamini and Hochberg [30] based on the false discovery rate. A gene was considered as differentially expressed when the \textit{p-value is < 0.05. The complete experimental data set was deposited in the GEO database with the accession number GSE29275.

Mapping of the transcriptional start sites by RACE-PCR

The initiation sites of transcription were determined from total RNA of \textit{C. difficile} using the 3‘ / 5‘ RACE kit (Roche Diagnostics) for rapid amplification of cDNA ends as recommended by the manufacturer. The primers used are presented in Table S1.

\textbf{Overexpression of flgM in \textit{C. difficile 630\Delta{}erm}}

The promoter region of \textit{flgM} in \textit{C. difficile} was amplified using primers P2767F-P2767R and primers \textit{flgM}-\textit{flgMR} respectively (Table S1). Both PCR products were then digested by EcoRI and ligated with each other. Ligation product was amplified using primers P2767F and \textit{flgMR}, digested and cloned into the Xhol and PvuI restriction sites of pMTL007. The resulting plasmid was transformed into \textit{E. coli} HB101 (RP4) and then transferred via conjuction into \textit{C. difficile 630\Delta{}erm}, giving the 630\Delta{}erm + pMTL::PCD2767-flgM.

\textbf{Overexpression of tcdR in \textit{C. difficile 630\Delta{}erm} and in the sigD mutant}

The \textit{tcdR} gene with its own promoter region (-810 to +825 from the translational start site) was amplified by PCR using OS314 and OS315 primers (Table S1). The PCR fragment was cloned into the BamHI and HindIII sites of pMTL84121 [31] to produce plasmid pDIA5941. Using the \textit{E. coli} HB101 (RP4) as donor, this plasmid was transferred by conjuction into both \textit{C. difficile 630\Delta{}erm} and its derivative \textit{sigD} mutant to give 630\Delta{}erm + pDIA5941 and the \textit{sigD} mutant + pDIA5941.

\textbf{Cloning, expression, and purification of SigD-His-tagged and FlgM-His-tagged fusion proteins in \textit{E. coli}}

The pQE30 expression system (Qiagen) was used to overexpress the SigD and FlgM proteins in \textit{E. coli} M15 pREP4 as N-terminal hexa-His-tagged proteins. DNA fragments (obtained with chromosomal DNA of \textit{C. difficile 630\Delta{}erm} as the template) containing the \textit{sigD} or \textit{flgM} gene was generated using \textit{sigD-surF-sigD-surR} and \textit{flgM-surF-flgM-surR}, respectively (Table S1). The PCR products were then cloned into Xhol and HindIII of pQE30. \textit{E. coli} M15 competent cells were transformed with the resulting plasmids.

\textit{E. coli} recombinant strains were grown at 37{}°C in LB medium containing ampicillin and kanamycine. Protein expression was achieved by induction with 1mM IPTG and a subsequent incubation of the culture for 4 h at 37{}°C. Cells were then harvested by centrifugation. The His-tagged proteins were purified by affinity chromatography on Ni2+ -nitrilotriacetic acid agarose (Qiagen) using Poly-Prep columns (BioRad) according to the manufacturers’ recommendations. Polyclonal anti-SigD and anti-FlgM antibodies were obtained by BALB/c mouse immunization (agreement number Bl/11-03-01/2; AgroBio).

\textbf{Western blot analyses}

Total proteins were extracted from cultures in BHI or TY broth. \textit{C. difficile} cells were harvested and washed in 20 mM Tris-HCl (pH 8.0) solution. The cells were then resuspended in 4% (w/v) SDS solution, shaken for 60 min and sonicated twice on ice for 1 min. Extracts were heated at 100{}°C for 5 min and centrifuged at 11,000 g for 5 min.

Proteins separated by SDS-PAGE were electroblotted onto Hybond-enhanced chemiluminescence (ECL) nitrocellulose membranes (4{}°C for 1 h, 100 V) (Amersham Biosciences). Membranes were probed first with mouse antisera to SigD (this study), FlgM (this study) or TcdA (Santa Cruz biotechnology, inc), or with rabbit antisera to FiIC or \textit{B. subtilis} SigA provided by M. Fujiita [32] used at dilution of 1:1000 (SigD, FlgM) or at 1:10000 (TcdA, Flc, SigA). Primary antibodies were detected using a HRP-conjugated sheep \textit{\alpha}-mouse (GE healthcare) or goat \textit{\alpha}-rabbit secondary antibody (Jackson Immuno Research) at a dilution of 1:10000. Immunodetection of proteins was performed with the SuperSignal West Femto kit (Thermo Scientific) according to the manufacturer’s recommendations. Blots were exposed to CL-XPOSURE films (Thermo Scientific) and developed.
Gel retardation experiments

Fragment of 249 bp containing the tcdR promoter was amplified by PCR from genomic DNA of C. difficile 630 strain with primers tcdRup-F and tcdRup-R. For the radioactive labelling of the PtcDr PCR fragment, tcdRup-F primer was end-labelled with T4 polynucleotide kinase (Fermentas) and γ32-P-adenosine triphosphate (3000 Ci.mM⁻¹; Perkin Elmer) as recommended by the manufacturer. After PCR, amplified labelled fragment was then purified by QIAquick Nucleotide Removal kit (Qiagen®). E. coli RNA polymerase holoenzyme and core enzyme forms were purchased from Epicenter. The labeled fragment (0.2 nM) was incubated for 60 min at room temperature in 10 µl of glutamate buffer [6] containing SigD purified, E. coli $\sigma^{70}$ RNA polymerase holoenzyme, E. coli RNA polymerase core enzyme or E. coli RNA polymerase core enzyme preincubated with a four-fold molar excess of SigD. Four microliters of a heparin-dye solution (150 mg of heparin per ml, 0.1% bromophenol blue, 50% sucrose) in glutamate buffer was added and the mixture was loaded during electrophoresis on a 4.5% polyacrylamide gel prepared in Tris-base. After electrophoresis (2 h at 13 V/cm), the gel was dried, transferred to filter paper, and analyzed by autoradiography.

