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Infection and Immunity

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Adaptive Strategies and Pathogenesis of *Clostridium difficile* from *In Vivo* Transcriptomics

Claire Janoir,[a] Cécile Denève,[a] Sylvie Bouttier,[a] Frédéric Barbut,[b] Sandra Hoys,[a] Laxmee Caleechum,[c] Diana Chapetón-Montes,[d] Fátima C. Pereira,[e] Adriano O. Henriques,[e] Anne Collignon,[a,f] Marc Monot,[c] Bruno Dupuy[c]

EA 4043, Université Paris-Sud 11, Faculté de Pharmacie, Châtenay-Malabry, France[a]; Centre National de Référence de *Clostridium difficile*, Service de Microbiologie, Hôpital Saint-Antoine, Assistance Publique-Hôpitaux de Paris, Paris, France[b]; Laboratoire Pathogenèse des Bactéries Anaérobies, Département de Microbiologie, Institut Pasteur, Paris, France[c]; Unité de Recherche et d'Expertise Bactéries Anaérobies et Toxines, Département de Microbiologie, Institut Pasteur, Paris, France[d]; Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal[e]; Service de Microbiologie, Hôpital Jean Verdier, Assistance Publique-Hôpitaux de Paris, Paris, France[f]

***Clostridium difficile* is currently the major cause of nosocomial intestinal diseases associated with antibiotic therapy in adults. In order to improve our knowledge of *C. difficile*-host interactions, we analyzed the genome-wide temporal expression of *C. difficile* 630 genes during the first 38 h of mouse colonization to identify genes whose expression is modulated *in vivo*, suggesting that they may play a role in facilitating the colonization process. In the ceca of the *C. difficile*-monoassociated mice, 549 genes of the *C. difficile* genome were differentially expressed compared to their expression during *in vitro* growth, and they were distributed in several functional categories. Overall, our results emphasize the roles of genes involved in host adaptation. Colonization results in a metabolic shift, with genes responsible for the fermentation as well as several other metabolic pathways being regulated inversely to those involved in carbon metabolism. In addition, several genes involved in stress responses, such as ferrous iron uptake or the response to oxidative stress, were regulated *in vivo*. Interestingly, many genes encoding conserved hypothetical proteins (CHP) were highly and specifically upregulated *in vivo*. Moreover, genes for all stages of sporulation were quickly induced *in vivo*, highlighting the observation that sporulation is central to the persistence of *C. difficile* in the gut and to its ability to spread in the environment. Finally, we inactivated two genes that were differentially expressed *in vivo* and evaluated the relative colonization fitness of the wild-type and mutant strains in coinfection experiments. We identified a CHP as a putative colonization factor, supporting the suggestion that the *in vivo* transcriptomic approach can unravel new *C. difficile* virulence genes.**

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). Higher morbidity and mortality due to *C. difficile* have been described in recent years. This results mainly from the emergence of new isolates, such as those that have been assigned to the BI/NAP1/027 group of strains (1). The spectrum of diseases caused by *C. difficile* is highly variable. It depends on host factors, the level of toxin production (2), and the genome plasticity of *C. difficile*, all of which contribute to virulence (3).

The *C. difficile* virulence mechanism is thought to be a three-step process that begins by disruption of the colonic microbiota barrier due to antibiotic treatment, further allowing the colonization phase, which includes bacterial adhesion to the host and subsequent bacterial multiplication. The last phase corresponds to toxin production (4). Most virulent *C. difficile* strains produce two high-molecular-weight toxins: toxin A (TcdA) and toxin B (TcdB). Both toxins have glucosyltransferase activity and modify the actin skeleton of intestinal epithelial cells through UDP-glucosylation of members of the Rho family of small GTPases (1), thus conferring the symptoms of *C. difficile* infection (CDI) (diarrhea, epithelial apoptosis, and ulceration). Although the toxins are regarded as the primary virulence factors, additional factors intervene in the pathogenic process, notably to allow the establishment of the bacterium in its colonic niche. Indeed, the features of epidemic-associated *C. difficile* strains, such as the BI/NAP1/027 strains, suggest that factors that improve sporulation/germination, adhesion, and persistence of *C. difficile* in the gut (1) can play a role in *C. difficile* pathogenesis. Several putative colonization factors have already been characterized in strain 630, but specific bacterial processes involved in the early step of the infection are still poorly understood (1). Thus, analysis of gene expression during the infection process should allow the characterization of novel *C. difficile* virulence determinants.

Analysis of bacterial whole-genome expression within a susceptible host should lead not only to the identification of new genes critical for pathogenesis but also to a better understanding of the molecular events involved in infection. The technology of DNA arrays offers a high-throughput approach to measure gene expression levels on a genome-wide scale under different conditions. This approach has been applied to determine the gene expression profiles of several pathogens, such as *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, *Campylobacter jejuni*, *Borrelia burgdorferi*, *Listeria monocytogenes*, and *Yersinia pestis*, in different mouse or rabbit organs (5, 6). *C. difficile* arrays

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were also used for *in vitro* global analysis of gene expression after exposure to environmental and antibiotic stresses (7) or to human colorectal epithelial Caco-2 cells (8). The same approach was recently applied to unravel the gene expression profile of *C. difficile* in the pig ligated-loop model (9). However, the *C. difficile* transcriptome was never studied in the course of infection in the mouse model, which is widely used as a colonization model.

Here we present an analysis of the genome-wide temporal expression of *C. difficile* genes during the first hours after challenge in order to understand the adaptive strategies used by this pathogen in the course of the colonization process. We compared the expression of genes of *C. difficile* in bacteria recovered from monoxenic mouse ceca at 8, 14, and 38 h postinfection in order to identify genes which are modulated during the course of mouse colonization. We showed that 549 genes were differentially regulated during *in vivo* growth and were distributed in several functional categories. Moreover, we observed that the majority of genes differentially regulated *in vivo* compared to *in vitro* were upregulated. This work led to new insights into adaptive survival strategies in the host. Finally, we also identified a novel colonization gene, CD1581, whose expression was highly upregulated *in vivo*. A mutant inactivated in this gene was affected in the ability to colonize mice.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Clostridium difficile* 630 and 630 Δ*erm* strains were grown anaerobically (5% H_2, 5% CO_2, and 90% N_2) in tryptone-yeast extract medium (TY) at 37°C in an anaerobic chamber. Mutants obtained by use of ClosTron technology (10) (strains 630 Δ*erm*::*CD1581* and 630 Δ*erm*::*CD3145*) were grown on brain heart infusion (BHI) plates or TY broth supplemented with erythromycin (2.5 μg/ml) under anaerobic conditions.

Animal model. All animal experiments performed for this study were carried out using 6- to 9-week-old germfree C3H mice purchased from INRA, Orléans, France. Mice were housed in sterile isolators with *ad libitum* access to food and water. Before experiments, each animal was confirmed to be germfree by Gram staining of feces and by inoculating feces into BHI broth and incubating the broth for 48 h, either aerobically or anaerobically. Infection was performed by oral gavage with an appropriate amount of *C. difficile* vegetative cells, and colonization was followed by enumeration of *C. difficile* in feces sampled throughout time. The *C. difficile* transcriptome was analyzed on total bacterial RNA extracted from cecal content after the sacrifice of mice at different time points.

Ethics statement. All procedures were performed in agreement with the guidelines of the European Commission for the Handling of Laboratory Animals. The animal experiments were approved by the Central Animal Care Facilities and Use Committee of University Paris-Sud (agreement number 92-019-01).

Transcriptomic analyses. (i) Mouse experiments. Three groups, each with eight germfree male C3H mice, were challenged by the oral route with 7×10^5 CFU of vegetative cells of the *C. difficile* 630 strain obtained from a 9-h culture in TY medium. In parallel, TY broth was inoculated with the same amount of bacteria. The mice were then sacrificed at 8, 14, and 38 h postchallenge, and the ceca were sampled. The luminal content containing nonadherent bacteria, as well as the mucosal adherent bacteria (collected by mucosal scraping), was then quickly collected and placed in an RNA-stabilizing solution (RNAlater; Ambion) before extraction.

(ii) RNA extraction. In order to minimize RNA degradation and/or changes in the gene expression level, extraction of total RNA was performed immediately by using a FastPrep instrument and an RNA ProBlue kit (QBiogene) according to the manufacturer's instructions. The contaminating eukaryotic RNA was further discarded to obtain pure bacterial RNA by use of a MicrobEnrich kit (Ambion). Total RNAs from the independent *in vitro* cultures were extracted from strain 630 grown following the same *in vivo* kinetics (8, 14, and 38 h) in TY medium, using the same method, but without removing eukaryotic RNA. RNA quality was further assessed by analysis on an Agilent Bioanalyser and by quantitative reverse transcription-PCR (qRT-PCR) on the housekeeping genes *gyrA* and *rpoA*. For each time point of the *in vivo* kinetics experiment, eight RNA samples were prepared from mice. The four RNA samples displaying the best RNA integrity numbers (RINs) were conserved for the microarray experiments (see Fig. S1 in the supplemental material). The others were used for qRT-PCR validation.

