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Highlight

Clostridium difficile: A bad bug goes into defensive mode

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Clostridium difficile was first isolated in 1935 from the stool sample of a healthy infant and was originally described as *Bacillus difficilus* (Hall and O'Toole, 1935). The difficulties experienced during the isolation and maintenance of this microorganism in the laboratory stuck with its name when it was re-classified as a member of the genus *Clostridium*. Recent taxonomic considerations that are based upon an expanded 16S gene sequence hierarchical framework (Collins *et al*., 1994) lead to its further re-classification as *Clostridioides difficile* (Lawson *et al*., 2016). This name was chosen to reflect (i) its similarity to *Clostridium* (*Clostridioides* = organisms similar to *Clostridium*) but (ii) without causing wide-ranging ramifications that would ensue when the use of the well-established abbreviations (*C. difficile*, or *C. diff*) for its name would no longer be possible in commercial and clinical setting (Lawson *et al*., 2016). This will happen if the proposal to taxonomically affiliate *C. difficile* with the genus *Peptoclostridium* is followed (Yutin and Galperin, 2013).

Leaving taxonomic considerations and controversies aside, *C. difficile* is a rising star among unsavory microorganisms, causing hundreds of thousands of infections and thousands of deaths and burdening European and North American health care systems with billions of dollars for its treatment. About half a million cases of infections with *C. difficile* are estimated for the United States alone for the year 2011, leading to about 29 000 deaths and costs of 4.8 billion dollars for acute care facilities (Lessa *et al*., 2015). This dire situation is acerbated by the appearance of hyper-virulent variants of *C. difficile* that spread into human populations and the increase in the number of strains resistant to commonly used antibiotics (Abt *et al*., 2016; Dingle *et al*., 2017). The determination of a very large number of *C. difficile* genome sequences paints a picture of a rather diverse gene content of this species, with an estimated pan-genome of about 9600 genes but only a restricted (15–20%) core genome (Knight *et al*., 2015). The genus-level core genome includes about 550 protein families. Based on these data, a metabolic network comprising proteins, RNAs and metabolites has recently been constructed that is crucial for a deeper understanding of the varied biology and pathogenic potential of members of the genus *Clostridium* (Udaondo *et al*., 2017).

C. difficile is a Gram-positive anaerobic spore-forming rod-shaped bacterium (Fig. 1) that can be found both in terrestrial and marine ecosystems and in the mammalian intestinal tract. Most human infants are colonized with it without exhibiting any negative symptoms, and the number of *C. difficile* carriers subsequently drops to about 3% in healthy adults (Bartlett and Perl, 2005). However, in hospital settings, a very large percentage of patients (20–40%) are carriers of *C. difficile*, and the ability of *C. difficile* to form highly stress- and desiccation-resistant endospores (Fimlaid and Shen, 2015; Shen, 2015; Bhattacharjee *et al*., 2016) certainly contributes greatly to its dissemination in this environment and to the ensuing infection cycle (Abt *et al*., 2016). As a enteropathogen, *C. difficile* is a major cause of antibiotic-treatment-associated diarrhoea and the potentially deadly disease pseudomembranous colitis (Abt *et al*., 2016). Although great attention is focused on the considerable number of hospital acquired infections, the majority of reported cases of *C. difficile* infections actually occur outside clinical settings and in the absence of antibiotic use (Warriner *et al*., 2017), a treatment that fosters the colonization of the intestine by *C. difficile* (Shen, 2015; Abt *et al*., 2016). The sources of community-acquired *C. difficile* infections are open for debate but food-based reservoirs seem likely (Warriner *et al*., 2017). Since *C. difficile* is a strict anaerobe that used Stickland reactions for the generation of its energy, its metabolism needs to be carefully taken into considerations when issues related to its persistence in the environment, infection, and virulence are discussed (Bouillaut *et al*., 2015).

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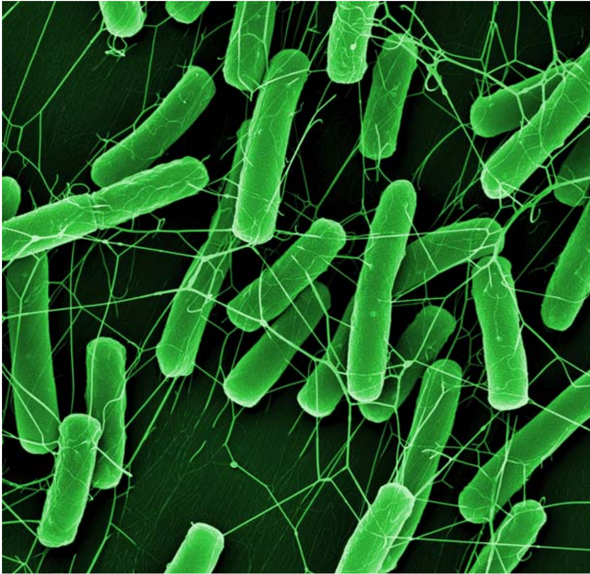