Relative quantification of toxin expression

Total toxin amounts were quantified in supernatants from TY cultures using the commercial RIDASCREEN®-ELISA (R-Biopharm) as previously described and according to the manufacture’s protocol [8,11].

Motility assays

Motility assays were performed using BHI motility agar tubes (0.175% agar), inoculated and grown anaerobically for 24 hours at 37 °C, as previously described [33].

Triton X-100 autolysis assay

C. difficile cultures grown until exponential, late exponential or stationary phases were harvested, washed twice, and resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing 0.01% of Triton X-100 (Triton X-100 acts as a detergent and forms micelles with lipoteichoic acids known to inhibit the autolytic activity in the peptidoglycan). The cells were then incubated anaerobically at 37 °C and the lysis monitored by measuring the absorbance at O.D. 600 nm at regular time intervals (Ultraspec 1100 Pro, Amersham Biosciences).

Results

Impact of sigD inactivation in C. difficile 630Δerm

The C. difficile 630 genome encodes putative SigD (CD0266) and anti-SigD (CD0229) factors homologous to SigD and FlgM of B. subtilis, with 34% and 43% identity, respectively. Both sigD and flgM genes are located in the region encoding flagellar apparatus [19]. To analyze the global role of SigD in C. difficile, we inactivated the sigD gene in C. difficile 630Δerm using the Clostron system [24]. Insertion of the group II intron into the target gene was verified by PCR using sigD and intron specific primers (Table S1, Figure S2). Moreover, Southern blot analysis confirmed that only one insertion occurred in the sigD mutant (Figure S2).

We first analyzed the impact of sigD inactivation on growth and on autolysis of C. difficile, since SigD regulates autolysis in B. subtilis [23,34]. The inactivation of sigD had no effect on the growth kinetics of C. difficile in BHI medium (Figure 1A). In addition, as shown in phase contrast microscopy, the sigD mutant was not impaired in cell separation (Figure 1B). These results suggest, that unlike B. subtilis, SigD does not control expression of autolysins involved in cell separation during vegetative growth of C. difficile. We also explored the possible implication of SigD in global autolysis of C. difficile by performing Triton X-100 autolysis assays [35]. The wild-type and mutant strains did not show significant difference in autolysis at mid- and late exponential growth phases. However, the sigD mutant lysed at a slower rate compared to the wild type in stationary phase (Figure 1C). Meanwhile, as shown in a recent study [19], the sigD mutant also displayed a loss of motility and flagellin synthesis (see below). Thus, the inactivation of sigD in C. difficile impairs motility and decreases autolysis at the stationary phase, but does not impair cell septation during the vegetative growth phase. We also examined the sporulation and germination yields by following the development of heat-resistant colonies, but we observed no difference between the sigD mutant and wild-type strains. This result suggests that, like in B. subtilis, the contribution of SigD to sporulation, if any, is modest [36].

Transcriptional and translational expression levels of sigD, flgM and fliC during growth phases of C. difficile 630Δerm

In order to find appropriate growth conditions to study and to identify the SigD regulon, transcription of sigD, flgM (which encodes a putative anti-SigD factor) and fliC (which encodes flagellin) was analyzed by qRT-PCR during growth of C. difficile 630Δerm in BHI medium. The levels of transcription of sigD were similar at mid- and late exponential phases, but decreased at early stationary phase (Figure 2A). Consistent with the sigD transcriptional level we showed by Western blot experiments using anti-SigD antibodies, that the level of SigD protein is stable during the exponential phase and decreases at early stationary phase (Figure 2B).

Transcription of flgM was maximal at early exponential phase and decreased from late exponential phase to reach the lowest level in stationary phase, which is also consistent with the level of the FlgM protein during the growth phases (Figure 2). Indeed, we showed by Western blot analysis using anti-FlgM antibodies that the level of FlgM was higher during exponential phase and decreased during late exponential and stationary phases of growth. Finally, although the transcriptional expression of fliC decreased along the growth, the level of FliC protein remained the same (Figure 2).
Comparative transcriptomic analysis of gene expression profiles of C. difficile 630Δerm and the sigD mutant

Based on the expression kinetics of sigD and flgM, we decided to compare the expression profiles of the 630Δerm and the sigD mutant at the late exponential phase (i.e. 6 h of growth) in BHI medium. In total, 35 genes were up-regulated and 68 genes down-regulated in the sigD mutant when compared to the wild-type strain (p≤0.05). We observed that SigD regulates genes involved in various functions such as motility, membrane transport, metabolism, regulation and toxin synthesis (Table S2). To validate the transcriptomic profile data, we selected a subset of 20 genes related to various functions, and tested their transcription level by qRT-PCR (Table 2). qRT-PCR results and microarrays data exhibited high correlation coefficient (R2=0.88) (Table 2).

Figure 1. Phenotypic analysis of the sigD mutant. A: Growth curves in BHI medium showing no differences between sigD mutant (○) and 630Δerm strain (◊). B: Contrast phase microscopy during exponential phase in BHI medium showing the lack of impact of sigD inactivation on cells separation. C: Triton X-100 induced autolysis of 630Δerm (○) and sigD mutant (○) strains at stationary phase showing that sigD mutant lyses more slowly than 630Δerm. The autolysis is expressed in percent initial absorbance at an optical density of 600 nm. Error bars indicate standard deviation.

