

The Key Sigma Factor of Transition Phase, SigH, Controls Sporulation, Metabolism, and Virulence Factor Expression in *Clostridium difficile*^{▽†}

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Toxin synthesis in *Clostridium difficile* increases as cells enter into stationary phase. We first compared the expression profiles of strain 630E during exponential growth and at the onset of stationary phase and showed that genes involved in sporulation, cellular division, and motility, as well as carbon and amino acid metabolism, were differentially expressed under these conditions. We inactivated the *sigH* gene, which encodes an alternative sigma factor involved in the transition to post-exponential phase in *Bacillus subtilis*. Then, we compared the expression profiles of strain 630E and the *sigH* mutant after 10 h of growth. About 60% of the genes that were differentially expressed between exponential and stationary phases, including genes involved in motility, sporulation, and metabolism, were regulated by SigH, which thus appears to be a key regulator of the transition phase in *C. difficile*. SigH positively controls several genes required for sporulation. Accordingly, *sigH* inactivation results in an asporogeneous phenotype. The *spo0A* and *CD2492* genes, encoding the master regulator of sporulation and one of its associated kinases, and the *spoIIA* operon were transcribed from a SigH-dependent promoter. The expression of *tcdA* and *tcdB*, encoding the toxins, and of *tcdR*, encoding the sigma factor required for toxin production, increased in a *sigH* mutant. Finally, SigH regulates the expression of genes encoding surface-associated proteins, such as the Cwp66 adhesin, the S-layer precursor, and the flagellum components. Among the 286 genes positively regulated by SigH, about 40 transcriptional units presenting a SigH consensus in their promoter regions are good candidates for direct SigH targets.

Clostridium difficile, a Gram-positive, anaerobic, spore-forming bacterium, is a major cause of nosocomial infections associated with antibiotic therapy. This enteropathogen can lead to antibiotic-associated diarrhea and pseudomembranous colitis, a potentially lethal disease. Transmission of *C. difficile* is mediated by contamination of the gut by spores (54). Spores also contribute to its survival during antibiotic therapy. The disruption of the colonic microflora by antimicrobial therapy precipitates *C. difficile* infection and colonization of the intestinal tract (54). Thus, spores germinate, vegetative forms multiply, and the major virulence factors, the two large toxins TcdA and TcdB, are produced. The toxin-encoding genes, *tcdA* and *tcdB*, are localized in the 19.6-kb pathogenicity locus with three other accessory genes, *tcdR*, *tcdC*, and *tcdE*. TcdR is an alternative sigma factor that directs transcription from the *tcdA*, *tcdB*, and *tcdR* promoters, while TcdC is an anti-sigma factor that negatively regulates TcdR-dependent transcription by destabilizing the TcdR-holoenzyme complex (33, 34). The *tcdE* gene encodes a holin-like protein that is required for the release of the toxins from the cell (R. Govind and B. Dupuy, unpublished results).

The spectrum of diseases caused by *C. difficile* is highly variable and depends on the level of toxin produced for the severe forms (1). This supports the hypothesis that regulation

of toxin synthesis is a critical determinant of *C. difficile* pathogenicity. Toxin synthesis increases as cells enter into stationary phase, and many environmental factors influence their production. In the presence of phosphotransferase system (PTS) sugars, such as glucose, and of certain amino acids, like cysteine or proline, toxin production is inhibited (16, 30). Environmental stresses, such as alteration of the redox potential, high temperature, or limitation of biotin, also modulate toxin production. Three regulators involved in the control of toxin synthesis have already been identified: CcpA, mediating glucose-dependent repression (5), and two regulators controlling pre- or post-exponential events, CodY and Spo0A (14, 52).

To cope with nutrient limitations, bacterial cells have to change their metabolism and physiology when they enter into stationary phase. To adapt and survive, they can also express functions like competence, biofilm formation, or antibiotic production. Bacilli and clostridia also have the ability to undergo a cellular differentiation process leading to the formation of a spore that will germinate when growth-favoring conditions return. During transition from exponential to stationary growth phase, a complex regulatory network will determine which option, stationary phase or initiation of sporulation, is most suitable under the given conditions. The regulatory mechanisms involved in the transition phase have been extensively studied in *Bacillus subtilis* (38). In this bacterium, it has been shown that during the exponential growth phase, repressors such as AbrB, CodY, and SinR prevent the expression of functions that are only needed during stationary phase or sporulation (38, 43). Sporulation initiation in *B. subtilis* is controlled by the response regulator, Spo0A, and a phosphorelay involving five kinases (KinA to KinE), intermediary phosphorylated proteins (Spo0F and Spo0B), and several phosphatases

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(38, 45). Environmental and cellular stress signals are sensed by the sensor kinases that autophosphorylate. They transfer their phosphoryl group to Spo0F and then to the Spo0A regulator via the intermediary phosphorylation of Spo0B (38, 45). Phosphorylated Spo0A (Spo0A-P) activates or represses the expression of target genes by binding to a specific target site (TGNCGAA) in their promoter regions (35). If the Spo0A-P level reaches a critical threshold, Spo0A-P activates the transcription of genes required for sporulation, such as the *spoIIAA-spoIIAB-sigF* operon and the *spoIIG* and *spoIIE* genes (35, 38, 43). The alternative sigma factor, SigH, is also a key element in the control of transition phase and of the initiation of sporulation. The RNA polymerase containing SigH transcribes several genes that function during the transition state, including genes required for sporulation, like *spo0A*, *spo0F*, *kinA*, *kinE*, and *spoIIAA*. The consensus sequence of genes transcribed by SigH in *B. subtilis* is (A/G)NAGGA(A/T)₃-N₁₁₋₁₂-(A/G)NNGAAT (8).

Compared to *B. subtilis*, less is known about the complex regulatory network controlling post-exponential events, including the initiation of sporulation, in clostridia (37). The AbrB repressor is absent in the *C. difficile* genome, while CodY and a SinR-like repressor are present (41). Spo0A and SigH are present in all clostridia. In contrast, the phosphorelay is absent and the sporulation initiation pathway remains in clostridia as a two-component system with Spo0A and associated kinases which can phosphorylate Spo0A directly in the absence of Spo0F and Spo0B (37, 44, 56). A recent report showed that in *C. difficile*, Spo0A is phosphorylated by the CD1579 kinase *in vitro*. Moreover, inactivation of CD2492, a second kinase, reduces the sporulation capacity of *C. difficile*, while a *spo0A* mutant does not sporulate (52).

Whether the production of toxins in *C. difficile* is a stationary-phase event or is associated with sporulation remains to be clearly established. Among the regulators involved in the control of the transition from exponential growth to stationary phase, it has recently been shown that CodY represses toxin gene expression via direct binding to the *tcdR* promoter region (14). In addition, TcdA production decreases in *spo0A* and *CD2492* mutants (52). Finally, the role of SigH in the stationary phase and in the initiation of sporulation, as well as in regulation of the toxin genes, has never been studied in clostridia.

In the present study, we analyzed the expression profile modifications between exponential growth and the onset of stationary phase in *C. difficile* and the role of SigH in these transcriptional changes. We then investigated the involvement of SigH in sporulation and virulence factor expression, including toxin production, in *C. difficile*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *C. difficile* strains and plasmids used in this study are presented in Table S1 in the supplemental material. *C. difficile* strains were grown anaerobically (10% H₂, 10% CO₂, 80% N₂) in TY (16) or brain heart infusion (BHI) medium (Difco). Sporulation assays were performed with BHI supplemented with 5 g/liter yeast extract and 0.1% L-cysteine (BHIS). When necessary, cefoxitin (25 µg/ml), thiamphenicol (15 µg/ml), or erythromycin (2.5 µg/ml) was added to *C. difficile* cultures. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth. When indicated, ampicillin (100 µg/ml) or chloramphenicol (15 µg/ml) was added to the culture medium. All routine plasmid constructions were carried out using standard procedures (40).