(iii) Competitive hybridization assays. The *C. difficile* microarray based on the genome sequence of strain 630 was obtained from the Bacterial Microarray Group at St. George's Hospital, University of London (BμG@S). It can be found in ArrayExpress (accession number A-BUG-20). The microarray contains 3,679 coding sequences (CDSs). A Genisphere 3DNA Array 900 MPX microarray kit was used for cDNA synthesis, labeling, and hybridization, with specific protocol modifications as described by O'Connor et al. (11). Ten micrograms of starting RNA was used for the cDNA synthesis reactions. The microarray slides were hybridized competitively with each *in vivo* or *in vitro* cDNA, and the cDNA synthesized from the RNA was extracted from strain 630 grown for 8 h *in vitro*, to allow further direct comparisons between all slides hybridized. A dye swap was performed for each hybridization.

(iv) Data analysis. The microarray slides were scanned using a ScanArray 4000 instrument (Packard Instrument Co.), and fluorescence intensities were quantified using ImaGene (BioDiscovery) software. Raw expression data from the four arrays were analyzed with R and Limma (linear model for microarray data) software from the Bioconductor project (www.bioconductor.org). Further data processing and statistical analysis were performed according to the method of Saujet et al. (12). Briefly, the background was corrected with the Normexp method (13), resulting in strictly positive values and reducing variability in the log ratios for genes with low hybridization signals. We then normalized each slide by using the Loess method (14). In order to identify differentially expressed genes, we used the Bayesian adjusted *t* statistics and performed the multiple-testing corrections of Benjamini and Hochberg (15), which are based on the false discovery rate. A gene was considered differentially expressed when the *P* value was <0.05.

The 8-h *in vivo* values were chosen as a reference for reporting the expression data during the kinetics experiment. We confirmed the microarray data by qRT-PCR, using 14 genes with 4 biological replicates distinct from the RNA samples used in transcriptomic experiments.

Construction of gene knockout mutants in *C. difficile*. The ClosTron gene knockout system (10) was used to inactivate the CD1581 and CD3145 genes, giving strains 630 Δ*erm*::*CD1581* and 630 Δ*erm*::*CD3145*, respectively. Primers to retarget the group II intron of pMTL007 to these genes (see Table S1 in the supplemental material) were designed by Targetron design software (Sigma-Aldrich) and used with the EBS universal primer to generate a PCR product for intron retargeting for each gene. These PCR products were cloned into pMTL007, giving plasmids pMTL007::Cdi-*CD1581*235s and pMTL007::Cdi-*CD3145*1083a. The derivative pMTL007 plasmids were transformed into *Escherichia coli* HB101 (RP4) and subsequently mated with *C. difficile* 630E (Δ*erm*). *C. difficile* transconjugants were selected, and their chromosomal DNAs were isolated as previously described (12). In order to verify the integration of the Ll.LtrB intron into the correct gene targets, we performed PCR with primers flanking the *CD1581* (OBD504-CD505) and *CD3145* (FibF-FibR) genes and with primers inside *CD1581* (OBD504) and *CD3145* (FibR), associated with the intron primer EBSu (see Table S1).

Enumeration of bacteria from mouse ceca. Preliminary experiments were performed to check that the stool content accurately reflects the cecal content and to ensure that freezing does not alter the results of counts of either vegetative cells or spores in a significant manner. In all experiments, each fecal sample was suspended in phosphate-buffered saline (PBS) as a 10-mg/ml suspension and was further serially diluted in PBS. Appropriate

dilutions were plated either on BHI, to numerate vegetative cells, or, after a heat shock treatment (60°C, 30 min), on BHI containing 0.1% taurocholate sodium salt to enumerate the spores.

***In vivo* and *in vitro* kinetics of sporulation.** Six germfree male C3H mice were challenged by the oral route with 1×10^6 CFU of vegetative cells of the *C. difficile* strain 630 obtained from a 9-h culture in TY medium (early stationary phase; spore rate of <0.1% of vegetative cells). At 8, 14, 24, and 48 h, feces were sampled from each mouse, and both vegetative cells and spores were further enumerated as described above. Bacteria from *in vitro* cultures were also collected at 14 and 38 h of growth, and vegetative cells and spores were counted as well, to enable comparison of the sporulation rates *in vitro* and *in vivo* in monoxenic mice.

Competitive colonization assays. For each competitive assay, eight germfree female mice were challenged orally with equal proportions ($\approx 5 \times 10^5$ CFU) of the parental wild-type (WT) strain and each of the respective mutant strains (630 Δ*erm*::*CD1581* or 630 Δ*erm*::*CD3145*). Gavage suspensions were obtained by the appropriate dilution of 9-h TY cultures of each strain after cell counting by microscopic examination and were further enumerated by plating of dilutions. Feces from each mouse were collected at 38 h postchallenge. Vegetative cells of the WT and mutant strains together were enumerated on BHI, while enumerations of mutant strains alone were performed on BHI containing erythromycin (2.5 μg/ml).

Microarray data accession number. The complete experimental data set was deposited in the Gene Expression Omnibus (GEO) database under accession number GSE43305.

RESULTS AND DISCUSSION

***In vivo* transcriptome approach.** Microarray technology is a powerful approach to investigate the gene expression profile of pathogens during infection. However, the performance of such *in vivo* expression studies depends on the choice of the animal model, which, in the case of *C. difficile*, must reflect the lifestyle of the pathogen in the intestine during survival, colonization, and the initial stages of pathogenesis, but also on the recovery of a sufficient quantity of high-quality mRNA to ensure acquisition of an accurate and specific transcriptome profile. As a first step toward understanding the complex *C. difficile*-host interactions, we chose the germfree mouse, a simplified animal model that lacks the complex microbiota of the host. This model mimics the onset of infection and some of the histopathological lesions observed in humans (16) and allows a moderate host inflammatory response (16) as well as a partial immune response (17). Bacteria were sampled from cecal contents because *C. difficile* colonization reaches its maximum in this section of the intestine and because epithelial lesions first appear in the cecum of the mouse digestive tract (18).

Challenge of germfree mice with *C. difficile* did not result in severe diarrhea or death, but it did allow a high colonization level. In preliminary animal experiments, we established that *C. difficile* vegetative cells quickly colonized the mouse ceca, with low interanimal variability. Specifically, maximum colonization was reached at 48 h postchallenge, preceding a low and gradual decrease in the colonization rate (see Table S2A in the supplemental material). We used microarrays containing oligonucleotides representing approximately 98% of the annotated open reading frames of *C. difficile* strain 630 (19). The microarrays were used to analyze *C. difficile* transcription profiles during *in vitro* growth (in TY medium) in the late exponential (8 h) and stationary (14 and 38 h) phases and during *in vivo* growth (mouse cecum) at 8, 14, and 38 h postinfection (see Table S2B). The time points were chosen to establish a set of factors whose expression was induced or repressed during the first steps of *C. difficile* infection, before

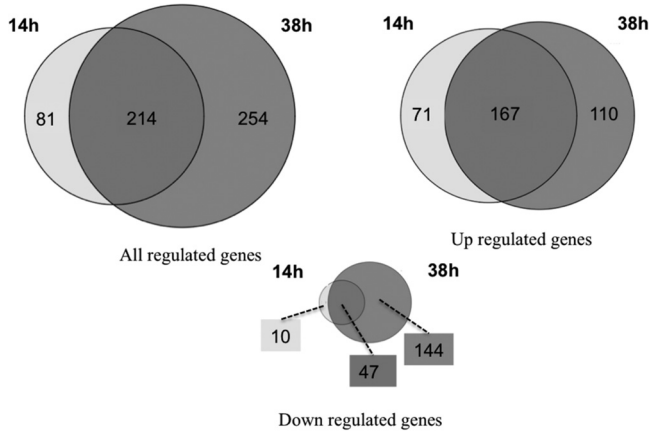

FIG 1 Venn diagrams showing the distributions of all genes that were up- and downregulated (with *P* values of <0.05) at the two *in vivo* infection time points (14 and 38 h) compared to their expression at 8 h.

maximum colonization was reached at 48 h (see Table S2A). For each time point, we used four biological replicates with a technical replicate in order to provide enough data for a robust statistical analysis of the microarray experiments. Genes showing statistically significant changes in expression level, with *P* values of <0.05, were considered in our analysis. To validate the transcriptomic profile data, we selected 14 genes and performed qRT-PCR on RNAs extracted from cecal bacteria at 8, 14, and 38 h postinfection. The qRT-PCR results and microarray data exhibited high correlation coefficients (R^2) for both 14- and 38-h analyses (0.88 and 0.95, respectively) (see Fig. S2). However, these two technologies revealed a quantitative difference in fold changes, which has also been reported by others (6, 20). This indicates that the fold changes in gene expression presented in the *in vivo* study are probably underestimated. Based on their expression profiles, genes can be separated into the following three categories: (i) genes upregulated early (14 h) or late (38 h) during infection, (ii) genes upregulated *in vivo* compared to *in vitro* kinetics; and (iii) genes inversely regulated *in vivo* and *in vitro* during analysis of growth kinetics.