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The microarray data highlighted that most of the motility genes were controlled by SigD, as observed in B. subtilis [37]. Indeed, the expression of most genes encoding flagellar hook-associated proteins as well as the flagellin and the flagellum cap protein (CD0226 to CD0240) and the expression of the flagellar glycosylation genes (CD0241 to CD0244) (Figure S1) was highly decreased in the sigD mutant (magnitude of change ranged from 11-fold to 50-fold) (Table S2). We confirmed by Western blot analysis that FliC was not detected in the sigD mutant (see below), as described previously [19] and that is consistent with the absence of fliC gene transcription (Table S2) and the loss of motility in the sigD mutant (see below). The expression of most genes encoding the hook basal body (flgB to flgH) (Figure S1) was only slightly decreased (magnitude of change ranged from 1.58-fold to 1.96-fold), suggesting that they could still be transcribed from another sigma factor.
Actually, when RACE-PCR experiment was conducted to map a putative promoter upstream of $flgB$, we identified a transcriptional start site located 261 nucleotides upstream of the starting codon of $flgB$ with a consensus sequence probably recognized by SigA ($\text{ATAACA-N}_{17}-\text{CATAA}$) (divergent bases are in bold). Whereas expression of genes upstream of $\text{sigD}$ is slightly affected by the $\text{sigD}$ mutation, genes directly downstream of $\text{sigD}$ ($\text{CD0267}$ to $\text{CD0272}$) (Figure S1) were found highly downregulated. However, no putative promoter sequence was found upstream of $\text{CD0267}$ suggesting a probable polar effect of the $\text{sigD}$ mutation on the expression of genes downstream of $\text{sigD}$ ($\text{CD0267}$ to $\text{CD0272}$). Finally, we observed that the expression of $flgM$ (the putative anti-SigD factor) decreased (50-fold) in the $\text{sigD}$ mutant (Table S2). Therefore we further investigated below the mechanism of the positive control of SigD on the expression of $flgM$.

Concerning cell wall proteins, the expression of $\text{cbpA}$ encoding a surface exposed adhesion [38], $\text{CD0514}$, encoding a cell surface protein, and $\text{CD0211}$, encoding a CTP:phosphocholine citidylyltransferase decreased in the $\text{sigD}$ mutant. Although SigD does not significantly regulate $\text{CD1036}$ and $\text{CD1304}$, which encode cell wall autolysins, the expression of $\text{CD0226}$, encoding a putative lytic transglycosylase, decreased dramatically in the $\text{sigD}$ mutant. Interestingly, lytic transglycosylases (enzymes degrading glycan chains of peptidoglycan) are considered to be autolytic [39] and have been recently shown as required for full motility of several Gram positive or Gram negative species [40].
Many genes encoding membrane transport associated proteins are differentially expressed in the sigD mutant (Table S2). For example, the expression of CD3525-CD3527, encoding putative ABC transport system proteins and CD3373 and CD3375, encoding putative magnesium transporters, decreased in the sigD mutant. Conversely, the expression of CD0206-CD0208 and CD0764-CD0767, encoding phosphotransferase sugar (PTS) transport systems of fructose and sorbitol-specific respectively, increased (Table S2).

### Complementation of sigD mutation

The sigD gene is located in the 3' region of the large operon that encodes proteins constituting the hook basal body and starting with the flgB gene. To determine whether SigD is expressed independently from genes upstream, we performed a RACE-PCR experiment to localize a putative promoter of sigD. However we did not find transcriptional start upstream of sigD, suggesting that sigD is part of a larger operonic structure. Owing to the complex regulation of flagella expression and to confirm that the defect of motility was directly due to the disruption of sigD, the complementation of the sigD mutant was undertaken. For this purpose we constructed two plasmids, one carrying only the wild type sigD gene and another one carrying the wild type sigD plus genes downstream until CD0272 (Figure S1). We used a tetracycline inducible promoter AtC in both plasmids to control gene expression (see Experimental procedures). Both complemented strains were restored for SigD and FliC synthesis (Figure 3). Interestingly, the sigD complemented strain is partially restored for motility, whereas the sigD-CD0272 complemented strain appears as motile as the wild-type strain, suggesting that the expression of genes downstream sigD seems to be required for full motility of C. difficile (Figure 3). Overall, these data strongly support evidence that SigD controls expression of flagellar genes in C. difficile.
SigD modulates Paloc genes expression

The transcriptomic analysis showed a decrease of tcdA and tcdR expression (4.16-fold and 1.85-fold, respectively) in the sigD mutant compared to the wild type grown in glucose-containing BHI medium (Table S2). We did not see differences in tcdB expression between the wild-type and the sigD mutant strains, probably due to the low level of tcdB transcripts at 6 hours of growth, as previously observed [46]. However, when we further analyzed by qRT-PCR the expression of the PaLoc genes in the wild type and the sigD mutant, we found that, in addition to tcdA and tcdR expression, the expression of tcdB also decreased in the sigD mutant grown in BHI medium (8.13-, 13.76- and 5.87-fold respectively) (Table 2). Furthermore, the same effect of sigD mutation on the Paloc genes transcription was observed in the optimal growth conditions for C. difficile to toxin production, i.e. when cells are grown in glucose-free TY medium at the stationary phase (Figure 4A). Western blot analysis of crude extracts, using antibodies raised against TcdA (Figure 4B) and ELISA quantification of toxins A and B in the supernatant of 10 and 24 hours cultures (Figure 4C) confirmed the loss of toxin synthesis in the sigD mutant. As complementation of the sigD mutant by both SigD-expressing plasmids restore toxin genes expression and production (Figure 4). Taken together, these data indicate that SigD positively controls the expression of C. difficile toxin genes, as recently suggested by several groups [19,47], whereas the mode of action of SigD was not described. Therefore we further investigated the mechanism of this regulation (see below).