Construction of gene knockout mutants in *C. difficile*. The CloStron gene knockout system (23) was used to inactivate the *sigH* and *spo0A* genes, giving strains 630E *sigH::erm* (CDIP8) and 630E *spo0A::erm* (CDIP3), respectively (see Table S1 in the supplemental material). Primers to retarget the group II intron of pMTL007 to these genes (see Table S2 in the supplemental material) were designed with Targetron design software (Sigma-Aldrich). The PCR primer sets were used with the EBS universal primer and intron template DNA to generate by overlap extension PCR a 353-bp product for each gene that would facilitate intron retargeting. These PCR products were cloned into the HindIII and BsrGI restriction sites of pMTL007 (see Fig. S1 in the supplemental material), giving plasmids pMTL007::Cdi-*spo0A*178a and pMTL007::Cdi-*sigH*123s. DNA sequencing was performed to verify plasmid constructs, using the pMTL007-specific primers pMTL007-F and pMTL007-R (see Table S2 in the supplemental material). The derivative pMTL007 plasmids were transformed into *E. coli* HB101(RP4) and subsequently mated with *C. difficile* 630E (Δerm) (27). *C. difficile* transconjugants were selected by subculturing on BHI agar containing thiamphenicol (15 µg/ml) and cefoxitin (25 µg/ml) and then plated on BHI agar containing erythromycin (2.5 µg/ml). Chromosomal DNA of transconjugants was isolated as previously described (5). PCR using the Erm^r primers confirmed that the Erm^r phenotype was due to the splicing of the group I intron from the group II intron following integration (see Fig. S1 in the supplemental material). In order to verify the integration of the L.LtrB intron into the right gene targets, we performed PCR with two primers flanking the *sigH* (IMV358-IMV359) or *spo0A* (OBD530-OBD529) gene and, on one hand, a primer in *sigH* (IMV358) or *spo0A* (OBD530) and, on the other hand, with the intron primer EBSu (see Table S2 and Fig. S1 in the supplemental material).

To complement the *sigH* mutant, the *sigH* gene with its promoter (-297 to +720 from the translational start site) was amplified by PCR. The PCR fragment was cloned into the XhoI and BamHI sites of pMTL84121 (24) to produce plasmid pDIA5932. Using the *E. coli* HB101(RP4) strain as donor, pDIA5932 was transferred by conjugation into the *C. difficile* 630E *sigH::erm* mutant, giving strain CDIP12 (see Table S1 in the supplemental material).

Sporulation assay. Overnight cultures grown at 37°C in BHI were used to inoculate BHIS medium. After 72 h of growth, 1 ml of culture was divided into two samples. To determine the total number of CFU, the first sample was serially diluted and plated on BHI with 0.1% taurocholate (Sigma-Aldrich). Taurocholate is required for the germination of *C. difficile* spores (55). To determine the number of spores, the vegetative cells of the second sample were heat killed by incubation for 20 min at 65°C prior to plating on BHI with 0.1% taurocholate. The percentage of sporulation was determined as the ratio of the number of spores/ml and the total number of bacteria/ml times 100. The mean values for at least two independent experiments are presented.

Crude extract preparation and protein dot blot analysis. To obtain *C. difficile* crude extracts, 10 ml of culture was centrifuged. Cells were washed with phosphate-buffered saline (PBS), resuspended in 1 ml of PBS, and disrupted in a FastPrep apparatus (2 times for 30 s at speed 6.5) in the presence of glass beads at 4°C. The soluble fractions were obtained by centrifugation to remove cells debris. For dot blot analysis, we directly spotted 200 ng of protein of each sample onto a nitrocellulose membrane (Hybond-C extra; Amersham Biosciences). The membranes were blocked with 5% (wt/vol) nonfat dried milk in Tris 50 mM, pH 7.5, containing 150 mM NaCl and then incubated overnight at 4°C with the primary anti-TcdA antibody. Following washing, membranes were incubated with the secondary antibody at 37°C for 60 min, and the TcdA protein was detected by using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific) according to the manufacturer's instructions. Dot blots were quantified and normalized by Image Gauge (FujiFilm).

RNA extraction, quantitative RT-PCR analysis, and 5' RACE. We extracted total RNA from strains 630E, 630E *sigH::erm*, 630E *spo0A::erm*, and 630E *sigH::erm* complemented with *sigH* in *trans* grown in TY medium. Cells were harvested after 4 h or 10 h of culture by centrifugation for 2 min at 4°C. RNA extraction was performed as previously described (4), cDNA were synthesized from 5 µg of total RNA with 1 µl of hexamer oligonucleotide primers (5 µg/µl pdN₆; Roche), 4 µl of deoxynucleoside triphosphates (dNTP; 10 mM each), and 6 µl of reverse transcription (RT) buffer in a final volume of 30 µl. Samples were heated for 5 min at 80°C. After a slow cooling, cDNAs were synthesized for 2 h at 42°C with avian myeloblastosis virus (AMV) reverse transcriptase (Promega). Real-time quantitative PCR was performed in a 20-µl reaction volume containing 10 ng or 10 pg (for 16S rRNA) of cDNAs, 12.75 µl of the SYBR PCR master mix (Applied Biosystems), and 400 nM gene-specific primers. Amplification and detection were performed as previously described (3). In each sample, the quantity of cDNAs of a gene was normalized to the quantity of cDNAs of the 16S rRNA gene. The relative change in gene expression was recorded as the ratio of normalized target concentrations (threshold cycle [$\Delta\Delta C_T$] method) (32). The

transcriptional start sites of the *spo0A*, *sigH*, *CD2492*, *spoIIAA*, *sigA2*, *dnaG*, and *sigG* genes were determined using the 5' rapid amplification of cDNA ends (RACE) system (Invitrogen) according to the manufacturer's recommendations. RNA preparations extracted from strains 630E or 630E *sigH::erm* were used as the template. The primers used for 5' RACE analysis are listed in Table S2 in the supplemental material.

Microarray design for the *C. difficile* genome, DNA array hybridization, and data analysis. The *C. difficile* strain 630 genome was obtained from the EMBL database. Probe design for the microarray was performed by using OligoArray 2.0 software (39). One or 2 oligonucleotides were designed for each 3,785 genes. We were unable to design oligonucleotides for 28 genes. Agilent produced the microarrays. Probes were replicated twice on the array to reach a final density of 14,224 probes per array. Five hundred thirty-six positive controls and 984 negative controls were also included. The description of the microarray design was submitted to the GEO database (accession number GPL10556).

Total RNA was extracted from cells of 4 independent cultures for each growth condition. RNA was labeled with either Cy3 or Cy5 fluorescent dye (GE Healthcare, Little Chalfont, United Kingdom) using a SuperScript Indirect cDNA labeling kit (Invitrogen) as previously described (4). The cDNAs were then mixed with Cy3 or Cy5 dyes, incubated for 1 h at room temperature in the dark, and purified on SNAP columns (Invitrogen). An amount of 200 pmol of Cy3- and Cy5-labeled cDNAs was mixed. Hybridization was performed for 17 h at 65°C according to the manufacturer's recommendations. Four differential hybridizations were performed for each experiment. The array was then washed successively with gene expression wash buffers 1 and 2 (Agilent). Scanning of arrays was performed 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 (7), 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 (42). In order to identify differentially expressed genes, we used the Bayesian adjusted *t* statistics and performed the multiple testing correction of Benjamini and Hochberg (6) that is based on the false discovery rate. A gene was considered differentially expressed when the *P* value was <0.05.

Microarray data accession number. The complete experimental data set was deposited in the Gene Expression Omnibus (GEO) database with the accession number GSE25521.