Global changes in *C. difficile* gene expression during *in vivo* growth. A total of 549 genes exhibited differential expression during mouse infection compared to *in vivo* growth at 8 h. Among these genes, 348 (63.4%) were upregulated and 201 (36.6%) were downregulated during the course of mouse colonization (see Table S6 in the supplemental material). A total of 167 genes were upregulated at both 14 and 38 h postinfection, whereas 71 or 110 genes were upregulated only at 14 h or 38 h, respectively (Fig. 1). Interestingly, among the genes inversely regulated *in vivo* compared to *in vitro*, 187 genes were upregulated. All genes found to be expressed differentially were then assigned to functional categories (Fig. 2). Notably, as shown in Fig. 2, the major *in vivo* gene expression changes concerned genes encoding proteins involved in metabolism (fermentation and amino acid and lipid metabolism), regulation, cell processes, stress response, pathogenicity, and sporulation, but there was also a large number of genes of unknown function. All biological processes whose constituent genes were found to be either upregulated or inversely upregulated *in vivo* compared to *in vitro* growth could indicate a possible role in *C. difficile* physiopathology and were given special attention.

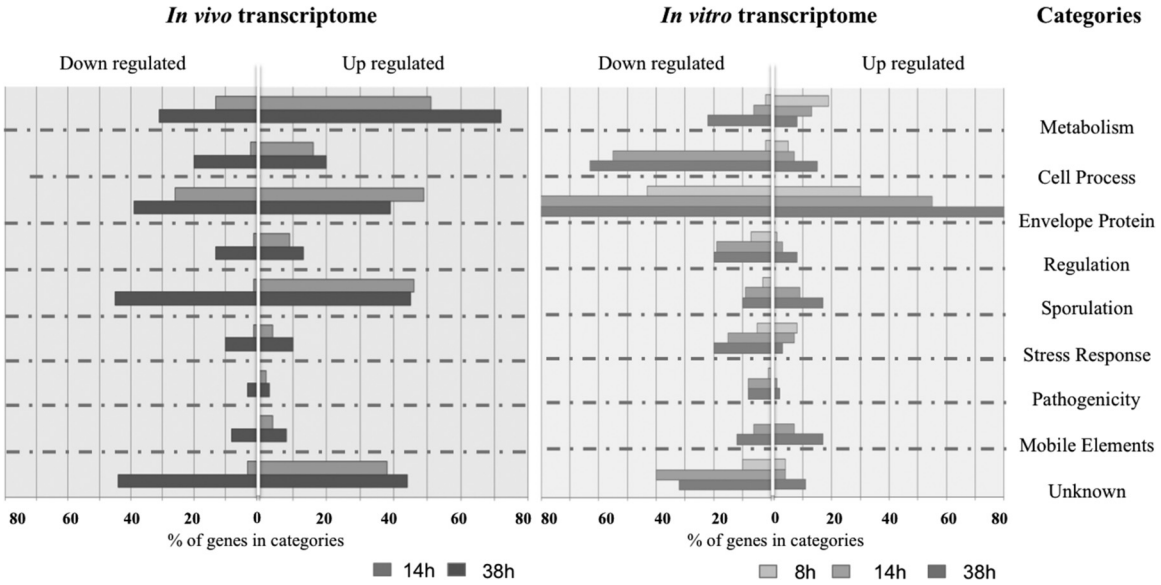

FIG 2 Functional categories of differentially expressed genes ($P < 0.05$) in the *C. difficile in vivo* (monoxenic mice) and *in vitro* (static TY medium) transcriptomes. The genes significantly upregulated or downregulated during growth *in vivo* (left) and *in vitro* (right) relative to their 8-h *in vivo* values are categorized based on the known or predicted functions of the encoded proteins.

Virulence factors. (i) Toxins. The transcriptional kinetic profiles of *tcdA* and *tcdB*, encoding the two major toxins, were similar in the monoxenic mice and in broth medium. Their expression increased moderately after 38 h both in mice and in TY (see Table S6 in the supplemental material). This is consistent with previous results showing that *in vitro*, the toxin genes are highly expressed at a late stage of growth (21). However, a recent *C. difficile* transcriptomic study performed on a pig ligated-loop model showed an early upregulation of the toxin genes, in particular of *tcdA*, which could be explained by the animal model used. In the study of Scaria and coauthors (9), pigs were fasted overnight before injection of *C. difficile* vegetative cells into the ligated loops, and they were kept anesthetized for the whole experiment. In our experiment, mice were housed with *ad libitum* access to food and were not stressed by artificial procedures such as anesthesia. Importantly, it has been shown that the level of toxin production is inversely correlated to nutrient availability (22), and it is likely that fasting pigs favored the early production of toxins.

(ii) Other virulence factors. Several surface proteins probably play a role in the colonization process of *C. difficile* (4). Among these putative colonization factors, the best characterized are the two S-layer proteins, encoded by a unique *slpA* gene (CD2793) (23). *slpA* was expressed at the early step of colonization (14 h), since its expression was downregulated at the late stage of infection (38 h). The same pattern of expression was observed for 3 of the 28 *slpA* paralogs (CD0440, CD2767/*cwp19*, and CD2796), which encode potential colonization factors (24). Among the other putative surface-associated proteins identified from an *in silico* analysis, the expression of a few of them decreased late during infection. This was the case for CD3145, encoding a fibrinogen binding protein. It has been assumed that the expression of genes that are needed in a particular situation will be upregulated, whereas genes with unnecessary functions in the same situation will be downregulated (25). The decreased transcription kinetics for some of the surface protein-encoding genes suggests that their expression is required mostly at the early stage of colonization, i.e., 8 and 14 h. Thereafter, a shift in gene expression can occur from colonization genes to strictly virulence genes, such as the toxin-encoding genes. This coordinated regulation has been documented previously for some pathogenic organisms, such as group A *Streptococcus* (26). It is noteworthy that expression of some of the *slpA* paralogs is downregulated along the *in vitro* kinetics, especially that of CD2787, encoding the cysteine protease Cwp84 (27), which plays a major role in the maturation of the surface layer, and CD0514, encoding the autoaggregation-promoting adhesin CwpV (28). In contrast, these corresponding genes are likely expressed constitutively *in vivo* throughout the duration of the experiment, since their expression was not downregulated compared to that *in vitro*. In support of this, antibodies against Cwp84 are frequently found in sera from patients suffering from *C. difficile*-associated disease (29). *In vivo* expression of CwpV could be related to the formation of clumps or microcolonies on the mouse intestinal epithelium, as previously suggested (30).

Flagella are probably involved in colonization of the gut by *C. difficile* (31). Surprisingly, in our *in vivo* experiment, no modulation of the three flagellum assembly operons was observed over time (see Table S6 in the supplemental material), apart from a moderate downregulation of *fliC*, encoding the major flagellum subunit flagellin. This result is in accordance with previous observations suggesting that motility should not be required for the virulence of *C. difficile* (3, 32). However, FliC and FliD are important antigens recognized during human infection (29), and thereby, flagella should be expressed at a given time of the infection. It is possible that flagellum-encoding genes are expressed during the very early colonization phase, i.e., before 8 h, and that their expression is downregulated later, facilitating the host immune system escaping, as also suggested for *Campylobacter jejuni* (6). To support this hypothesis, some of the flagellum-encoding genes were shown to be upregulated 4 h after infection in the pig

ligated-loop transcriptomic study, and most were shown to be downregulated thereafter (9).

Cellular activity and cell factors. During *in vivo* growth, the expression of most ribosomal genes was stable or was weakly downregulated (see Table S6 in the supplemental material) compared to the highly decreasing kinetics *in vitro*. Most likely, this reflects a higher cellular activity during *in vivo* growth than that *in vitro*, which is consistent with continuous culture, with a constant renewal of the nutrients (see Table S2). In addition, the expression of the ribosome recycling factor (RRF)- and elongation factor G (EF-G)-encoding genes (CD1486 and CD0022, respectively) was highly increased at 14 and/or 38 h postinfection (see Table S6). In connection with EF-G, RRF *in vitro* catalyzes the fourth step of protein synthesis, i.e., the disassembly of the ribosome-mRNA-tRNA posttermination complex (33). Increased expression of RRF at any given time could reflect the ability of *C. difficile* to synthesize proteins from newly transcribed mRNAs. This could include TcdA, TcdB, and sporulation proteins as well as any other protein with known or unknown function (see below), since their corresponding mRNAs were overexpressed at 38 h postinfection (see Table S6).

In vitro, 20% of genes encoding proteins annotated "cell factors" were downregulated at 14 and/or 38 h of growth, whereas only 8% of them were moderately upregulated. In contrast, *in vivo*, only 3% of cell factor-encoding genes were downregulated over time, whereas 17% were upregulated. Among the latter, we found genes encoding several hydrolases (CD0704 and CD2864), putative helicases (CD0749 and CD3298), peptidases (CD1086, CD3652, and CD3489), and proteins belonging to the radical SAM superfamily (CD0392 and CD0731). The latter may participate in critical processes such as DNA replication and transcription, DNA repair, or biosynthesis of vitamins and coenzymes (see Table S6 in the supplemental material). Thus, the kinetics of expression of many "cell factors" also reflects the differences between *in vivo* continuous culture and discontinuous *in vitro* culture.