Identification of direct target genes of SigD

In the transcriptome analysis, 68 genes showed decreased expression in the sigD mutant, indicating that SigD exerts direct or indirect positive control on these genes in the wild-type. To find potential direct target genes controlled by SigD, we looked for the presence of the consensus sequence of B. subtilis SigD-dependent promoters (TAAA-N13-16GCC#G#ATAW) in the 300 bp region upstream of start codons of C. difficile genes using the GenoList web server (http://genodb.pasteur.fr/cgi-bin/WebObjects/GenoList), allowing three mismatches. Among the genes found to contain a B. subtilis SigD-like consensus sequence in their promoter regions, only 11 genes and operons are significantly and positively regulated by SigD, as observed in the comparative transcriptomic analysis (Table S2). This includes 5 late flagellar genes and 2 early flagellar genes, suggesting that multiple sigD-dependent promoters are implicated in the expression of the flagella regulon (Table S2, Table 3).

RACE-PCR experiments were then performed to confirm the promoter sequences for 5 out of the 11 genes identified. We found a transcription initiation site located 28 nucleotides upstream of the flgM start codon (Figure 5, Figure S1), which displays a B. subtilis SigD-like consensus sequence in its promoter region. Direct control of flgM by SigD is consistent with the dramatic decrease of the flgM transcription in the sigD mutant (Table S2). We also identified transcription initiation sites located 152, 68 and 164 nucleotides upstream of the CD0226, fliC and CD3527 start codons, respectively, with a B. subtilis SigD-like consensus sequence in their promoter regions (Figure 5 Figure S1). These results strongly suggest that C. difficile SigD directly controls the expression of these genes. Interestingly, we also found a B. subtilis SigD-like
consensus sequence in the promoter region of the \textit{tcdR} gene as recently proposed [47] (Table 3). Indeed, we identified by RACE-PCR a transcription initiation site located 76 nucleotides upstream of the \textit{tcdR} start codon, which displays a consensus sequence of the \textit{B. subtilis} SigD-dependent promoters (TAAA – N\textsubscript{13} – GCCGAT – TA) (divergent base is in bold) (Figure 5). The alignment of all probable SigD-dependent promoters using the WebLogo website (\url{http://weblogo.berkeley.edu}) and listed in Table 3, allowed to propose a consensus sequence of \textit{C. difficile} SigD-dependent promoters, which contains two conserved motifs TAAA and CG separated by 15 to 18 bases (Figure 6). Surprisingly, when we used the consensus sequence of \textit{C. difficile} SigD-dependent promoters to find more genes under direct control of SigD in the \textit{C. difficile} 630 genome, we did not find more than the eleven genes and operons previously cited in Table 3.

Since, \textit{C. difficile} SigD-dependent promoter sequence was only found in the promoter regions of \textit{tcdR} and not in \textit{tcdA} and \textit{tcdB} promoter regions, the decreased expression of \textit{tcdA}, \textit{tcdB} and \textit{tcdR} in the \textit{sigD} mutant suggests that the regulation of toxin genes by SigD must be controlled via TcdR.

\textbf{SigD directly controls tcdR transcription}

To determine whether SigD directly control \textit{tcdR} transcription, a plasmid containing the \textit{tcdR} gene with its promoter region (pDIA5941) was introduced into both 630\textsubscript{erm} and CD0272 strains grown in TY medium. SigA antibodies were used as an internal control. C: TcdA and TcdB expression levels in supernatants of \textit{C. difficile} 630\textsubscript{erm} + pRPF185, \textit{sigD}\textsubscript{::erm} + pRPF185, \textit{sigD}\textsubscript{::erm} + pRPF-sigD and \textit{sigD}\textsubscript{::erm} + pRPF-sigD to CD0272 Strains were quantified using ELISA test after 10 and 24 hours growth in TY medium. Error bars correspond to standard deviation from at least three biological replicates.
Table 3. *C. difficile* genes which expression significantly decreased by SigD and displaying a SigD consensus sequence in their promoter region.

| Gene     | Function                                           | Expression ratio sigD mutant/630.urem | Consensus sequence                  |
|----------|----------------------------------------------------|---------------------------------------|-------------------------------------|
| CD0226   | putative lytic transglycosylase                    | 0.08                                  | aTAAAttttttttttttttCCGATAAt         |
| flgM     | negative regulator of flagellin synthesis (anti-SigD factor) | 0.02                                  | aTAAAttttttttttGCCTGATAAt           |
| flgK     | flagellar hook-associated protein FlgK (or HAP1)   | 0.03                                  | aTAAAgaaaacattttttCAGAAAa           |
| flgC     | flagellar C                                       | 0.02                                  | aTAAAgtagttagaacttcgCCGATAAt        |
| moaA     | flagellar motor rotation protein MotA             | 0.53                                  | aTAAAlgagtttagagagagCCGAAAa         |
| CD0230   | putative flagellar biosynthesis protein           | 0.05                                  | cTAAAtagagagagagagCCGAAAt           |
| fliQ     | FlQflagellar biosynthetic protein                 | 0.51                                  | ITAAGaagaagaaatagTCGTGAAa           |
| tcrR     | toxin transcriptional regulator                   | 0.53                                  | aTAAAttaatttttyCCGATTAI            |
| CD29668  | transcription antiterminator, LicT family         | 0.45                                  | aTAAAttgagaatccaaatGCGTTAc          |
| CD3022   | putative phosphosugar isomerase                   | 0.43                                  | ITAAAgagttataataACGATTGa            |
| CD3527   | putative iron ABC transporter, ATP-binding protein | 0.04                                  | aTAAAtgaaataattttgaCCGATTAI         |

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Figure 5. Identification of the SigD-dependent promoter sequence by RACE-PCR. SigD-dependent transcription start sites upstream of start codons of genes involved in motility (CD0226, flgM, fliC), membrane transport (CD3527) and virulence (tcdR). The transcriptional start sites are indicated in bold and underlined. The -35 and -10 boxes corresponding to SigD-dependent promoters are indicated in bold.