RESULTS AND DISCUSSION

Comparative analysis of gene expression profiles of strain 630E after 4 h and 10 h of growth. We first performed growth kinetics of strain 630E in TY medium, and we observed that the transition between exponential growth phase and stationary phase occurred around 8 h of growth. Then, we analyzed by quantitative RT-PCR (qRT-PCR) the expression of transition-phase markers (*spo0A*, *sigH*, and *spoIIAA*) at different times of growth (4 h, 6 h, 8 h, 10 h, and 12 h). We showed that the expression of *spo0A* and *sigH* increased from 6 h to reach a plateau between 8 h and 10 h of growth, while the expression of *spoIIAA* occurred later, becoming enhanced after 8 h of growth. These patterns of transcription are in agreement with data obtained for *Clostridium acetobutylicum* (28). However, *sigH* expression is only slightly increased during transition phase in *C. acetobutylicum* (2), while in *C. difficile*, *sigH* expression is 15-fold induced at 10 h compared to its expression at 4 h.

To identify global changes in gene expression between exponential growth phase and stationary phase, we compared the gene expression profiles of strain 630E grown in TY medium for 4 h (exponential growth) and 10 h (early stationary phase). Seven hundred fifty-two genes were differentially expressed with a factor of ≥ 2 . Three hundred twenty-five genes were expressed at higher levels and 427 were expressed at lower levels at 4 h than at 10 h (Table 1; also see Table S3 in the supplemental material). Genes involved in motility or in trans-

lation or encoding transporters and membrane proteins were expressed at higher levels during exponential growth than at the beginning of stationary phase, as reported for *C. acetobutylicum* (28). Conversely, several genes which participate in sporulation, cellular division, carbon metabolism, fermentation pathways, amino acid metabolism, and protein catabolism were induced at the onset of stationary phase (see Fig. S2 and S3 and Table S3 in the supplemental material). Actually, genes involved in the downstream part of glycolysis were induced in stationary phase in *C. difficile*, as already observed in *C. acetobutylicum* (2). In addition, the expression of genes involved in the biosynthesis of threonine, glycine, cysteine, aspartate, and glutamine, as well as genes encoding 17 putative proteases and peptidases or peptide (Opp, CD1404) and amino acid transporters (CD1259, CD1260, CD2693), increased after 10 h of growth (see Fig. S3 in the supplemental material). Finally, at the beginning of stationary phase, the expression of genes involved in the response to stress stimuli, including the oxidative stress response and the HrcA and CtsR regulons, were also induced (see Table S3 in the supplemental material) (2).

Changes in fermentation pathways from acidogenesis to solventogenesis occur at the onset of stationary phase in *C. acetobutylicum* (2). In the nonsolventogenic *C. difficile*, modifications in the expression of genes required for fermentation and energy metabolism were also observed between 4 h and 10 h of growth (see Fig. S2 in the supplemental material). Actually, the expression of the *cat1* operon, involved in crotonyl-coenzyme A (CoA) production from succinate, and of two *adhE* genes (*CD2966* and *CD3006*), encoding alcohol dehydrogenases, decreased after 10 h of growth. In contrast, the expression of the *bcd2* operon, involved in acetyl-CoA to butyryl-CoA synthesis, and of genes encoding two phosphate butyryl-transferases (*CD0112* and *CD0715*) and two butyrate kinases (*CD0113* and *CD2379*) increased 5- to 10-fold at the onset of stationary phase. The genes involved in amino acid fermentations were not differentially expressed, with the exception of the *ldhA* gene and the *hadA* operon required for L-leucine reduction, which were induced after 10 h of growth (31). Finally, several genes involved in acetyl-CoA production from pyruvate, CO₂, or CO or related to electron transfer systems, including genes encoding flavodoxins, ferredoxins, flavoproteins, oxidoreductases, ATP synthases, and hydrogenases, were also induced at the onset of stationary phase. All these results suggest a rerouting of energy metabolism at the end of exponential growth in *C. difficile*.

The large set of genes that are differentially expressed during exponential growth and at the beginning of stationary phase is required for a general adaptive response and survival-enhancing properties associated with entry into stationary phase. This also indicates the existence of complex regulatory networks modulating gene expression between these two growth phases, in agreement with the 29 genes encoding transcriptional regulators that are differentially expressed in these two conditions. Interestingly, the *agrD* gene, encoding an autoinducer prepeptide, was 2.5-fold induced at 10 h, suggesting that a system of quorum sensing could play a role at the end of exponential growth, as observed in several Gram-positive bacteria, including *Clostridium perfringens* and *Clostridium botulinum* (11, 36). Finally, the expression of genes encoding six sigma factors (*sigA2*, *sigB*, *sigH*, *sigF*, *sigE*, and *sigG*), including

TABLE 1. Genes involved in sporulation controlled by SigH or differentially expressed at 4 h and 10 h of growth

Locus tag	Gene name ^a	Function ^a	Expression ratio in transcriptome ^b		Associated sigma factor(s) and/or Spo0A box(es) ^c
			<i>sigH</i> strain/630E	4 h/10 h	
CD0057	<i>spo0H</i>	RNA polymerase sigma-H factor	D	0.19	SigA*, Spo0A
CD1214	<i>spo0A</i>	Stage 0 sporulation protein A	0.20	0.07	SigA*, SigH*, Spo0A
CD2492		Histidine kinase associated to Spo0A	0.35	0.55	SigH*
CD3670		Putative selenocysteine lyase	0.34	0.38	
CD3671	<i>spo0J</i>	Spo0J, stage 0 sporulation protein J	0.46	—	
CD3672	<i>soj</i>	Sporulation initiation inhibitor	0.56	—	SigH
CD3673		Putative stage 0 sporulation protein, Spo0J-like	0.21	0.28	SigH
CD0770	<i>spoIIAA</i>	Anti-sigma F factor antagonist	0.03	0.04	SigH*, Spo0A
CD0771	<i>spoIIAB</i>	Anti-sigma F factor	0.03	0.04	
CD0772	<i>sigF</i>	RNA polymerase sigma-F factor	0.05	0.08	
CD2469	<i>spoIIP</i>	Stage II sporulation protein P	0.50	0.33	
CD2470	<i>gpr</i>	Spore endopeptidase	0.48	0.51	
CD2641		Putative sporulation protein	0.48	0.59	
CD2642	<i>sigG</i>	RNA polymerase sigma-G factor	0.20	0.09	SigF*
CD2643	<i>sigE</i>	RNA polymerase sigma-E factor	0.17	0.18	
CD2644	<i>spoIIGA</i>	Sporulation sigma-E factor processing peptidase	0.20	0.22	
CD3490	<i>spoIIE</i>	Phosphoprotein phosphatase	0.03	0.02	Spo0A
CD1192	<i>spoIIIAA</i>	Stage III sporulation protein AA	0.31	0.42	
CD1193	<i>spoIIIAB</i>	Stage III sporulation protein AB	0.25	0.31	
CD1194	<i>spoIIIAC</i>	Stage III sporulation protein AC	0.16	0.18	
CD1195	<i>spoIIIAD</i>	Stage III sporulation protein AD	0.32	0.38	
CD1196	<i>spoIIIAE</i>	Stage III sporulation protein AE	0.35	0.41	
CD1197	<i>spoIIIAF</i>	Stage III sporulation protein AF	0.50	0.48	
CD1198	<i>spoIIIAG</i>	Stage III sporulation protein AG	0.14	0.15	
CD1199	<i>spoIIIAH</i>	Stage III sporulation protein AH	0.19	0.18	
CD0126	<i>spoIIID</i>	Stage III sporulation protein D	0.07	0.12	
CD2629	<i>spoIVA</i>	Stage IV sporulation protein AA	0.11	0.16	
CD2656	<i>spoVD</i>	Stage V sporulation protein D (penicillin-binding protein)	0.15	0.18	
CD2652	<i>spoVE</i>	Cell division/stage V sporulation protein	0.20	0.28	
CD3516	<i>spoVG</i>	Regulator required for spore cortex synthesis	0.01	0.23	SigH
CD1935	<i>spoVS</i>	Stage V sporulation protein S	0.03	0.08	SigH
CD3499	<i>spoVT</i>	Stage V sporulation protein T	—	0.08	
CD2967	<i>spoVFB</i>	Dipicolinate synthase subunit B	—	2.20	
CD1230	<i>sigK</i>	Fragment of RNA polymerase sigma-K factor (part 1)	—	2.73	
CD3563		Putative spore cortex-lytic enzyme	0.47	0.28	
CD3494		Putative spore protein	0.50	0.42	
CD0106	<i>cwlD</i>	Germination N-acetylmuramoyl-L-alanine amidase	0.31	0.31	
CD3349	<i>bclA3</i>	Putative exosporium glycoprotein	0.38	3.51	Spo0A
CD3569		Sporulation-specific protease	0.33	—	SigH, Spo0A
CD1068		Putative polysaccharide biosynthesis/sporulation protein	0.39	0.4	
CD3541	<i>spmB</i>	Spore maturation protein B	0.43	—	
CD2247	<i>cspBA</i>	Serine peptidase	0.50	0.43	

^a Gene names and functions correspond to those indicated in the MaGe database Clostriscope (<https://www.genoscope.cns.fr>).