Biosynthesis of PG. The deacetylation of the GlcNAc moiety, in association with an unusually high level of L,D-transpeptidation, is the main peptidoglycan (PG) structural characteristic of vegetative *C. difficile* cells (34). No upregulation was observed for the three genes encoding known or putative L,D-transpeptidases (CD2713, CD2963, and CD3007). Among the 10 putative polysaccharide deacetylase genes identified *in silico*, only CD3257, encoding a protein with significant homology to the catalytic domain of the PG GlcNac deacetylase of *S. pneumoniae* (PgdA) and of other Gram-positive bacteria, was upregulated during the course of infection (see Table S6 in the supplemental material). A high level of deacetylation is related to resistance of pathogenic bacteria to exogenous lysozyme, produced in abundance on mucosal surfaces by the host (35). Moreover, enzymes with PG deacetylation activity like that of PgdA of *S. pneumoniae* or of the major swine pathogen *Streptococcus suis* have been reported as putative virulence factors (36, 37). In *S. suis*, although the level of deacetylation of PG sugar moieties is very low during *in vitro* growth (less than 1%), the *pgdA* gene expression is upregulated upon interaction with swine neutrophils. The mechanism of resistance to lysozyme by PG deacetylation seems to be favored by *C. difficile* in the mouse model over the recently described mechanism shown to be required for efficient colonization in a hamster model (38) and involving a set of extracytoplasmic function σ factors (CD0677, CD1558, and CD1887). Indeed, expression of these extracytoplasmic function σ factors was not modulated during the course of infection in our experimental model.

Note that CD3463, encoding an alanine racemase (Alr2), was significantly upregulated at 14 and 38 h postinfection, together with CD3464, encoding a conserved hypothetical protein. Alanine racemase catalyzes the conversion of L-alanine into D-alanine, a key component of the bacterial peptidoglycan. However, expression of other genes involved in PG biosynthesis, such as CD1408 and CD1626, encoding D-Ala-D-Ala ligase, was not differentially regulated throughout time, suggesting that D-alanine could be used in a different cell process. One tempting hypothesis is that Alr2 could be present on the exosporium of the *C. difficile* spore, as already described for some bacilli. Indeed, in *Bacillus anthracis*, D-alanine is known to be an inhibitor of germination, and production of D-alanine prevents premature germination of the spore (39). To strengthen this hypothesis, the CD3463 gene was downregulated 100-fold *in vitro* in a *sigE* mutant, inactivated for the mother cell-specific RNA polymerase sigma factor σ^E, compared to the wild type (L. Saujet et al., submitted for publication).

Membrane proteins. In addition to the membrane sugar transporters (see below), the *C. difficile* genome codes for around 200 membrane proteins of known, predicted, or unknown function. Transcription of only a few of the corresponding genes was upregulated during *in vivo* growth (see Table S6 in the supplemental material). Most of these genes code for proteins with little or no homology to proteins in databases. Nevertheless, for some, a function could be assigned provisionally. In fact, the CD0393 gene might encode the S component of an energy-coupling factor (ECF)-type riboflavin transporter (40). Riboflavin is a precursor of essential coenzymes which are involved in oxidative metabolism and other essential processes. The riboflavin uptake systems are mostly ECF-type transporters, with an integral membrane S component as the substrate binding protein, coupled to an energy-coupling module, frequently an AT module (ATP binding cassette [A] associated with the T component, a transmembrane protein). The CD0393 gene is not genetically linked to a gene encoding an AT module, but it was previously shown that S components could use AT modules scattered elsewhere in the genome, particularly in the *Firmicutes* phylum (41). In bacteria, riboflavin could either be synthesized from purine metabolism or the pentose phosphate pathway (42) or be taken from the environment. Interestingly, the expression of the riboflavin biosynthesis pathway (CD1697 and CD1698 to CD1700) was not differentially expressed *in vivo*, while it was moderately upregulated *in vitro* at 14 and 38 h. This suggests that extracellular riboflavin is present in sufficient amounts during the course of mouse colonization, while the riboflavin present in TY medium is quickly consumed by *C. difficile*, which needs to synthesize it *de novo*. Therefore, it is coherent to note that the bifunctional riboflavin kinase/FMN adenylyltransferase (CD1315), which catalyzes the conversion of riboflavin into flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), was also upregulated *in vivo* throughout time, emphasizing the essential role of these cofactors for bacterial growth and viability.

Global metabolism changes during infection. Twenty percent of genes differentially expressed *in vivo* are involved in *C. difficile* metabolic pathways. This somehow reflects specific adjustments of the bacterial cellular metabolism to nutrients and conditions present in the cecum of the axenic mouse. Most of

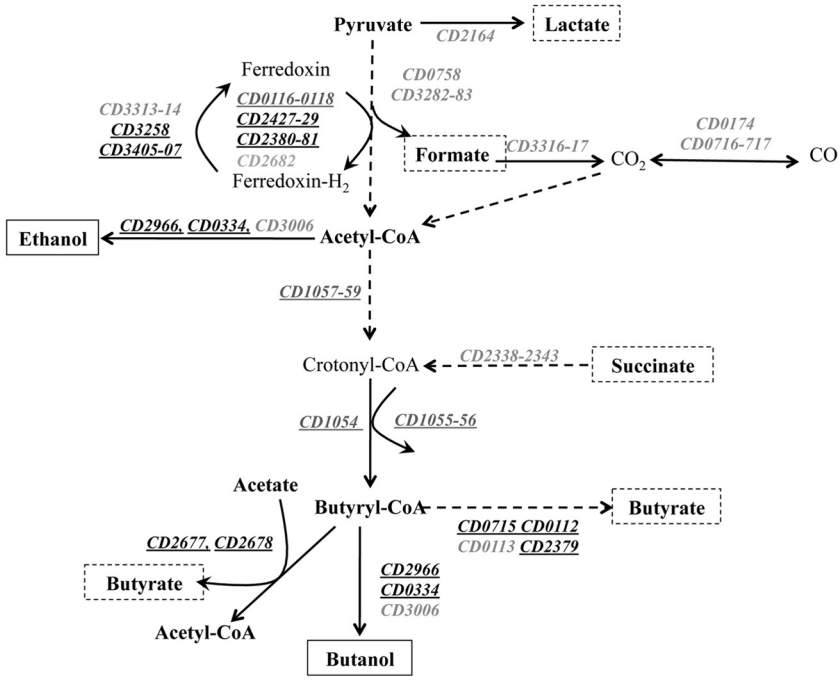

FIG 3 Overview of the fermentation pathway involved in the production of butyrate, butanol, and ethanol. Genes upregulated (in black) or constitutively regulated (in gray) *in vivo* are underlined. Genes that are not expressed are shown in gray and not underlined. Assignments of enzymes to genes which are regulated *in vivo* are as follows: CD3258 and CD3405 to CD3407, iron hydrogenases; CD2427 to CD2429 and CD2380-CD2381, ferredoxin oxidoreductases; CD2966 and CD0334, aldehyde-alcohol dehydrogenases; CD1059, acetyl-CoA acetyltransferase; CD1058, 3-hydroxybutyryl-CoA-dehydrogenase; CD1057, 3-hydroxybutyryl-CoA dehydratase; CD1054, butyryl-CoA dehydrogenase; CD1055-CD1056, electron transfer flavoproteins; CD0116 to CD0118, ferredoxin oxidoreductases; CD0715 and CD0112, phosphate butyryltransferases; CD2379, butyrate kinase; CD2677 and CD2678, succinyl CoA:3-oxoacid CoA transferase subunits A and B.

these genes were upregulated (70%) and are involved in transport and metabolism of lipids and carbohydrates as well as in the fermentation pathways (see Table S3 in the supplemental material).

Compared to *in vitro* conditions, we observed an *in vivo* upregulation of several genes involved in degradation and synthesis of polysaccharides (see Table S3 in the supplemental material). Specifically, we observed an upregulation of a glycosyltransferase gene (CD3350), whose product probably catalyzes glycosyl transfer reactions during the biosynthesis of di-, oligo-, or polysaccharides. Moreover, *glgA* (CD0884) and the *glgC* and *glgD* genes (CD0882-CD0883), whose products together catalyze the biosynthesis of glycogen and regulate the flux of carbon to glycogen, were also upregulated *in vivo* compared to *in vitro* (see Table S3). However, *glgB* (CD2526), which is essential for glycogen accumulation (43), did not appear to be expressed differentially *in vivo* compared to *in vitro*. Thus, *glgB* is either expressed constitutively or not expressed. If *glgB* is not expressed, we cannot exclude the possibility that another enzyme, which remains to be identified, replaces the GlgB activity in *C. difficile*. Nevertheless, the upregulation of the *glgCDA* operon strongly suggests that *C. difficile* accumulates glycogen *in vivo*, providing stored sources of energy and carbon surplus, which might be one of the strategies used by this bacterium to cope with starvation conditions temporarily present during the course of gut colonization.