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and sigD mutant strains (see Experimental procedures). As expected, transcriptional analysis showed an overexpression of tcdR in both strains containing pDIA5941 (Figure 7A). However, the pDIA5941-containing 630 ∆erm strain expressing SigD, displayed a higher level (4.9 fold) of tcdR than the pDIA5941-containing sigD mutant (Figure 7A), confirming that SigD controls positively tcdR expression. We noted that difference of tcdA expression is lesser than that of tcdR expression in the pDIA5941-containing sigD mutant when compared to the pDIA5941-containing 630 ∆erm strain. This result is consistent with the fact that expression of tcdA is not directly linked to SigD but through the TcdR sigma factor (Figure 7A, 7B). Thus, SigD positively controls toxin gene expression by directly regulating tcdR transcription likely via the SigD-dependent promoter sequence present upstream of the promoter region of tcdR.

**RNA polymerase containing SigD binds specifically to tcdR promoter region**

Generally, Sigma factors like SigD are sequence-specific, DNA-binding subunits of RNA polymerase, ensuring the recognition of appropriate promote sites. Thus to determine whether RNA polymerase containing SigD activates tcdR transcription, we performed a gel mobility shift assay with the tcdR promoter DNA fragment and the RNA polymerase core enzyme purified from E. coli (Epicentre) with or without addition of SigD and challenged the complexes with heparin. Neither core enzyme nor SigD alone was able to shift the mobility of the tcdR promoter-containing fragment (Figure 8). However, when we mixed SigD with the core enzyme, the reconstituted RNA polymerase is able to form heparin-resistant complex at the tcdR promoter in a dose-dependent manner (Figure 8). The RNA polymerase containing the major vegetative sigma factor SigA was unable to bind to the promoter region of tcdR (Figure 8). Moreover, the addition of an excess of unlabelled heterologous DNA [1 mg of poly (dI-dC)] did not prevent DNA binding (data not shown), while the addition of an excess of unlabelled homologous DNA effectively prevented DNA binding (Figure 8). Thus, it is clear that sigD directly activates tcdR expression by directing RNA polymerase core enzyme to recognize tcdR promoter and activate its transcription.

**FlgM turns off the positive regulation of SigD on flagella and toxins expression**

To support that SigD act as an alternative sigma factor on the positive regulation of flagella and toxins expression, we investigated the role of FlgM, the putative anti-SigD. In B. subtilis and Salmonella typhimurium, FlgM binds to SigD, thereby inhibiting premature expression of late flagellar gene [48,49]. We first tried to inactivate the flgM gene using the Clostron system, but repetitive attempts using different intron sites remained unsuccessful. Instead, flgM was overexpressed in the 630 ∆erm strain by cloning the flgM gene downstream of the CD2767 promoter (under the control of the domestic sigma factor SigA; unpublished data) in pMTL007. Overexpression of flgM (130-folds) led to a decrease of the sigD expression (Figure 9B), indicated that FlgM interferes with the SigD protein to initiate transcription from its promoters, ie SigD-dependent fliQ promoter located 5 genes upstream. Moreover, although SigD is still present at a significant level, overexpression of FlgM leads to a complete loss of motility in the corresponding strain, which is related to the absence of fliC transcription and flagellin production (Figure 9). In addition, transcriptional analysis revealed that the expression of tcdR, tcdA and tcdB was also decreased in the presence of high level of FlgM (Figure 9A) and consequently on TcdA production as confirmed by a Western blot analysis (Figure 9B). Thus, overexpressed FlgM leads to a down-regulation of genes under positive
control of SigD, and strongly support that SigD act as a sigma factor on the flagella and toxin genes expression.

Discussion

Among Gram-positive bacteria, the regulatory properties of the SigD factor have been extensively studied in B. Subtilis where it controls flagellar synthesis, motility and vegetative autolysins [23,34,50]. The aim of our study was to characterize the regulatory properties of SigD in C. difficile, by comparing phenotypic properties and transcriptomic profiles of C. difficile 630Δerm and its sigD mutant.

In B. subtilis, SigD has been shown to play a critical role in the cell separation. Indeed, the major autolysins LytC, LytD and LytF [23,51] are under transcriptional control of SigD. Consequently, a sigD mutant does not form separate cells and grows constitutively in chains. In C. difficile, the inactivation of sigD does not have any impact on cell separation but a significant decreased autolysis is observed at the stationary phase. Among the 37 putative peptidoglycan hydrolases identified on the genome of C. difficile [52], only the genes CD2141, encoding a putative D-Ala-D-Ala carboxypeptidase, and CD0226, encoding a putative transglycosylase have been shown transcriptionally deregulated in the microarray analysis. However, CD2141 is upregulated in the sigD mutant strain and carboxypeptidase are known to not destroy the peptidoglycan mesh and are generally considered as peptidoglycan maturation enzymes [53]. Conversely, CD0226 is downregulated in the sigD mutant. Transglycosylases are not true hydrolases because they cleave the glycosidic bond with a concomitant intramolecular transglycosylation reaction, but they are able to act as autolysins [39]. Furthermore, a SigD consensus sequence was identified in the promoter region of CD0226. Thus, control of CD0226 by SigD could explain the lysis defect in the sigD mutant. Nevertheless, unlike B. subtilis,
the role of SigD of *C. difficile* in the control of the autolysins appears to be very limited.