^b A gene is considered differentially expressed when the *P* value is <0.05 using the statistical analysis described in Materials and Methods. The cutoff for biologically significant change was set at 2-fold. However, some genes had a fold change of less than 2-fold but were included because they appeared to be in the same transcription units with regulated genes for which the fold change was ≥2. Genes not differentially expressed between 4 h and 10 h of growth or between the 630E strain and the *sigH* mutant are indicated by a dash. D, deleted in the *sigH* mutant.

^c Using the *B. subtilis* SigH or Spo0A consensus, we searched for SigH-dependent promoters or Spo0A boxes. An asterisk indicates that we performed promoter cartography by 5' RACE.

4 out of 5 sigma factors involved in the sporulation process and one sigma factor sharing similarities with SigB, which is involved in the general stress response in Gram-positive bacteria, was induced at 10 h in our transcriptome analysis, in agreement with the major changes detected in the transcriptional patterns. Surprisingly, the expression of the first part of the *sigK* gene, which is inactivated by a *sigK* intervening (skin) element, as observed in *B. subtilis* (21), was downregulated at the onset of stationary phase.

Comparative analysis of gene expression profiles of strain 630E and the *sigH* mutant at the onset of stationary phase. In *B. subtilis*, SigH plays a key role in the control of the transition

phase and in the initiation of sporulation (8). The *C. difficile* SigH protein shares 63% identity with SigH of *B. subtilis*. To analyze the role of SigH in *C. difficile*, we inactivated the *sigH* gene using the ClosTron system (23). The group II intron of pMTL007 was retargeted to insert into the *sigH* gene in sense orientation immediately after the 123rd nucleotide in its coding sequence. Then, the derivative plasmid pMTL007::Cdi-*sigH*123s was transferred to *C. difficile* strain 630E (Erm^s) by conjugation and erythromycin-resistant transconjugants were isolated. To verify the insertion of the group II intron into *sigH*, PCRs were carried out using internal primers of *sigH*, the intron-specific primer EBSu, and the

RAM-F and RAM-R primer pair from both wild-type and transconjugant DNA templates (Materials and Methods; also see Fig. S1 in the supplemental material). The results obtained confirmed the integration of the intron into *sigH*.

In order to identify genes regulated by SigH, we compared the expression profiles of strain 630E and the *sigH* mutant at the onset of stationary phase (10 h). Four hundred seventy-six genes were differentially expressed between these two strains with a factor of ≥ 2 : 190 genes were upregulated and 286 were downregulated in the *sigH* mutant compared to their expression in strain 630E (see Table S3 in the supplemental material). Of the genes regulated by SigH, 85% were differentially expressed between 4 h and 10 h of growth. We observed that SigH regulates numerous genes involved in various functions, such as motility, sporulation, cellular division, and protein catabolism, as well as carbon, amino acid, energy, and cell wall metabolism, virulence, and regulation. Thus, eight genes encoding peptidases or proteases and some genes involved in amino acid metabolism (a serine acetyl-transferase, an OAS-thiol-lyase, an aspartate aminotransferase, a glutamate synthase, a homoserine kinase, and a racemase) were induced via a SigH-dependent mechanism after 10 h of growth. Except for *gapN*, all genes involved in glycolysis that were regulated in a growth-dependent manner were negatively controlled by SigH (see Fig. S2 in the supplemental material). In addition, several genes implicated in fermentation and energy metabolism pathways were positively controlled by SigH (see Fig. S2 in the supplemental material). This includes the *bcd2* operon that is involved in butyrate production, genes encoding a formate dehydrogenase, the bifunctional CO dehydrogenase/acetyl-CoA synthase, a hydrogenase (*CD3313-CD3314*), and an ATP synthase (*CD2960-CD2954*). Among the genes not differentially expressed during the growth phase, we observed that the expression of the *gnd* operon, encoding the glycine reductase, a key enzyme of Stickland reactions, was 5- to 15-fold reduced in the *sigH* mutant compared to its expression in strain 630E (see Fig. S2 in the supplemental material). Finally, about 50 genes encoding proteins of unknown function were highly induced at the onset of stationary phase by a SigH-dependent mechanism, while most of the genes involved in the stress response were not regulated by SigH (see Table S3 in the supplemental material).

SigH positively controls the transcription of many genes involved in sporulation and cellular division. In strain 630E, a total of 49 genes involved in sporulation and cellular division were induced at the onset of stationary phase, and most of these genes were expressed at lower levels in the *sigH* mutant than in strain 630E (Table 1). The *spo0A* and *CD2492* genes, which encode the master regulator of the sporulation initiation and one of its associated kinases, respectively (52), were expressed at lower levels in the *sigH* mutant. Genes involved in chromosomal segregation during asymmetric division and prespore engulfment (*spo0J*, *soj*, and *spoIIP*) were positively controlled by SigH. Moreover, genes encoding sigma factors of sporulation SigF, SigE, and SigG and proteins controlling their activity (SpoIIAA, SpoIIAB, SpoIIIE, and SpoIIIGA) were expressed 5- to 30-fold less in the *sigH* mutant than in strain 630E. Finally, many genes involved in later stages of sporulation (*spoIIIAA* to *spoIIIAH*, *spoIIID*, *spoIVA*, *spoVD*, *spoVE*,

spoVG, and *spoVS*) were also induced after 10 h of growth and positively regulated by SigH (Table 1).

Thus, as already observed in *B. subtilis* (8), SigH positively controls the expression of a large set of sporulation genes in *C. difficile*. To confirm the crucial role of SigH in the sporulation process, we compared the sporulation abilities of strain 630E, the *sigH* mutant, and a *spo0A* mutant inactivated with the ClosTron system and used as a control. After 72 h of incubation in BHIS medium, the percentage of sporulation in strain 630E was $9.3\% \pm 0.7\%$ (mean \pm standard deviation). Inactivation of the *spo0A* or *sigH* gene resulted in a complete inability of *C. difficile* to sporulate [$< 2 \times 10^{-4}\%$ sporulation]. When we complemented the *sigH* mutant using a pMTL84121-*sigH* plasmid, which gave strain CDIP12, we obtained a sporulation efficiency of $17\% \pm 0.5\%$, indicating that the lack of sporulation is only due to the *sigH* gene disruption. Thus, in *C. difficile*, SigH is as essential as Spo0A for sporulation (52).

We also observed that 11 genes involved in cell division were induced at the onset of stationary phase and were less expressed in the *sigH* mutant (see Table S3 in the supplemental material). In *B. subtilis*, the formation of an asymmetric septum during sporulation is related to the control of several cellular division genes by sporulation factors (18, 26). The *ftsZ* gene, involved in the initiation of septum formation, and the *minC*, *minD*, and *minE* genes, required for proper placement of septum division, were expressed at 3- to 10-fold-lower levels in the *sigH* mutant. In *B. subtilis*, genes like *ftsZ* or *minCD* are also regulated by SigH (8). Using qRT-PCR, we confirmed the positive control of *minC* transcription by SigH. In addition, the expression of *minC* was restored in a *sigH* mutant complemented by pMTL84121-*sigH*. Finally, genes encoding DivIVA- and FtsQ-type proteins as well as FtsH, SepF, and MrdB were induced at the onset of stationary phase and positively controlled by SigH.