Indeed, the principal source of energy in *C. difficile* comes from the fermentation of carbohydrates and amino acids. *C. difficile* has a large range of genes dedicated to carbohydrate transport and metabolism (19), suggesting that the ability to use a variety of carbohydrates might be an important feature related to its ability to colonize the host gut. We observed that genes encoding the phosphoenolpyruvate-dependent phosphotransferase system (PTS), which catalyzes the concomitant uptake and phosphorylation of glucose (CD2666-CD2667), were upregulated *in vivo*, supporting the notion that glucose is present in the mouse intestinal tract (44). Interestingly, other specific PTS-encoding genes were upregulated during early (14 h) or late (38 h) colonization, such as those involved in transport of sorbitol (CD0763 to CD0768) or fructose (CD0206 to CD0208), respectively. Inversely, genes encoding maltose-, lactose-, cellobiose-, and glucoside-specific PTS were downregulated *in vivo* compared to *in vitro* (see Table S6 in the supplemental material). This may be explained either by the absence of these PTS sugars in the gut content or by glucose being preferred over these substrates in *C. difficile*.

The final step of the glycolytic pathway leads to pyruvate, which is then metabolized by fermentation pathways for energy production or used for anabolic reactions (Fig. 3). Thus, *C. difficile* can form a variety of fermentation end products, such as lactate, acetate butyrate, and several other short-chain fatty acids, and can produce ethanol and butanol (45). The relative proportions of the different products formed depend on the specific environmental growth conditions. Several genes involved in the fermentation pathways were induced late and differentially expressed *in vivo* compared to *in vitro* (Fig. 3). The pyruvate ferredoxin oxidoreductase (CD2682), which catalyzes the decarboxylation of pyruvate to acetyl-coenzyme A (acetyl-CoA), was not regulated *in vivo*. However, the indolepyruvate ferredoxin oxidoreductase

(CD2380) and the putative ferredoxin oxidoreductases (CD0116 to CD0118 and CD2427 to CD2429), whose substrates have not been identified, were upregulated at 38 h (see Table S3 in the supplemental material) and could substitute for the activity of the pyruvate ferredoxin oxidoreductase. We observed that the operon encoding the enzymes involved in the conversion of acetyl-CoA into butyryl-CoA (CD1054 to CD1059) was constitutively expressed, since this operon was downregulated at 38 h *in vitro* (see Table S3). Concomitantly, we observed that two phosphate butyryltransferases (CD0112 and CD0715) and a butyrate kinase (CD2379), involved in butyrate synthesis from butyryl-CoA, were upregulated *in vivo*. However, butyryl-CoA may also yield butyrate via a butyryl-CoA:acetate-CoA transferase (Fig. 3). In this reaction, butyryl-CoA is exchanged with exogenously derived acetate to generate acetyl-CoA and butyrate. The genes involved in the incorporation of acetate into butyrate (CD2677-CD2678) were similarly upregulated early *in vivo* and *in vitro*, in contrast to the *in vivo* and *in vitro* expression of the butyrate kinase-encoding gene. This suggests that *in vitro*, *C. difficile* seems to preferentially utilize the butyryl-CoA:acetate-CoA transferase route to produce butyrate, while *in vivo C. difficile* also favors the butyryl-CoA:acetate-CoA transferase route but later uses the butyrate kinase during growth. This is consistent with the case for butyrate-producing human gut isolates, covering a wide range of representative clostridial strains, in which the butyryl-CoA:acetate-CoA transferase route is more prevalent than the butyrate kinase route (46). Finally, we found that genes encoding the aldehyde-alcohol dehydrogenase (CD2966), probably involved in ethanol and butanol production from acetyl-CoA and butyryl-CoA, respectively, were expressed more *in vivo* than *in vitro* (see Table S3). Together, the data suggest that alcohol and butyrate pathways are used successively during pyruvate fermentation in the course of *C. difficile* infection. Interestingly, addition of butanol or butyrate suppressed or enhanced toxin production, respectively (45). This is consistent with the late expression of the toxin genes during infection. Moreover, toxin expression was downregulated early, in contrast to that of butanol, but upregulated later (38 h), like that of butyrate. Thus, toxins and pyruvate fermentation pathways are closely controlled by the same regulatory mechanism, such as CcpA, which was recently shown to directly regulate expression of genes encoding toxins and for the key steps of fermentation (47).

C. difficile uses amino acids as an energy source, through a mechanism called Stickland reactions. These reactions consist of the coupled fermentation of two amino acids, in which one acts as an electron donor (Stickland donor) and the other acts as an electron acceptor (Stickland acceptor) (see Fig. S3 in the supplemental material). It has been shown in *C. difficile* that even in the presence of glucose, the availability of amino acids as electron acceptors is a key limiting factor in growth yields (48). This indicates that Stickland reactions might be the main chemical reactions by which *C. difficile* obtains its energy. Among the major Stickland donors (leucine, isoleucine, valine, and alanine), we observed that genes encoding enzymes involved in the biosynthesis of leucine (CD0989 to CD0992) were highly upregulated earlier during infection (14 h) than valine or isoleucine biosynthesis genes (CD1565-CD1566). This suggests that there is a preferred use of leucine in Stickland reactions *in vivo*. Among the two reductases that catalyze the reduction of the main Stickland acceptors (glycine and proline), genes encoding subunits of the D-proline reductase (CD3236 to CD3244) were upregulated during the course of infection, whereas those encoding subunits of the glycine reductase (CD2348 to CD2358) were downregulated (see Fig. S3 and Table S3). Thus, it appears that leucine-proline could be a Stickland pair preferentially used *in vivo* by *C. difficile* to generate ATP.

From the *in vivo* transcriptome analysis, we found several specific metabolic pathways inherent to the infection of the host gut, such as the ethanolamine or *N*-acetylglucosamine catabolism operon. Ethanolamine is a product of the catabolism of phosphatidylethanolamine, an abundant phospholipid in both mammalian and bacterial membranes which is provided by the host dietary intake and the bacterial and epithelial cells in the intestine. Like a variety of bacteria from the gut, *C. difficile* can use ethanolamine as a sole source of carbon and/or nitrogen (19). The *eut* operon (CD1907 to CD1925), involved in ethanolamine utilization, is induced mainly late during the infection process and is strongly downregulated *in vitro* (see Table S3 in the supplemental material). This is in agreement with the probable role of ethanolamine as a carbon and/or nitrogen source in the *C. difficile* gastrointestinal lifestyle (19), which in turn could contribute to pathogenesis by promoting successful gut colonization by *C. difficile*. *N*-Acetylglucosamine is ubiquitous in the biosphere. It is present, for instance, as side chains of gastrointestinal mucins or as a component of bacterial and fungal cell walls. It can also be used as a carbon, energy, and nitrogen source by *C. difficile*, and previously, competition for this nutrient has been proposed as a part of the barrier effect of the colonic microbiota against *C. difficile* (49). The *nagAB* genes (CD1010-CD1011), whose enzymes are responsible for *N*-acetylglucosamine degradation into fructose-6P, a glycolysis intermediate, were expressed more *in vivo* than *in vitro* (see Table S6). Thus, in response to the different access of free glucose or amino acids *in vivo* versus *in vitro*, *C. difficile* has to develop different ways to catabolize complex molecules in the host for its survival.

Expression of sporulation genes *in vivo*. Our *in vivo* transcriptome analysis showed that many genes known to have roles in sporulation were upregulated early after infection (see Table S4 in the supplemental material). The first major morphological manifestation of sporulation is an asymmetric division that divides the sporulating cell into a larger mother cell and a smaller forespore (the future spore) (Fig. 4). The mother cell then engulfs the forespore. At later stages, a layer of peptidoglycan known as the spore cortex is deposited between the inner and outer forespore membranes, and this layer is further encased within a protective proteinaceous coat. Following a period of maturation, the spore is released into the environment through lysis of the mother cell. Spore differentiation follows essentially the same morphological sequence in all endospore-forming organisms that have been examined (50; F. Pereira et al., submitted for publication; L. Saujet et al., submitted).