Recently, a link was established between transglycosylase activity and motility of *Helicobacter pylori* and *Salmonella typhimurium*, and between glucosaminidase activity and motility in *Listeria monocytogenes* [40]. Indeed, proper anchoring and functional integrity of the flagellar motor could involve the maturation of the surrounding peptidoglycan by a hydrolytic enzyme. Interestingly, CD0226, encoding a putative lytic transglycosylase is the first gene of the late-stage flagellar genes. Further analysis should explore if a similar link exists in *C. difficile*.

Control of motility by SigD has been studied and demonstrated both in Gram negative (where it is usually called FliA), such as *Escherichia coli* [54] or *Salmonella typhimurium* [55] and in Gram positive bacteria, such as *Bacillus subtilis* [51]. Very recently, SigD has also been shown implicated in the positive regulation of motility in *C. difficile* [19] that is widely confirmed in this present study. Moreover, our microarray analysis combined to the identification of promoters regions by RACE-PCR and by in-silico analysis allows us to bring new elements on the transcription initiation of sigD and the flagellar regulon. First, transcriptional analysis shows that sigD inactivation in *C. difficile* affects only slightly the expression of genes encoding the hook basal body (early flagellar genes). This is consistent with the SigA-like consensus sequence identified by RACE-PCR upstream of the starting codon of fliB, the first gene of this operonic structure, indicating that the expression of the early flagellar genes is partly independent of the expression of SigD. From our in-silico analysis, the first probable SigD-dependent promoter in this operon is located upstream motA and another one is then found upstream fliQ (Figure S1). In *B. subtilis*, the fia-che transcription unit resembles the early flagellar genes element of *C. difficile* and a SigA-dependent promoter P\_P\_0,\_D\_0, dispensable for motility has also been identified upstream of the primary P\_P\_0,\_D\_0 promoter [22,57]. Two others SigD-dependent promoters have also been found within the fla-che transcription unit [58] of *B. subtilis*, the P\_P\_0,\_D\_0 promoter governing partly the expression of sigD [59] and the P\_P\_sigD promoter, residing immediately upstream of sigD itself but its activity is not clearly demonstrated [22,58]. In contrast to the early-stage flagellar genes, transcription of the late-stage flagellar genes is strongly affected by the sigD inactivation. In agreement with this observation, RACE-PCR experiments led to the identification of a SigD-dependent promoter upstream CD0226, the first gene of this cluster, whereas no SigA-dependent promoter could be found. Moreover, two others SigD-dependent promoters were identified within this region, one upstream of flgM and the other upstream fliC. In support of this, we showed a complete loss of flIC and flgM transcription in the sigD mutant and a restoration of their expression expression after complementation of sigD mutation. This is similar to *B. subtilis*, where the hag gene encoding flagellin and the flgM gene possesse a SigD-dependent promoter and is transcribed by the SigD containing RNA polymerase [57,60]. The flagellar glycosylation genes cluster is located 717 bp downstream from CD0240 (the last gene of the late-flagellar genes region) [19] and its transcriptional expression is also strongly downregulated in a sigD mutant. Yet, no SigD-dependent promoter could be identified immediately upstream or within this cluster by our in-silico analysis, suggesting that these genes are cotranscribed with fliC and CD0240 from the SigD-dependent promoter residing upstream fliC.

In *B. subtilis*, the expression of sigD is necessary for the transcription of genes involved in flagellar synthesis and chemotaxis [59,61] and the SigD-dependent transcription of late flagellar genes is repressed by FlgM, the anti-SigD factor, through a post-translational control [49]. FlgM directly binds to SigD and antagonizes its activity in the early stage of growth [62]. However, when the formation of the hook basal body is completed, SigD is released due to the secretion of FlgM from the cells through the assembled flagellar motor structure and genes under SigD-dependency are then transcribed [63]. In *C. difficile*, the overexpression of flgM inhibited SigD activity and consequently suppressed, like in the sigD mutant, motility and flagellin expression. Thus, we confirm that SigD is a positive regulator of motility in *C. difficile*, and further show the role of FlgM as an anti-SigD factor participating in the flagellar regulation. Other studies will be undertaken in future in our lab to analyze the probable secretion of FlgM in the culture supernatant.

The inactivation of sigD decreases dramatically the expression of tcdA, tcdB and tcdR [19] and it has been recently shown that sigD expression is negatively regulated by increasing intracellular level of the second messenger cyclic diguanilate (c-di-GMP), which impacts the expression of toxin genes [64]. Indeed, the regulation of *C. difficile* toxin production...
by the level of c-di-GMP, via the control of SigD, was recently established and a mechanism for the SigD-dependent regulation of toxin expression has been proposed [47]. However, the mode of action of SigD on the regulation of tcdR expression was not experimentally determined. In our study, we demonstrated the regulation of toxin genes by SigD through TcdR. Moreover, a SigD-dependent promoter predicted by the in-silico analysis is present upstream of the 5' region of tcdR and has been confirmed by RACE-PCR. Most importantly, electrophoretic mobility shifts assays demonstrated the direct binding of SigD-containing RNA polymerase to the tcdR promoter. Therefore, this is the first study that unambiguously demonstrates the role of SigD in the controls of toxin synthesis via a direct regulation of the tcdR promoter. Thus SigD, which has never been reported as a positive regulator of toxin synthesis in other bacteria, appears as a key positive regulator of both motility and toxin synthesis in C. difficile.