Modulation of PaLoc gene expression by SigH and growth phase. In the VPI10463 strain, the expression of *tcdA* and *tcdB* increases as cells enter stationary phase (16). We tested whether the same regulation exists in strain 630E and the possible role of SigH in this regulation. The transcriptome analysis showed that the *tcdA* gene was expressed 4-fold more after 10 h than after 4 h of growth (see Table S3 in the supplemental material). Using qRT-PCR, we confirmed that *tcdA* expression increased 10-fold at the onset of stationary phase. Furthermore, *tcdA* is expressed 3.7- and 9-fold more in the *sigH* mutant than in strain 630E, as shown by transcriptome and qRT-PCR analysis, respectively (Fig. 1A; also see Table S3 in the supplemental material). In the *sigH* mutant complemented by pMTL84121-*sigH*, *tcdA* expression was restored to the level observed in strain 630E (Fig. 1A). So, *tcdA* expression is induced at the onset of stationary phase and is negatively controlled by SigH. We further measured the effect of SigH inactivation on TcdA protein synthesis. Crude extracts were obtained from the *sigH* mutant and strains 630E and CDIP12 grown in TY medium. TcdA production was assayed by dot blot assay using specific antibodies raised against TcdA (Fig. 1B). We observed an 8- and a 4-fold-increase in TcdA quantity after 10 h and 48 h of culture, respectively, in the *sigH* mutant compared to the levels in strain 630E. TcdA production decreased when the *sigH* gene was introduced in *trans* into the *sigH* mutant (Fig. 1B). The variation in the level of TcdA

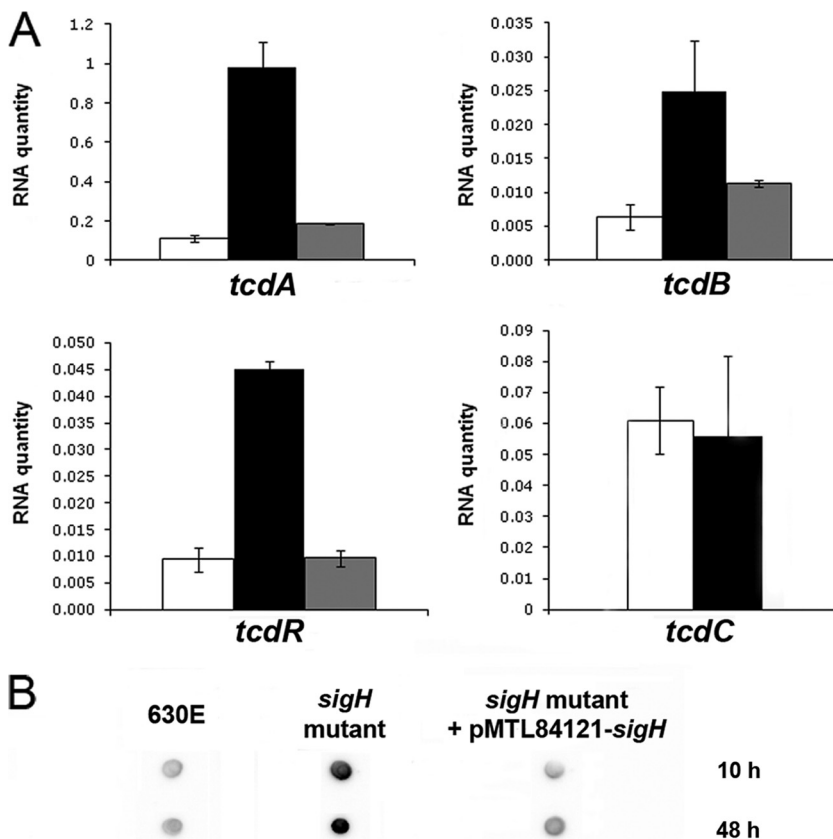


FIG. 1. Effects of SigH inactivation on the expression of pathogenicity locus genes. (A) Expression of *tcdA*, *tcdB*, *tcdR*, and *tcdC* in strain 630E (white), in a *sigH* mutant (black), and in strain CDIP12 (*sigH* mutant complemented with pMTL84121-*sigH*) (gray) after 10 h of growth in TY medium. After reverse transcription, specific cDNAs were quantified by qRT-PCR, using the 16S rRNA gene for normalization and standard curve. The mean normalized quantity of RNA is indicated in arbitrary units. Error bars correspond to standard deviations from at least two biological replicates. (B) The TcdA level was estimated from crude extracts by protein dot blot analysis using specific monoclonal antibodies raised against TcdA. Crude extracts were obtained after 10 h and 48 h of growth in TY. The results shown are representative of at least three biological replicates of *C. difficile* crude extracts.

produced is in agreement with the increase of *tcdA* transcription in the *sigH* mutant.

By qRT-PCR, we observed that the expression of *tcdB* and *tcdR* in strain 630E also increased 4- and 3-fold at the onset of stationary phase, respectively. In addition, *tcdB* and *tcdR* were expressed 5-fold more in the *sigH* mutant than in strain 630E (Fig. 1A). The levels of *tcdB* and *tcdR* expression were restored when the *sigH* gene was introduced in *trans* into the *sigH* mutant. In contrast, *tcdC* was transcribed similarly in strain 630E and in the *sigH* mutant (Fig. 1A). The absence of modulation of *tcdC* transcription in the *sigH* mutant compared to its expression in strain 630E indicates that the SigH-dependent control of *tcdA*, *tcdB*, and *tcdR* expression is not mediated by the regulation of TcdC synthesis. However, we cannot exclude the possibility that SigH might influence factors controlling TcdC stability or activity.

Surprisingly, the effects of SigH or Spo0A inactivation on toxin production differ. While a *spo0A* mutation reduces the amount of TcdA produced (52), a *sigH* mutation stimulates TcdA production via the control of its transcription. However, neither a Spo0A-binding motif nor a SigH promoter is present upstream of *tcdA*, *tcdB*, and *tcdR*, indicating an indirect, still-

uncharacterized effect of these two proteins on toxin gene expression.

Control of surface-associated proteins and flagellum synthesis by SigH. Surface-associated proteins and factors, which play a role in cell adherence and could be involved in intestinal colonization, have been identified in *C. difficile* (12, 57). Several genes encoding surface-associated proteins and adhesion factors were differentially expressed between 4 h and 10 h of growth and/or controlled by SigH (see Table S3 in the supplemental material). The expression of *CD2789* (*cwp66*), encoding a cell surface protein with adhesive properties (53), was induced at the onset of stationary phase and positively regulated by SigH. The *slpA* gene, encoding the S-layer precursor (19), was upregulated in the *sigH* mutant. The high-molecular-weight S-layer protein (HMW-SLP) binds to epithelial cells and plays a role in adherence (9). The *C. difficile* genome contains 28 genes encoding proteins with domains similar to the HMW-SLP (41). Some of them are located on the cell surface (57). *CD0514*, a gene controlled by phase variation that encodes the cell wall protein CwpV (17), was upregulated in the *sigH* mutant, as observed for *slpA*. The expression of some other genes, *CD2713*, *CD1803* and *CD2784*, encoding SLP-like

proteins, dropped at the onset of stationary phase and was not controlled by SigH. In addition, two genes encoding SLP-like proteins (*CD2518* and *CD0440*) were expressed more at the onset of stationary phase and were positively regulated by SigH, similarly to *CD2789*. We confirmed by qRT-PCR 4-, 82-, and 500-fold decreases in transcription in the *sigH* mutant for the *cwp66*, *CD0440*, and *CD2518* genes, respectively. Their transcription was restored when the *sigH* mutant was complemented with pMTL84121-*sigH*. Interestingly, we also observed that the expression of *CD3145*, encoding a protein that may bind to extracellular matrix components of eukaryotic cells, was higher after 4 h of growth and in the *sigH* mutant. So, the expression of several genes encoding surface-associated proteins considered putative accessory virulence factors is growth phase dependent and/or regulated by SigH.