Fig. 1. Scanning electron micrograph of *C. difficile*. The primary magnification is ×8000; the micrograph was taken and kindly provided by M. Rohde (Helmholtz Centre for Infection Research, Braunschweig, Germany).

Undisturbed consortia of intestinal microbiota provide protection against *C. difficile* but the disruption of their composition and the reduction in number of gut microorganisms (e.g. through antibiotic treatment) allows *C. difficile* to flourish (Theriot *et al*., 2014; Shen, 2015). Changes in metabolite pools of microbiota allow the germination of the ingested *C. difficile* spores and the colonization of the intestinal tract by vegetative cells; bile acids (e.g. taucholate) serve as a specific trigger for spore germination by *C. difficile* (Shen, 2015; Abt *et al*., 2016; Bhattacharjee *et al*., 2016; Stoltz *et al*., 2017). Efforts to elucidate the sporulation network of *C. difficile* and defining cues for spore germination profited greatly from extensive studies on these topic with *Bacillus subtilis* (de Hoon *et al*., 2010; Higgins and Dworkin, 2012; Setlow, 2014) and established in both bacterial species the central role of the transcription factor Spo0A in controlling sporulation. However, the genetic wiring of the signal transduction cascade leading to the Spo0A-dependent onset and progression of this cellular differentiation process to spore-formation and subsequent spore germination clearly differs between the two species (Fimlaid and Shen, 2015; Bhattacharjee *et al*., 2016). Taking advantage of this information, bile acid analogues are currently evaluated to specifically inhibit germination of *C. difficile* spores, thereby minimizing the suppression of growth and colonization of health-promoting gut microbiota that occurs through treatment with antibiotics (Stoltz *et al*., 2017).

Once vegetative *C. difficile* cells have developed from the ingested dormant spores (Bhattacharjee *et al*., 2016), they colonize the intestinal tract and their proliferation in compromised gut ecological niches causes intensive local inflammatory processes (Peniche *et al*., 2013; Shen, 2015; Abt *et al*., 2016; Jose and Madan, 2016). *C. difficile* produces two toxins (TcdA, TcdB) that are delivered into host cells and that cause deactivation of Rho- and Ras-family GTPases via glycosylation and eventually lead to depolymerization of the cytoskeleton of intestinal epithelial cells and their subsequent death. A third, binary toxin (CDT) that is produced by a substantial subgroup of *C. difficile* strains, also contributes to the inflammatory process and acts as an actin-specific ADP-ribosyltransferase whose activity results in the destruction of the cytoskeleton as well (Aktories, 2015; Abt *et al*., 2016; Janoir, 2016; Martin-Verstraete *et al*., 2016). The disruption of the cytoskeleton causes the disassociation of tight junctions between colon epithelial cells, and necrosis provides an opportunity for *C. difficile* to overcome the barrier-function of the gut epithelium and allows it to spread throughout the body (Abt *et al*., 2016; Janoir, 2016). The ensuing activation of the host inflammatory responses and sepsis is often life threatening. Chronic gut infections (colitis) by *C. difficile* are difficult to combat through treatment with antibiotics but fecal stool transplants seem to offer new therapeutic opportunities as these help to re-establish a healthy gut microbiome that in turn will restrict growth and long-term colonization by the pathogen (Yurist-Doutsch *et al*., 2014; Shen, 2015).

While the toxins produced by *C. difficile* are certainly prime virulence determinants (Martin-Verstraete *et al*., 2016), other factors such as adhesins, surface layer proteins, fibronecting-binding proteins, and flagella also seem to contribute to the colonization process of the intestine by *C. difficile*. Metabolic adaptations aid this pathogen in conquering ecological niches in this challenging environment (Bouillaut *et al*., 2015; Shen, 2015; Janoir, 2016). Great strides have been made in understanding the roles played by toxins, the microbiome, and host immunity for *C. difficile* infection and pathogenesis (Peniche *et al*., 2013; Aktories, 2015; Abt *et al*., 2016; Janoir, 2016; Martin-Verstraete *et al*., 2016), but much still needs to be learned. In particular, further insight is urgently needed to elucidate additional virulence and colonization factors used by *C. difficile* during the infection process and to uncover the molecular mechanisms that allow this pathogen to perceive and respond to environmental and nutritional cues in the gut and to persist in food and in terrestrial and marine ecosystems.