Supporting Information

Figure S1. (adapted from Aubry et al [19]): Flagellar locus from C. difficile 630, with location of the three SigD promoter sites identified by RACE-PCR (arrows above the flagellar locus). Dashed arrows indicate genes which posses a SigD consensus sequence and which significantly regulated by SigD. White triangle: mutagenesis of sigD gene using the Clostron system. (TIF)
Figure S2. Inactivation of sigD gene. A: Schematic presentation of pMTL-based knock-out plasmid. a: parental plasmid pMTL007. b: wild-type target gene. c: mutated target gene. Group II intron (black arrow), internal RAM conferring erythromycin resistance (white arrow) are represented. The locations of primers used for screening mutants are indicated. B: Confirmation of gene knockouts using PCR. Amplifications were performed on 630Δerm and 630Δerm sigD::intron-erm using: sigD target specific primers F and R (sigD-F and sigD-R), sigD-F and EBSu primers and ErmRAM-F and ErmRAM-R primers. C: Southern blot analysis of genomic DNA from C. difficile 630Δerm and C. difficile 630Δerm sigD::intron-erm with an intron probe. Chromosomal DNA (6μg in each reaction) was digested with HindIII

(TIF)

Table S1. Oligonucleotides used in this study.

(DOCX)

Table S2. Genes positively or negatively controlled by SigD according to the expression ratio in transcriptomic analysis of sigD mutant/strain 630Δerm after 6h of growth.

References

1. Johnson S, Gerding DN (1998) Clostridium difficile associated diarrhea. Clin Infect Dis 26: 1026-1035; quiz:
2. Walters JR, Pattni SS (2010)Managing bile acidic diarrhea. Therap Adv Gastroenterol 3: 349-357.
3. Voht DE, Ballard JD (2005) Clostridium difficile toxins: mechanisms of action and role in disease. Clin Microbiol Rev 18: 247-263. doi:10.1128/CMR.18.2.247-263.2005. PubMed: 15831824.
4. Warny M, Pepin J, Fang A, Killgore G, Thompson A et al. (2005)Toxin production by an emerging strain of Clostridium difficile associated with outbreaks of severe disease in North America and Europe. Lancet 366: 1079-1084. doi:10.1016/S0140-6736(05)67420-X. PubMed: 16182895.
5. Hundsieberger T, Braun V, Weidmann M, Leukel P, Sauerborn M et al. (1997)Transcription analysis of the genes tcdA-E of the pathogenicity locus of Clostridium difficile. Eur J Biochem 244: 735-742. doi:10.1007/BF00192488. PubMed: 9108241.
6. Mani N, Dupuy B (2001) Regulation of toxin synthesis in Clostridium difficile by an alternative RNA polymerase sigma factor. Proc Natl Acad Sci U S A 98: 5844-5849. doi:10.1073/pnas.111268998. PubMed: 11310220.
7. Matamouros S, England P, Dupuy B (2007) Clostridium difficile toxin expression is inhibited by the novel regulator TcdC. Mol Microbiol 64: 1274-1286. doi:10.1111/j.1365-2958.2007.07539.x. PubMed: 17542920.
8. Bakker D, Smits WK, Kuijper EJ, Corver J (2012) TcdC does not significantly repress toxin expression in Clostridium difficile 630Δerm. PLOS ONE 7: e43247. doi:10.1371/journal.pone.0043247. PubMed: 22912837.
9. Carter GP, Douce GR, Govind R, Howarth PM, Mackin KE et al. (2011) The anti-sigma factor TcdC modulates hypervirulence in an epidemic BI/NAP1/027 clinical isolate of Clostridium difficile. PLOS Pathog 7: e1002317.
10. Govind R, Dupuy B (2012) Secretion of Clostridium difficile Toxins A and B Requires the Holin-like Protein TcdE. Plos Pathog 8: e1002727.
11. Olling A, Seehase S, Minton NP, Tatge H, Schröter S et al. (2012) Release of TcdA and TcdB from Clostridium difficile cdi 630 is not affected by functional inactivation of the tcdE gene. Microb Pathog 52: 92-100. doi:10.1016/j.micpath.2011.10.009. PubMed: 22107968.
12. Antunes A, Martin-Verstraete I, Dupuy B (2011) CcpA-mediated repression of Clostridium difficile toxin gene expression. Mol Microbiol 79: 882-899. PubMed: 21299645.
13. Antunes A, Camiade E, Monot M, Courtois E, Barbut F et al. (2012) Global transcriptional control by glucose and carbon regulator CcpA in Clostridium difficile. Nucleic Acids Res 40: 10701-10718. doi:10.1093/nar/gks864. PubMed: 22989714.
14. Sonenshein AL (2005)CodY, a global regulator of stationary phase and virulence in Gram-positive bacteria. Curr Opin Microbiol 8: 203-207. doi:10.1016/j.mib.2005.01.001. PubMed: 15802253.
15. Dineen SS, Villapakkam AC, Nordman JT, Sonenshein AL (2007) Repression of Clostridium difficile toxin gene expression by CodY. Mol Microbiol 66: 206-219. doi:10.1111/j.1365-2958.2007.05906.x. PubMed: 17725558.
16. Underwood S, Guan S, Vijayasubash B, Vaines SD, Graham L et al. (2009) Characterization of the sporelation initiation pathway of Clostridium difficile and its role in toxin production. J Bacteriol 191: 7296-7305. doi:10.1128/JB.00272-11. PubMed: 19783633.
17. Rosenbusch KE, Bakker D, Kuijper EJ, Smits WK (2012) C. difficile 630Δerm Spo0A regulates sporulation, but does not contribute to toxin production, by direct high-affinity binding to target. DNA - PLOS ONE 7: e48608. doi:10.1371/journal.pone.0048608.
18. Sajuet L, Monot M, Dupuy B, Soutourina O, Martin-Verstraete I (2011) The key sigma factor of transition phase, SigH, controls sporulation, metabolism, and virulence factor expression in Clostridium difficile. J Bacteriol 193: 3186-3196. doi:10.1128/JB.00272-11. PubMed: 21572003.
19. Aubry A, Hussack G, Chen W, KuoLee R, Twine SM et al. (2012)Modulation of toxin production by the flagellar regulator in Clostridium difficile. Infect Immun 80: 3521-3532. doi:10.1128/IAI.00224-12. PubMed: 22851750.
20. Courtney CR, Cozy LM, Kearns DB (2012) Molecular characterization of the flagellar hook in Bacillus subtilis. J Bacteriol 194: 4619-4629. doi:10.1128/JB.00444-12. PubMed: 22730131.
21. Guttenplan SB, Shaw S, Kearns DB (2013) The cell biology of peritrichous flagella in Bacillus subtilis. Mol Microbiol 87: 211-229. PubMed: 23190039.
22. West JT, Estacio W, Márquez-Magaña L (2000) Relative roles of the fla/che P(D-3) and P(D+AP) promoters in regulating motility and sigD expression in Bacillus subtilis. J Bacteriol 182: 4841-4848. doi:10.1128/JB.182.17.4841-4848.2000. PubMed: 10940026.
23. Chen R, Guttenplan SB, Blair KM, Kearns DB (2009) Role of the sigmA-dependent autolytos in Bacillus subtilis population heterogeneity. J Bacteriol 191: 5775-5784. doi:10.1128/JB.00521-09. PubMed: 19542270.
24. Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP (2007) The Clostron: a universal gene knock-out system for the genus Clostridium. J Microbiol Methods 70: 452-464. doi:10.1016/j.mimet.2007.05.021. PubMed: 17658189.