The role of flagellum in adherence has also been shown in *C. difficile* (49). Most of the genes of the flagellum locus (43 genes) were downregulated at the onset of stationary phase and upregulated in a *sigH* mutant (see Table S3 in the supplemental material). The levels of expression of the *fliC* and *fliD* genes, encoding the flagellin and the flagellum cap protein, were 6.4 and 8.3-fold higher, respectively, in the *sigH* mutant in the transcriptome and we confirmed by qRT-PCR a 13-fold increase of the *fliC* mRNA in this mutant. Furthermore, the expression of *CD0240*, encoding a glycosyl-transferase enzyme involved in flagellin glycosylation (51), was also 4-fold increased in a *sigH* mutant. However, we failed to detect an increased motility of the *sigH* mutant under the conditions used (BHI plates or tubes containing 0.3% agar) (data not shown). In *C. acetobutylicum*, Spo0A negatively controls a large set of genes involved in motility (50), suggesting that both SigH and Spo0A repress motility-associated genes at the onset of the stationary phase in clostridia, as observed in *B. subtilis* (8, 20). In *B. subtilis*, the effect of SigH and Spo0A on flagellum synthesis is mediated by SinR. Interestingly, the expression of the *C. difficile* *sinR*-like gene, *CD2214*, was 16-fold higher in a *sigH* mutant than in strain 630E, while the expression of the *sigD* gene, encoding the SigD sigma factor of late flagellum genes, hardly increased (1.5-fold). In *C. difficile*, SinR might be involved in the SigH-dependent control of flagellin synthesis.

Transcriptional control of *sigH*, *spo0A*, *CD2492*, and *spoIIAA* by SigH and Spo0A. To test whether SigH and Spo0A mutually regulate their transcription, we performed qRT-PCR using RNAs extracted from strain 630E, the *sigH* or *spo0A* mutant, and CDIP12 grown for 10 h in TY medium (Fig. 2). We also tested the transcriptional regulation of *CD2492*, encoding the Spo0A-associated kinase and the *spoIIAA* operon, by SigH and Spo0A. In the *spo0A* mutant, we observed that the transcript levels of *sigH*, *CD2492*, and *spoIIAA* were 2-, 3-, and 40-fold lower, respectively, than in strain 630E. In the *sigH* mutant, the *spo0A*, *CD2492*, and *spoIIAA* transcript levels were 4.5-, 3-, and 200-fold lower, respectively, than in strain 630E (Fig. 2). Using antibodies raised against Spo0A of *B. subtilis*, we also showed that the quantity of Spo0A decreased in a *sigH* mutant (data not shown). In all cases, when we complemented the *sigH* mutant using a pMTL84121-*sigH* plasmid, we restored the expression of these genes to the level observed in strain 630E (Fig. 2). Thus, SigH positively controls *spo0A*, *CD2492*, and *spoIIAA* transcription, in agreement with our transcriptome data (Table 1), while Spo0A activates the expression of *sigH*,

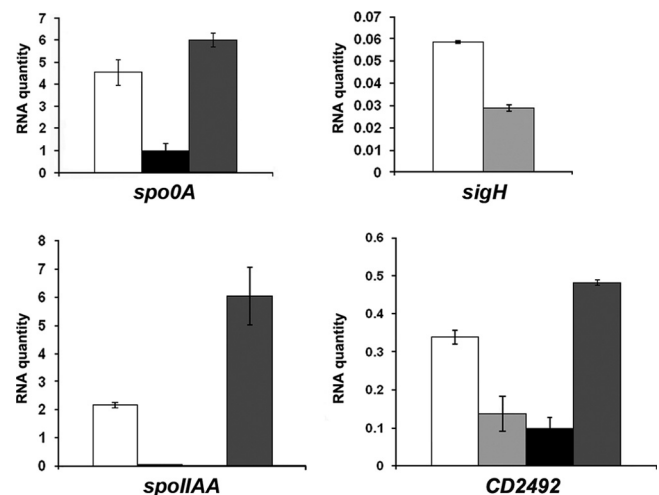


FIG. 2. qRT-PCR analysis of the role of SigH and Spo0A in the control of *spo0A*, *sigH*, *CD2492*, and *spoIIAA* gene expression. Total RNAs were extracted from *C. difficile* strain 630E (white), the *spo0A* mutant (pale gray), the *sigH* mutant (black), and strain CDIP12 (*sigH* mutant complemented with pMTL84121-*sigH*) (dark gray) grown in TY medium. After reverse transcription, specific cDNAs were quantified by qRT-PCR, using the 16S rRNA gene for normalization and standard curve. Error bars correspond to standard deviations from at least two biological replicates.

CD2492, and *spoIIAA*. In *C. acetobutylicum*, Spo0A also positively controls the expression of the *spoIIAA* operon, of the *cac3319* and *cac0903* genes, encoding Spo0A-associated histidine kinases, and of *cac0437*, encoding a protein that dephosphorylates Spo0A-P (44, 50).

We further characterized the *sigH*, *spo0A*, *CD2492*, and *spoIIAA* transcription initiation sites by the 5' RACE technique and looked for the presence of a Spo0A binding motif using the consensus sequence TGNCGAA of *B. subtilis* (35). We identified a transcription initiation site 32 bases upstream of the *sigH* gene start codon. The deduced -35 (TTGAAA) and -10 (TATAAT) boxes correspond to the consensus sequence of a SigA-dependent promoter. A Spo0A box is present 67 bases upstream of the transcription initiation site (Fig. 3A), in agreement with the positive control of *sigH* transcription by Spo0A. Transcription of *spo0A* is initiated 65 bases upstream of its start codon. A sequence sharing similarities with the consensus of SigH-type promoters of *B. subtilis* (GGAGGATAT-N₁₁-GTCTGAAT) is present upstream of this transcriptional start site (Fig. 3B). This is in accordance with the positive control of *spo0A* transcription by SigH. As found in *B. subtilis* (48), a Spo0A binding site overlaps the -10 box of this promoter, suggesting a repression of its own transcription by Spo0A. We noted that *spo0A* transcription was still detectable in the *sigH* mutant (Fig. 2), suggesting the existence of a second promoter. So, we performed a 5' RACE experiment using RNAs extracted from the *sigH* mutant. We identified a second transcription initiation site 139 bases upstream of the start codon. A consensus sequence (TGTTAT-N₁₇-TAAAAT) probably recognized by SigA is present (Fig. 3B).

The transcription initiation site of *CD2492* was mapped 35 bases upstream of the start codon. We found a SigH-dependent promoter (GAAGGAATT-N₁₂-ATCGTAT), in agree-



FIG. 3. Promoter regions of *sigH* (A), *spo0A* (B), *spoIIAA* (C), and *CD2492* (D) genes. The transcriptional start sites are indicated by gray backgrounds and “+1.” The -35 and -10 boxes corresponding to SigA or the conserved sequences of SigH-dependent promoters are underlined. The Spo0A binding sites are boxed, while the translational start sites are indicated in bold.

ment with its positive control by SigH (Fig. 3D). For the *spoIIAA* operon, the transcription initiation site was located 39 bases upstream of the start codon. A consensus sequence recognized by SigH (GAAGGAATA-N₁₁-ATAGAAT) and a Spo0A box located 63 bases upstream of the transcription initiation site are present (Fig. 3C). This is in accordance with the positive regulation of *spoIIAA* transcription by both Spo0A and SigH (Fig. 2).