Sporulation begins when Spo0A is phosphorylated in response to environmental and physiological signals. Once activated, Spo0A-P controls the expression of many genes and operons in the predivisional cell at the onset of sporulation, including those involved in the switch to asymmetric division. A cascade of cell type-specific RNA polymerase sigma factors then controls gene expression in the forespore and in the mother cell. Studies in the model organism *Bacillus subtilis* have shown that σ^F in the forespore and σ^E in the mother cell control early stages of development and that these sigma factors are replaced by σ^G and σ^K, respectively, following completion of engulfment (51). Recent studies

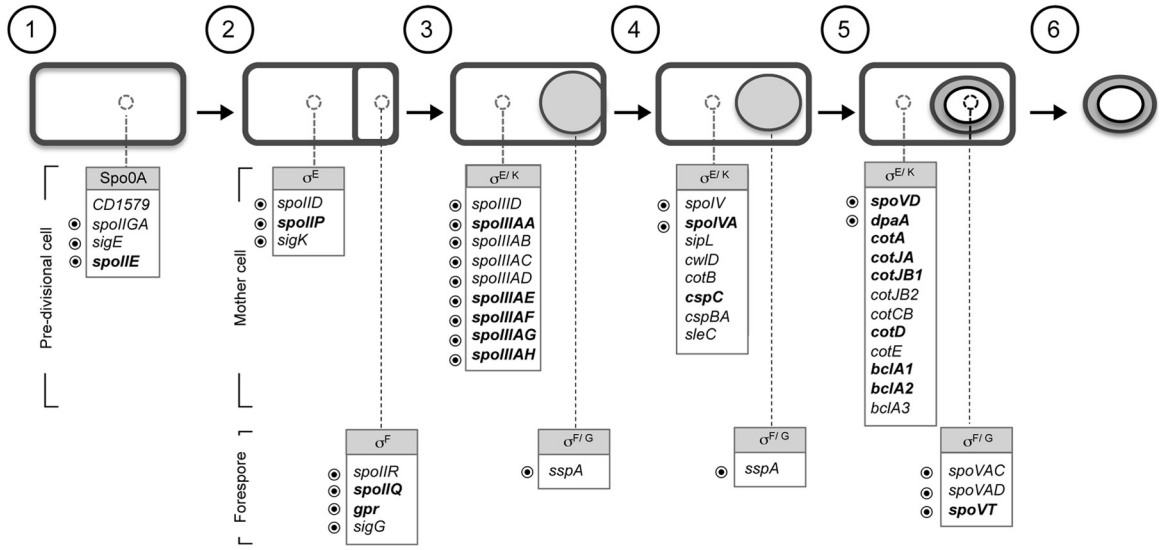

FIG 4 *C. difficile* sporulation gene expression *in vivo*. The schematic representation shows the main stages of sporulation in *C. difficile*. (1) Predivisional cell; (2) asymmetric division; (3) intermediate stage in the process of engulfment of the forespore (the future spore) by the larger mother cell; (4) completion of engulfment; (5) synthesis of the spore surface layers (cortex peptidoglycan, coats, and exosporium); (6) free, mature spore, which resumes growth upon germination. The sporulation genes found to be upregulated in our study are indicated in boxes for each stage of the process, with the presumed cellular compartment where their expression takes place. The genes found to be upregulated *in vitro* are shown in gray, whereas those upregulated both *in vivo* and *in vitro* are shown in black. The genes have been placed along the morphological sequence according to their time of expression following infection of mice. The black dots mark genes that belong to the sporulation core machinery (50).

have established that the main periods of activity of the four cell type-specific sigma factors are maintained in *C. difficile*, which allowed the definition of the genes belonging to each of the cell type-specific sigma factor regulons (F. Pereira et al., submitted; L. Saujet et al., submitted).

In our study, no modulation of the expression of the *spo0A* gene (CD1214) or of CD2492, coding for a potential sensor kinase required for sporulation (52), was detected. Two hypotheses could account for this result: either the expression of *spo0A* was stable over time *in vivo* or *spo0A* was not expressed at the sampling points chosen. However, results of qRT-PCR experiments are in favor of the stable expression of *spo0A* during the course of mouse colonization (see Fig. S4 in the supplemental material). In agreement, expression of many late sporulation genes was detected (see below), and several genes known to be dependent on Spo0A in *B. subtilis* were upregulated at this early time point. Among these genes we found CD3490, which codes for a homolog of the SpoIIE phosphatase (50), involved in the forespore-specific activation of σ^F, and the *spoIIG* operon, coding for σ^E (CD2643) and the membrane-bound SpoIIGA protease (CD2644), required for the proteolytic conversion of pro-σ^E to its active form. Expression of the *spoIIR* gene (CD3564), required for activation of SpoIIGA, was also detected *in vivo* at 8 h. Interestingly, expression of the *spoIIA* operon, coding for σ^F and two other proteins that control its activity (CD0770 and CD0772), was not detected. *spoIIA* is under the joint control of σ^H and Spo0A, whereas both *spoIIE* and *spoIIG* are under the control of σ^A and Spo0A. This suggests that the σ^H regulon, which also includes *spo0A*, is promptly induced *in vivo*, prior to 8 h. In line with this suggestion, we note that the expression of other σ^H-controlled genes, including *ftsZ* (CD2646), required for asymmetric division, was also not detected. Yet expression of several mother cell- and forespore-specific genes was also detected at 8 h, including orthologs of *spoIIQ* (CD0125), *spoIID* (CD0124), and *spoIIP* (CD2469), required for forespore engulfment in *B. subtilis*, and increased early after infection. This suggests that at our first *in vivo* sampling point, engulfment of the forespore by the mother cell was under way. Studies in *B. subtilis* also established that σ^E initiates assembly of the spore cortex and coat layers of the spore (53). Among the σ^E-controlled genes, the SpoIVA ATPase is essential for the biogenesis of these two layers (54), and we found that the *spoIVA* ortholog (CD2629) was strongly induced following 8 h of infection. In addition, the ortholog of *spoVD* (CD2656), a sporulation-specific high-molecular-weight class B penicillin-binding protein essential for synthesis of the spore cortex (55), was upregulated early *in vivo*.

After completion of engulfment, stability of the forespore and continued gene expression in this compartment require expression of the σ^E-dependent *spoIIIA* operon, *spoIIQ* (see above), and the *spoIIIJ* gene (56). The *spoIIIA* operon and *spoIIIJ* are conserved among spore formers, suggesting a central role in (endo)sporulation (50), and their orthologs (CD1192 through CD1199 and CD3678, respectively) were strongly induced *in vivo*. In the forespore, σ^G controls the synthesis of small acid-soluble proteins (SASP), which are required for protection of the forespore chromosome and used as a source of amino acids during spore germination and outgrowth when degraded by a specific protease. The expression of both *sspA* (CD2688) and *sspB* (CD3249), coding for orthologs of two major SASP of *B. subtilis*, as well as expression of the putative SASP protease Gpr (CD2470), was upregulated 38 h after challenge. Synthesis of muramic δ-lactam, a characteristic modification of the cortex peptidoglycan, requires the σ^G-controlled *pdaA* gene, and its *C. difficile* ortholog (CD1430) was upregulated at 14 and 38 h postchallenge. In *C. difficile*, σ^K is essential for synthesis of the spore coat, and presumably also of the exosporium (F. Pereira et al., submitted). The expression of several σ^K-controlled genes for spore coat or exosporium proteins (CD1433,

CD0597, CD2400, CD0598, CD0332, CD3230, and CD3349) (L. Saujet et al., submitted) was strongly upregulated from 14 to 38 h.

In all, the results show that during infection, genes involved in all stages of sporulation are induced early and differentially expressed and/or more strongly expressed in monoxenic mice than their expression in TY medium (see Table S4 in the supplemental material). Similar observations were also reported in the recent transcriptomic study using the pig ligated-loop model (9). Thus, both models suggest that sporulation is strongly and rapidly induced *in vivo*. The strong and early induction of the sporulation genes *in vivo* is consistent with the sporulation rates measured in monoxenic mice versus *in vitro* in TY medium for *C. difficile* 630. Indeed, we observed a gradual increase of spores relative to vegetative cells during infection, from 10% after 8 h of infection to 56%, 67%, and 78% after 14, 24, and 48 h of infection, respectively (see Fig. S4). In contrast, even after 38 h of growth in TY medium, spores did not exceed 1% of the total bacterial counts. TY medium is a low-spore-forming medium, but similar low rates of 630 sporulation were already observed *in vitro* in media such as BHI, in which the sporulation yield after 48 h was less than 1% (54; F. Pereira et al., submitted). Therefore, our results suggest that sporulation is rapidly induced *in vivo*, in line with the view that spores are important for the persistence of *C. difficile* in the host gut (and therefore linked to disease relapse) and for spreading in the environment (27, 54). Interestingly, the sporulation rates are lower in human microbiota-associated mice than in the monoxenic mouse model (data not shown), in agreement with the data obtained for conventional mice by Deakin and colleagues (57). Presumably, the presence of a complex microbiota can modulate the intestinal environment, i.e., the immune and inflammatory responses (16, 17) or the chemical conditions of the gut, and influence sporulation (58).

Stress responses. During the course of infection, *C. difficile* encounters and must adapt to several environmental changes. Adaptation to these stressful environments requires a wide range of proteins, whose expression is generally tightly regulated and coordinated. Several features of the *C. difficile* stress responses can be inferred from our transcriptomic study.