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Author Contributions

Conceived and designed the experiments: IEM JP JLP BD. Performed the experiments: IEM BD MM OS. Analyzed the data: IEM JP MM BD MPC. Contributed reagents/materials/analysis tools: OS. Wrote the manuscript: IEM JLP BD.
Practical and powerful approach to multiple testing. J R Stat Soc Ser B 2004; 66: 3057-3062

Systematic analysis of SigD-regulated genes in Bacillus subtilis by DNA microarray and Northern blotting analyses. Gene 2004; 329: 2700-2707. doi:10.1016/j.bmbcom.2012.09.012

Normalization of cDNA microarray data. Methods 2003; 31: 265–273. doi:10.1016/S1046-2023(03)00155-5

False discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B 1995; 57: 289–300.

Finding the right combinations of multiple sigma factors with RNA polymerase during sporulation in Bacillus subtilis. Genes Cells 2000; 5: 79-88. doi:10.1046/j.1365-2443.2000.00307.x

Global regulation of gene expression in response to cysteine availability factors with RNA polymerase during sporulation in human collagen. Cell Microbiol, 15: 1674–87. PubMed: 23517059.

The MerR family gene and is affected by a

Cyclic di-guanylate inversely regulates motility and aggregation in Salmonella typhimurium. Mol Gen Genet 221: 139-147. PubMed: 2196428.

Dual promoters are responsible for transcription initiation of the fla/che operon in Bacillus subtilis. J Bacteriol 2005; 187: 3548-3555. PubMed: 16957996.

Cell population heterogeneity during growth of Bacillus subtilis. Genes Dev 2009; 191: 7050-7062. doi:10.1128/JB.00861-09. PubMed: 19749038.

Completion of the hook-basal body complex of the Salmonella flagellum is coupled to FlgM secretion and FlgM glycosylation in Salmonella typhimurium. Mol Gen Genet 221: 130-139. PubMed: 2196428.

Generation of an erythromycin-sensitive derivative of Staphylococcus aureus. J Bacteriol. 1992; 174: 5123-5127. doi:10.1128/0000411.6975693.

Toxin production by controlling expression of toxin genes. Mol Microbiol 27: 107-120. doi: 10.1046/j.1365-2958.2000.07112.x

Motility development in Bacillus subtilis. Mol Microbiol 76: 273-285. doi: 10.1111/j.1365-2958.2007.01284.x

Motility and flagellar glycosylation in Bacillus subtilis. Mol Gen Genet 279: 739-749. PubMed: 15661000.

A master regulator for biofilm formation by Bacillus subtilis. Mol Microbiol 55: 739-749. PubMed: 15661000.

A novel surface exposed adhesin of Clostridium perfringens to toxin genes. Mol Microbiol 27: 107-120. doi:10.1111/j.1365-2958.1992.tb01771.x

Cyclic diguanylate activated complexes of the flagellar regulon of Salmonella typhimurium: an antisigma factor inhibits the activity of the flagellum-specific sigma factor, sigma F. Mol Microbiol 6: 3149-3157. doi: 10.1111/j.1365-2958.1992.tb01771.x

Rouillard JM, Zuker M, Gulari E (2003) OligoArray 2.0: design of gene and is affected by a

Global analysis of the sporulation pathway of Clostridium difficile. PLOS Genet 9: e1003660.

SigmaD Regulon in Clostridium difficile