Similar to *B. subtilis* (38, 43), there is reciprocal control of the synthesis of SigH and Spo0A in *C. difficile*. However, the positive control of *sigH* transcription by Spo0A in *B. subtilis* is indirect, with a negative effect of Spo0A-P on the synthesis of AbrB, which in turn represses *sigH* expression (46, 47). In the absence of the AbrB repressor in *C. difficile*, the Spo0A box that is located upstream of the SigA-dependent promoter of *sigH* (Fig. 3) strongly suggests direct positive control of *sigH* transcription by Spo0A. The transcription of *spo0A* itself is initiated from two promoters recognized by the RNA polymerase containing SigA or SigH. We further showed that the increase of *spo0A* expression at 10 h was abolished in a *sigH* mutant (data not shown). So, SigH contributes to the induction of *spo0A* expression at the onset of the stationary phase, as in *B. subtilis* (10). A second level of control of Spo0A activity probably occurs via the regulation of the synthesis of the CD2492 kinase by SigH. In contrast, the synthesis of the other putative Spo0A-associated kinases, including CD1579, which is able to phosphorylate Spo0A *in vitro*, and CD1492 (52) is not controlled by the growth phase or by SigH under our experimental conditions (Table 1). The possible regulation of the expression of CD1579 and CD1492 by Spo0A or by other regulators remains to be established.

Identification of potential direct target genes of SigH. In the transcriptome analysis, 286 genes are positively regulated by SigH. To identify genes directly controlled by SigH, we searched for the presence of the consensus sequence of *B. subtilis* SigH-dependent promoters (A/G)NAGGA(A/T)₃-N₁₁₋₁₂-(A/G)NNGAAT in the 200-bp region upstream of start codons of *C. difficile* genes using the GenoList web server (<http://genodb.pasteur.fr/cgi-bin/WebObjects/GenoList>) and allow-

ing two mismatches. About 40 genes and operons are positively regulated by SigH and contain a SigH-like consensus sequence in their promoter regions, suggesting their direct regulation by SigH (Table 2). In *B. subtilis*, there are about 50 transcriptional units directly controlled by SigH (8). Surprisingly, only eight of them are in common with *C. difficile*: *spo0A*, *spoIIAA*, *spoVG*, *spoVS*, *ftsZ*, *minC*, *glgC*, and *CD0142/lyvyD*. In addition, genes encoding Spo0A kinases in *B. subtilis* (*kinA* and *kinE*) and *C. difficile* (CD2492) are also transcribed by SigH in these two bacteria. Most of the common genes that are direct SigH targets are involved in sporulation or cell division, confirming the crucial role of this sigma factor in the early steps of the sporulation process in both microorganisms. Furthermore, in *C. difficile*, the *soj-spo0J-CD3670* operon and the *CD3673* gene, encoding a Spo0J-like protein, are probably transcribed by SigH (Table 2). The effects of SigH on the other sporulation genes are probably indirect and mediated at least partly by SigF and/or Spo0A. For example, a Spo0A box is present upstream of *spoIIE* in *C. difficile*, as in *B. subtilis* (58). Moreover, we mapped a SigF-type promoter upstream of the *sigG* gene (see Fig. S4 in the supplemental material), which was expressed 5-fold less in a *sigH* mutant (Table 1). In contrast, some other genes that are direct SigF targets in *B. subtilis*, such as *spoIIR* and *spoIVB*, are not induced in *C. difficile* after 10 h of growth compared to their expression at 4 h and are not controlled by SigH in the transcriptome. These differences could be due either to low expression after 10 h of growth or to a different circuit of control of these genes in *C. difficile* than in *B. subtilis*.

Among the genes that are potentially direct targets of SigH, we found genes involved in metabolism and translation and encoding membrane and surface-associated proteins or proteins of unknown function. The *glgCDAP-CD0886* operon, involved in a glycogen-type polymer synthesis and degradation (*glgP-CD0886*), is probably transcribed from a SigH-dependent promoter, as observed in *B. subtilis* (8). In *C. acetobutylicum*, there is no SigH-dependent promoter upstream of the *glgCDA* operon, which is involved in granulose production, while a promoter recognized by SigH is present upstream of *glgP* (2, 28). This suggests a diversity of control of the *glg* system among clostridia. In *C. difficile*, there are two copies of the *sigA* gene (CD1455/*sigA1* and CD1498/*sigA2*). The *sigA1* gene forms an operon with *dnaG*, as observed in *B. subtilis*. Interestingly, *sigA2* transcription increased at the onset of stationary phase and was strongly dependent on SigH (see Table S3 in the supplemental material). We confirmed by qRT-PCR a 180-fold decrease of *sigA2* transcription in a *sigH* mutant and the restoration of its expression in a *sigH* mutant complemented with pMTL84121-*sigH*. We further demonstrated the presence of a unique SigH-dependent promoter upstream of *sigA2*, while we mapped a SigA-dependent promoter upstream of the *dnaG-sigA1* operon (see Fig. S4 in the supplemental material). In *B. subtilis*, the *dnaG-sigA* operon is transcribed by several promoters, two of them being SigH dependent (8), while in *C. difficile*, one copy of *sigA* is transcribed by SigH and the other by SigA. Finally, it is worth noting that among the genes encoding surface-associated proteins with HMW-SLP-type domains that are induced at the onset of stationary phase in a SigH-dependent manner, three genes (*cwp66* [CD2789],

TABLE 2. Genes or operon positively controlled by SigH and with a SigH consensus sequence in their promoter region