The ability to acquire sufficient iron from the immediate environment is vital for the majority of prokaryotes. It has been shown that *C. difficile* is able to use both inorganic ferric and ferrous iron as well as organic sources of ferric iron (59). In its genome, we found several, possibly redundant, potential iron acquisition systems, i.e., 3 ABC-type transport systems (CD1647 to CD1649, CD2997 to CD2999, and CD3525 to CD3530) and 3 Feo-type systems (CD1477-CD1478, CD1517-CD1518, and CD3273-CD3274), involved in ferric and ferrous iron uptake, respectively (60). Only modulation of transcription of genes encoding the ferrous iron acquisition Feo systems was observed, with their levels either decreasing (CD1477-CD1478 and CD3273-CD3274) or increasing (CD1518) during the course of infection (see Table S6 in the supplemental material). The Feo systems, which in some enteric bacteria contribute to efficient gut colonization (61), are formed by at least two proteins: the large transmembrane protein FeoB and the small FeoA protein (60). FeoB acts as the permease through which ferrous iron is transported to the cell and displays a weak GTPase activity in its N-terminal domain. Surprisingly, in the Feo operon (CD1517-CD1518), only the *feoA* gene, CD1518, was strongly upregulated *in vivo* at 14 and 38 h. The exact role of the small cytoplasmic FeoA protein is not fully understood, but it was hypothesized from a recent study of *Salmonella enterica* serovar Typhimurium that FeoA could serve as an activator of GTPase activity of FeoB (62). This suggests that *in vivo*, the regulation of iron uptake could be controlled only by increasing expression of a single gene encoding a small protein, which is less energy consuming for bacteria. The increased cell requirement in iron must be related not only to the rise of the bacterial population observed in the cecum, from 4.4×10^7 CFU/g of feces at 8 h to 1.2×10^9 CFU/g of feces at 38 h of infection (see Table S2), but also to *de novo* synthesis of iron-dependent proteins such as EutG (ethanolamine iron-dependent alcohol dehydrogenase; CD1907), a putative ferredoxin oxido-reductase (CD0115), and a rubrerythrin (CD2848), all of whose expression was upregulated late during infection.

Little is known about the *C. difficile* response to oxidative stress, caused by reactive oxygen species (ROS). Among the most toxic ROS, hydrogen peroxide (H_2O_2) is generated intracellularly by exposure of bacteria to oxygen partially reduced by a superoxide dismutase, or extracellularly by the inflammatory process in the gut.

The genome of *C. difficile* 630 encodes several proteins putatively involved in the oxidative stress response (19), including a putative manganese superoxide dismutase (CD1631), several putative manganese catalases (CD1567, CD0598, and CD2401) (even though vegetative cells of *C. difficile* display no catalase activity), a putative thioredoxin-dependent thiol peroxidase (CD1822), and four rubrerythrins or rubrerythrin-like proteins (CD0825, CD1474, CD1524, and CD2845). The last proteins have been shown to display NADPH peroxidase activity and are broadly distributed among microaerophilic (i.e., *Campylobacter*) and anaerobic bacteria (63).

We observed from our *in vivo* transcriptomic study an increased transcription of the superoxide dismutase (Mn) (CD1631), potentially leading to an increased H_2O_2 level. Since the rubrerythrin encoded by CD2845 was significantly upregulated at 14 and 38 h postinfection (see Table S6 in the supplemental material), we suggest that this protein may have an important role in H_2O_2 detoxification and, by extension, in protection from oxidative stress in *C. difficile*. The same observation was made in *Clostridium acetobutylicum*, where transcription of genes encoding several rubrerythrin-like proteins quickly increases after exposure to oxygen and H_2O_2, suggesting a significant contribution to protection against oxidative stress (64). In the Gram-negative anaerobic bacterium *Porphyromonas gingivalis*, the rubrerythrins have also been shown to scavenge H_2O_2, but they are also possibly involved in protection against reactive nitric species (RNS), thus playing a role in nitrosative stress responses encountered during host inflammatory responses to gastrointestinal infection (65).

The same pleiotropic role of the rubrerythrins can occur in *C. difficile*. Actually, the recent proteomic data obtained on *C. difficile* suggested an overlapping role of the rubrerythrins in response to stresses such as heat or acid/alkali stress (7, 66). Although the function of rubrerythrins in the overall *C. difficile* stress response cannot be established accurately, our data, in addition to others (7, 66), are consistent with a role of the rubrerythrin proteins in host adaptation.

Oxidative stress conditions could promote lesions in proteins and nucleic acids. In particular, H_2O_2 damages DNA through the Fenton reaction. Oxidative stress conditions also lead to many metabolic failures due to inactivation of dehydratases and nonre-

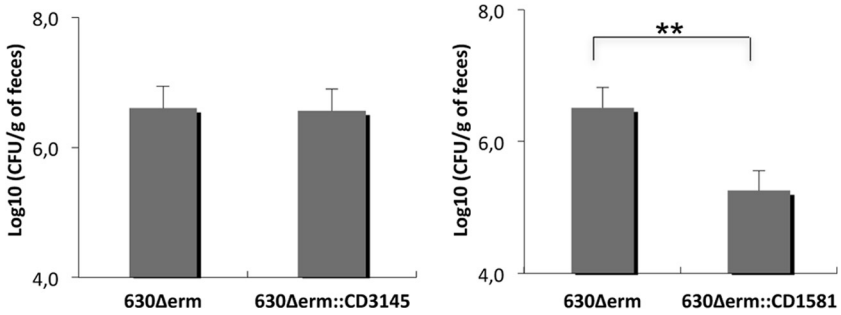

FIG 5 Competitive colonization assays between the parental wild-type strain and the 630 Δ*erm*::*CD1581* and 630 Δ*erm*::*CD3145* mutant strains. Mice were orally challenged with 5×10^5 CFU of both the WT and mutant strains. The number of bacteria in the feces was determined at 38 h postinfection. Eight mice were used for each competition experiment. Statistically significant differences compared to the wild type are indicated by asterisks (**, $P < 0.005$).

dox mononuclear enzymes (63). We observed a strong upregulation *in vivo* of genes involved in the metabolism of nucleic acids, and particularly of *recA* and *ssb* (CD1328 and CD3235), while they were downregulated *in vitro*. These genes are essential for the repair and maintenance of DNA, suggesting an important activity in recombination and repair of replication forks during the infection process.

Induction of regulators during infection. The genome of *C. difficile* contains many genes encoding transcriptional regulators (11% of the CDSs), which include more than 40 two-component regulatory systems (19). Surprisingly, very few regulatory genes were differentially expressed *in vivo* (0.3%). Among them, we found only one two-component system (CD0668-CD0669) and six orphan transcriptional regulators (CD2640, CD2214, CD0670, CD2048, CD0693, and CD2444), which were upregulated *in vivo* compared to *in vitro* (see Table S6 in the supplemental material). The expression of the genes encoding the two-component system and the orphan regulator CD0670 were regulated similarly to the toxin genes during infection. Interestingly, these genes are genetically linked on the chromosome, immediately downstream of the locus of pathogenicity (PaLoc). Among the other transcriptional regulators that were significantly upregulated *in vivo*, we found NrdR and SinR (CD2640 and CD2214, respectively) and regulators belonging to the MerR and RpiR families (CD0693 and CD2048, respectively). NrdR negatively regulates expression of the ribonucleotide reductases (RNRs), which provide the only *de novo* pathway for the biosynthesis of deoxynucleoside triphosphates (dNTPs) for DNA synthesis and repair (67). RNRs catalyze the controlled reduction of all four ribonucleotides to maintain a balanced pool of dNTPs during the cell cycle (68). These processes enable the cell to rapidly adapt to environmental changes such as oxygen tension. Finally, a transcriptional regulator of the MerR family (CD0693) was the most upregulated transcriptional regulator during infection (4.7-fold at 14 h). Members of the MerR family respond to environmental stimuli such as oxidative stress, heavy metals, or antibiotics. In *C. difficile*, the gene encoding the MerR-like regulator was also regulated early, suggesting that many stimuli should intervene *in vivo* to induce the rapid transcription of such a regulator.

Use of the *in vivo* transcriptomic approach to identify new *C. difficile* virulence factors. Bacterial genes with enhanced expression in the cecum could contribute to the survival of bacteria *in vivo* and to the progression of disease. Thus, the *in vivo* transcriptomic approach could be used to identify genes that encode proteins that may play a role in the infectious process. Such a strategy was successfully applied to the study of several pathogenic bacteria (6, 20) and led to the identification of a number of genes of unknown function whose expression was enhanced *in vivo*. Interestingly, we observed from our study that up to one-sixth of the *in vivo* upregulated genes encode proteins of unknown function, and some of them are highly expressed early and/or late in the mouse model (see Table S5 in the supplemental material). These genes might have a role in the adaptation of *C. difficile* during colonization or might function in an uncharacterized infection mechanism.

We next wanted to test whether our transcriptomic approach permitted the identification of new *C. difficile* virulence genes among those expressed early in or throughout infection. We hypothesized that genes showing high levels of expression *in vivo* would be good candidates for this analysis. Therefore, using the ClosTron system (10), we inactivated 2 genes that were moderately or highly regulated early or late during infection compared to their *in vitro* expression and that encoded proteins whose functions were not yet characterized. One of the disrupted genes codes for the CD3145 protein, a putative surface-associated protein that showed increased expression at an early time of colonization that may be related to its early role in the colonization process. The second gene codes for a protein of unknown function (CD1581) and was the most highly expressed gene *in vivo* throughout the duration of the experiment compared to any point considered *in vitro*. The CD1581 gene is found downstream of the gene for a putative homoserine dehydrogenase (Hom2) and just upstream of the gene for a putative two-component histidine kinase (HisD). The CD1581 gene itself codes for a small cysteine-rich protein that may be unstructured (not shown). Therefore, the genetic context of CD1581 hints at no functional relationship to the genes in its close vicinity. Moreover, CD1581 showed a much higher expression level *in vivo* than those of any of the genes in its vicinity (see Fig. S4 in the supplemental material), strongly suggesting that CD1581 is expressed independently of them, at least *in vivo*. The ability of these mutants to colonize the cecum was investigated by a competitive assay in a dixenic mouse model with the original wild-type strain 630 Δ*erm* and the isogenic mutant strains, enabling a direct fitness comparison *in vivo*. The number of bacteria in the feces was scored at 38 h postinfection. We observed no significant difference in the fitness colonization of 630 Δ*erm* and 630 Δ*erm*::*CD3145* (Fig. 5). This result is surprising, since the CD3145 protein was recently described as a collagen binding pro-

tein potentially involved in adhesion of *C. difficile* to the colonic epithelium (69), but it attests the multifactorial nature of the colonization process. In contrast, the bacterial counts for the 630 Δ*erm*::*CD1581* strain were significantly lower (≈1.8 log; $P < 0.005$) than those for the wild-type strain 630 Δ*erm* (Fig. 5). The *in vitro* growth rate of the mutant strain in BHI was comparable to that of the WT whether the mutant was grown alone or in competition with the WT (data not shown). This indicated that CD1581 might have a role in the ability of *C. difficile* to colonize the mouse cecum. In addition, the level of toxin synthesis detected from cells grown in TY medium was comparable to that of strain 630 Δ*erm*, and disruption of the CD1581 gene did not affect formation of heat-resistant spores *in vitro* (data not shown). Thus, the genetic inactivation of CD1581 results in a reduced fitness by a mechanism that does not involve modulation of growth or heat-resistant spore formation.

Complemented strains could not be analyzed *in vivo*, because a lack of antibiotic selection *in vivo* leads to loss of the plasmid during the infection process. This was already discussed when the *fliC* and *fliD* mutant strains were complemented (32). Additional work will be necessary to confirm the impact of CD1581 on the colonization process, which could be helped by the two-step allele-exchange technique recently developed for *C. difficile* (70). However, due to the monocistronic organization of CD1581, the reduced colonization phenotype observed in the mutant is unlikely due to a polar effect related to the intron insertion. In any event, although the real function *in vivo* of CD1581 requires further investigation, this analysis allowed the detection of potential novel colonization genes, thus establishing the value of the *in vivo* transcriptomic strategy. More than 40 genes of unknown function were found to be expressed differentially *in vivo* compared to *in vitro*. Their characterization is an important goal for future investigations.

Conclusions. All specific microbial factors that contribute to the success of the infection process should be considered virulence factors. This includes colonization factors such as adhesins, which tightly mediate the binding of bacteria to mucosal surfaces, factors that favor nutrient adaptation in the host microenvironment, and the mechanisms involved in stress resistance and/or sporulation, which contribute to resistance to host defenses and help in the persistence of *C. difficile*. The bacterial transcriptome is a dynamic entity that reflects the overall modification of bacterial gene expression in response to environmental changes and therefore contributes to a comprehensive understanding of the molecular processes leading to infection. Our data provide important new insights into strategies used by *C. difficile* to adapt to and persist in a gut environment. The late downregulation of some of the major adherence factors, such as the S-layer proteins, suggests that adhesion to mucosal epithelium is an early step of the infection that allows the establishment of *C. difficile* in its niche. Once set up, the organism seems to multiply *in vivo* continuously, in contrast to *in vitro* growth, as showed by the stable expression of cell factors throughout time. The large range of genes dedicated to carbohydrate transport and metabolism in the genome of *C. difficile* is certainly an important feature for host gut colonization. This was illustrated in this study by the probable switch from glucose to sorbitol uptake (upregulated at 14 h postinfection) and, further, to ethanolamine utilization (upregulated at 38 h postinfection) (see Table S4 in the supplemental material). Our data also underscore the importance of the *C. difficile* responses to different stresses that bacteria encounter in the gut, such as iron limitation or oxidative stress, which must be considered essential for its survival. Interestingly, we observed that the toxin-antitoxin (TA) system (CD3461-CD3462), which is homologous to the *E. coli* MazEF TA system, was also regulated *in vivo*. CD3461 encodes the MazF-cd stable toxin, with an endoribonuclease activity, and CD3462 encodes the MazE-cd unstable antitoxin (71). During the course of infection, *mazF-cd* is upregulated, although *mazE-cd* remains stably expressed over time. Moreover, the two genes are downregulated *in vitro*. Interestingly, the ClpC ATPase (CD0026) of the Clp complex, which has been proposed to participate in the regulation of the TA system by the degradation of the antitoxin (72), is upregulated at 38 h, suggesting that *in vivo*, the toxin/antitoxin ratio could be favored. Although the functional relevance of these systems is still subject to debate, the specific *in vivo* expression of the *C. difficile* TA system suggests a role in the virulence of the bacteria, facilitating bacterial survival during stresses, the persistence process, or niche-specific colonization, as proposed for other pathogenic bacteria (73).

Overall, our results are in agreement with the study of Scaria and coauthors (9), but some differences were observed between the two studies. Among them were differences in the timing of toxin production, PG biosynthesis, the production of surface proteins, and some carbohydrate degradation pathways, which are undoubtedly related to the specific features of the animal model and of experimental procedures used. To illustrate how both models can affect gene expression, we observed that for one of the two prophages carried on the *C. difficile* 630 genome (CD2889 to CD2952), the genes were not significantly modulated in our mouse model, whereas they were upregulated in the pig ligated-loop model (9). This discrepancy may be explained by the lack of antibiotic treatment in our model, since some antibiotics are able to trigger phage induction *in vivo* (73). Moreover, a recent study pointed out that *C. difficile* infection following antibiotic treatment promotes prophage induction (74).

One of the main findings of this study was the rapid induction of the sporulation process *in vivo* in our *C. difficile* monoassociated mouse model. In the pig ligated loop, which replicates the more complex environment that *C. difficile* encounters during natural infection in humans, genes involved in the sporulation cascade were also upregulated as early as 4 h after infection. Thus, it appears that signals triggering *C. difficile* sporulation are not exclusively associated with the microbiota activity or the lack of nutrients, since the mice were fed *ad libitum* during the duration of the experiment, without clinical signs that could cause a loss of appetite. Although the influence of both host factors and the microbiome on the germination of *C. difficile* spores has been studied (75), little is known about their impact on the sporulation process. Comparison of conventional and monoassociated mouse models should be of great interest for deciphering the complex signals of the host and the microbiome that promote *C. difficile* sporulation. Note that several of the sporulation genes detected code for enzymes involved in spore germination: *cspBAC* (CD2247-CD2246) and *sleC* (CD0551) encode enzymes required during germination for initiation and complete cortex hydrolysis (76). These enzymes associate with the spore at a late stage in development. Because they were upregulated *in vivo* at 14 h postchallenge, it is possible that a large fraction of the population had reached a late stage of sporulation at that time.

Finally, our work led us to identify one potential new coloni-

zation virulence factor (CD1581), confirming that we can use the *in vivo* transcriptomic approach to find new *C. difficile* virulence factors, and by extension highlighting the potential role of the conserved hypothetical proteins that are highly and differentially upregulated *in vivo* in regard to the *in vitro* transcriptome. Further analysis of these proteins will certainly enable the identification of new factors involved in the host adaptation process and/or virulence of *C. difficile*.

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AUTHOR CORRECTION

Adaptive Strategies and Pathogenesis of *Clostridium difficile* from *In Vivo* Transcriptomics

Claire Janoir, Cécile Denève, Sylvie Bouttier, Frédéric Barbut, Sandra Hoys, Laxmee Caleechum, Diana Chapetón-Montes, Fátima C. Pereira, Adriano O. Henriques, Anne Collignon, Marc Monot, Bruno Dupuy

EA 4043, Université Paris-Sud 11, Faculté de Pharmacie, Châtenay-Malabry, France; Centre National de Référence de *Clostridium difficile*, Service de Microbiologie, Hôpital Saint-Antoine, Assistance Publique-Hôpitaux de Paris, Paris, France; Laboratoire Pathogenèse des Bactéries Anaérobies, Département de Microbiologie, Institut Pasteur, Paris, France; Unité de Recherche et d'Expertise Bactéries Anaérobies et Toxines, Département de Microbiologie, Institut Pasteur, Paris, France; Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal; Service de Microbiologie, Hôpital Jean Verdier, Assistance Publique-Hôpitaux de Paris, Paris, France

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