Gene ^a	Promoter recognized by SigH ^b	Expression ratio in <i>sigH</i> mutant/strain 630E	Function
<i>spo0A</i> *	taGGAGGAATAtaat ^u tttggagtGTCGAATat	0.2	Stage 0 sporulation protein A
<i>CD2492</i>	aaGAAGGAATTataatgtcagatATCGTATAa	0.35	Histidine kinase associated to Spo0A
<i>soj</i>	atACAGGAATTgatgctaagtatAATGAAAta	0.56	Sporulation initiation inhibitor
<i>spoIIAA</i> *	ttGAAGGAATAaaaat-ataattATAGAATtg	0.03	Anti-sigma F factor antagonist
<i>spoVS</i> *	atAAAGGTTTTTcttaaaacgattATAGAAGTa	0.03	Stage V sporulation protein S
<i>spoVG</i> *	aaAGAGGATATccctagtgttcATAGAATta	0.01	Regulator required for spore cortex synthesis
<i>CD3673</i>	atAAAGGAATTattaaagtagatGCAGAAAAta	0.21	Putative stage 0 sporulation protein, Spo0J-like
<i>CD3569</i>	aaGAAGGGTTTagtgaagatgatATAGAAAag	0.33	Sporulation-specific protease
<i>ftsZ</i> *	aaAAAGGAAAAAttac-gttttGTGGAAATat	0.34	Cell division protein FtsZ
<i>minC</i> *	taAAAGGGTTTaaagc-gtattGAAGAATat	0.09	Cell division regulator
<i>CD2624</i>	taAAAGGGTTTaaagc-gtattGAAGAATat	0.33	Conserved hypothetical protein
<i>sigA2</i>	aaGGAGGATATgctg-ttagaaGTAGAATaa	0.05	Transcription
<i>CD0142</i> *	aaAGAGGATTAfgaga-gttcgtGTAGAATat	0.14	Putative RNA-binding protein
<i>CD0838</i>	taCAAGGATTTaaag-ataaatATAGAAAAtt	0.26	DNA-binding protein
<i>gapN</i>	ttATAGGtAAAcgtttatgaaaaAAAGAAAtg	0.48	Glyceraldehyde-3-P dehydrogenase
<i>glgC</i> *	agGAAGGATATggaaa-ttaaagGTGCAATta	0.19	Glucose-1-P adenylyltransferase
<i>fdhF</i>	taAGAGGAATTgtgag-aaaattGTTGAATtt	0.31	Formate dehydrogenase
<i>cobT</i>	atAAAGGAAATgaaca-taaaatGTAGAATaa	0.35	Nicotinate-nucleotide-dimethylbenzimidazole
<i>CD3456</i>	agGGAGGAAATtatttt-atgaaaAAAGAAAtt	0.49	5-Formyltetrahydrofolate cycloligase
<i>CD2738</i>	agGGAGGGATAatattgcaagagAGAGAAGaa	0.49	Putative cytosine permease
<i>CD0760</i>	gtTTAGGATAAataaat ^u tttattATGGAAAaa	0.4	Putative Ca ²⁺ /Na ⁺ antiporter
<i>CD2789</i>	caAGAGGAgAAgttattaagtgcAATGAATgc	0.39	Putative adhesin Cwp66
<i>CD0440</i>	atAAAGGAAAAAttac-ttttatGTAGAATta	0.25	Putative cell wall-binding protein
<i>CD2518</i>	aaGGAGGAAAAgaaa-tttaaGTAGAATta	0.14	Cell surface protein
<i>lplA</i>	ttAAAGGAgTTtttat-attattGTGCAATta	0.34	Lipoate protein-ligase
<i>CD3458</i>	ttAAAGGAAATttagtagtattATCGAATtg	0.07	Membrane protein
<i>CD2800</i>	ctATAGGAACtTgtaaatctaaaAAAGAAAGaa	0.06	Membrane protein
<i>CD2295</i>	taGGAGGGATTttatg-gattttGGGGAATtt	0.28	Membrane protein
<i>CD1590</i>	aaAAAGGAAAAcgtctaaagtatTGGAAATac	0.44	Membrane protein
<i>CD0865</i>	taACAGGATTTatgtaggtgttATAGAAAAta	0.08	Putative ADP-ribose binding protein
<i>CD2447</i>	taGAAGGAATTTgctataacatGTAGAAAAtt	0.06	Putative histidine triad (HIT) protein
<i>CD3654</i>	aaAGAGGAAGAcgtatagtaaGGAGAATat	0.24	Putative DNA replication protein
<i>CD0022</i>	aaAGAGGACAAttacccagatGTAGAAAAta	0.04	Elongation factor G (EF-G)
<i>CD2137 (frr)</i>	atAAAGGTATTtgagcttaacaAGAGAATat	0.4	Ribosome-recycling factor
<i>CD1941</i>	aaGTAGGTATAtataacaaggaaACAGAAcCcc	0.05	Unknown function
<i>CD1543A</i>	atAAAGGAAAAAcctctttaaGTAGAAAct	0.07	Unknown function
<i>CD1264</i>	aaAGAGGAAAAatcatt-ttaaGTAGAATaa	0.08	Unknown function
<i>CD1061</i>	ccAGAGGATATgaagagttaatATCGAAAtt	0.25	Unknown function
<i>CD1317</i>	taAAAGGAAAAGacaggat-aaatATAGAAAtt	0.42	Unknown function
<i>CD1622</i>	atAAAGGGTTTtagaggcataatATAGAATaa	0.46	Unknown function

^a The transcriptional start site was identified for underlined genes. We used the search pattern program of the GenoList Web server (<http://genodb.pasteur.fr/cgi-bin/WebObjects/GenoList>) to detect the presence of the consensus sequence of *B. subtilis* SigH-dependent promoters (A/G)NAGGA(A/T)₃-N₁₁₋₁₂-(A/G)NNGAAT in the 200-bp region upstream of *C. difficile* genes, allowing two mismatches. An asterisk indicates genes or operons with a SigH promoter in *B. subtilis* (9).

^b The -10 and -35 boxes are indicated by uppercase letters.

CD0440, and *CD2518*) are probably transcribed by SigH (Table 2).

The alignment of all the probable SigH-dependent promoters listed in Table 2 allows the proposal of a consensus for the promoters transcribed by the RNA polymerase associated with SigH in *C. difficile* (Fig. 4). This consensus includes two well-conserved motifs, AGG and GAA, that are separated by 18 to 19 bases and is rather similar to the promoter sequences of the SigH regulon of *B. subtilis* (8).

Conclusion. We showed that about 20% of *C. difficile* genes are differentially expressed during the exponential growth phase and the onset of stationary phase. About 60% of these genes are regulated directly or indirectly by SigH, demonstrating the crucial role of this sigma factor during the post-exponential growth phase in *C. difficile*. However, other regulators, such as CodY or Spo0A, also contribute to the transcriptional changes observed during the transition phase. Indeed, among

the genes whose expression varies depending on the growth phase, 80 (10.5%) are controlled by CodY in the transcriptome (13), and a putative Spo0A box is located upstream of 70 genes (9.5%). The regulatory network modulating gene expression at the end of exponential growth is probably more complex, since

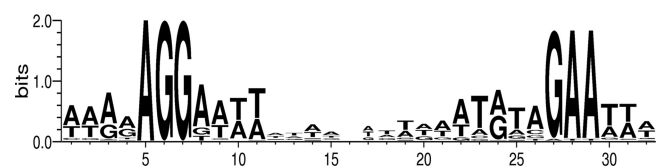


FIG. 4. Consensus of SigH-dependent promoters in *C. difficile*. Using an alignment of all the proposed direct targets of SigH listed in Table 2, the sequence logo was created on the WebLogo website (<http://weblogo.berkeley.edu>). The height of the letters is proportional to their frequency.

we showed that 29 additional regulatory genes were induced or repressed at the onset of stationary phase in the transcriptome.

Among the genes regulated by SigH, we found genes involved in sporulation, cellular division, motility, metabolism (carbon and energy, amino acids, and protein catabolism), and virulence, as well as genes encoding membrane and surface-associated proteins. Only a small number of genes activated by SigH in clostridia are found in *B. subtilis*, as proposed for Spo0A (35). This may be due to the fact that clostridia are anaerobes, leading to major differences in the physiology of these bacteria, and the fact that initiation of sporulation appears to occur via a different pathway with the lack of the phosphorelay (44).

Actually, the global impact of SigH on transcription in *C. difficile* largely exceeds the 40 genes or operons probably directly transcribed by SigH (Table 2). Most of the other genes, including those negatively controlled, are indirectly regulated by SigH via other regulators or changes in the pools of signaling molecules. We demonstrated that the regulation of SigH and Spo0A are intertwined, with SigH modulating *spo0A* transcription and Spo0A activating *sigH* expression. The identification of the Spo0A target genes would be helpful to better understand the global regulatory role of SigH during the transition phase. Part of the indirect effects of SigH may also be due to the alteration of the association of alternate sigma factors, such as SigB or SigD, with core RNA polymerase in the presence or absence of SigH. Competition between sigma factors for the core RNA polymerase has been observed within the σ^{70} family or between σ^S and σ^N in *E. coli* (15) and between SigA and SigH, SigE, or SigK in *B. subtilis* (25, 29). Finally, among the 14 genes encoding transcriptional regulators, including CggR and SinR, whose expression is modulated by SigH, only *CD0838*, encoding a DNA binding protein of unknown function, seems to be directly transcribed by SigH (Table 2). How this regulator may contribute to the control of gene expression by SigH will be interesting to explore in future investigations. Moreover, SinR, a repressor of genes expressed in stationary phase in *B. subtilis*, is also a good candidate for participation in the global regulatory effect of SigH.

Finally, the *sigH* mutant is unable to sporulate but still produces toxins, demonstrating that toxin synthesis is not associated with sporulation, contrary to the production of enterotoxin in *C. perfringens* (22). Toxin production in *C. difficile* is therefore clearly a stationary-phase event. The mechanism of control of toxin synthesis by SigH remains to be established. Several hypotheses could be suggested. This effect could be due to decreased competition between SigH and TcdR for the core RNA polymerase binding at the onset of stationary phase in the *sigH* mutant, to control of TcdC activity, or to a regulatory cascade involving other transcriptional factors. Further work will be required to analyze the molecular mechanisms of SigH-dependent control of toxin production.

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