In a recent issue of *Environmental Microbiology*, Kint *et al*. (2017) have studied the impact of the stress-responsive alternative sigma factor SigB on the transcriptional profile of *C. difficile* on a genome-wide scale and the role played by it for the pathophysiology of *C. difficile* (Kint *et al*., 2017). This study opens a new window into a deeper

understanding of the biology and stress responses of this medically very important human pathogen and provides a rich blueprint for further investigations. SigB is the central regulator of the general stress response system that operates in many Gram-positive bacteria (Hecker *et al*., 2007). Its induction provides pre-emptive stress resistance to a variety of either environmentally or cellular imposed constraints on growth and survival and battle-hardens cells in the face of uncertainty (Hecker *et al*., 2007; de Been *et al*., 2011; Guldimann *et al*., 2016). The genetics, physiology, and signal transduction mechanisms of the SigB-controlled general stress response system have most intensively been studied in *B. subtilis* (Hecker *et al*., 2007; Price, 2011; Pane-Farre *et al*., 2017), but these processes have also been explored in the important human pathogens *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus* (Bischoff *et al*., 2004; van Schaik *et al*., 2007; van der Veen and Abee, 2010; Mäder *et al*., 2016).

The SigB regulon of *B. subtilis* comprises about 200 genes and a variety of environmental (e.g. ethanol and salt exposure, some antibiotics, oxidative conditions, blue light, or growth temperature extremes) and cellular (e.g. energy limitation) stress conditions are known to trigger the coordinated transcriptional induction of the entire regulon (Nannapaneni *et al*., 2012; Nicolas *et al*., 2012). With the notable exceptions of sustained exposure to growth-restricting low or high growth temperatures (Brigulla *et al*., 2003; Holtmann *et al*., 2004), the environmentally dependent transcriptional activation of SigB-regulated genes in *B. subtilis* is typically short-lived, and consists of a single adaptive activity pulse whose amplitude depends on the rate at which the stress is increased (Young *et al*., 2013). Activation of the SigB-regulon in *B. subtilis* is coordinated by the stressosome, a cytoplasmic particle which acts as a signal integration and signal transduction hub (Pane-Farre *et al*., 2017). This 1.8 Megadalton supra-molecular complex forms a truncated icosahedron and comprises a number of signal-perception and signal transduction proteins (Marles-Wright *et al*., 2008) that communicate with each other through phosphorylation and dephosphorylation reactions. These events trigger structural transitions within the stressosome that eventually afford the liberation of SigB in a transcriptionally active form from a prior formed transcriptional inactive complex (Marles-Wright *et al*., 2008; Pane-Farre *et al*., 2017). It is beyond the scope of this *Opinion* contribution to detail the current status of understanding of this remarkable super-complex. I refer the interested reader to enlightening and thought-provoking overviews on this topic and point out that the composition of the stressosome and the way the cell relays information to it varies between different species (Hecker *et al*., 2007; de Been *et al*., 2011; Price, 2011; Pane-Farre *et al*., 2017). It suffices to state here that the signal-perception and transduction mechanism within the stressosome depends on a partner-switching modus of regulatory proteins that ultimately lead to the release of SigB from its anti-sigma-factor RsbW. SigB is then free to assemble with core RNA-polymerase to direct gene expression on a global scale. In *C. difficile* (Kint *et al*., 2017), the *sigB* gene is present in a genetic locus that also contains the gene for its anti-sigma factor SigW and the anti-anti-sigma Factor RsbV, a protein that traps SigW when the signal transduction cascade in the stressosome triggers the release of SigB from SigW (Hecker *et al*., 2007; de Been *et al*., 2011; Price, 2011; Guldimann *et al*., 2016).

The major aim of the study directed by Isabelle Martin-Verstraete (Institut Pasteur & Universite Paris Diderot; France) on the role played by SigB during gut infections by *C. difficile* was to elucidate processes and regulatory circuits that allow this pathogen to induce protective, detoxification, and repair systems to foster its survival in the gut (Kint *et al*., 2017). To this end, Kint *et al*. (2017) have compared the growth, physiology and transcriptional profile of an isogenic pair of *sigB*$^+$ and *sigB* mutant strains at the onset of the stationary phase, conditions that lead to the activation of SigB. The authors have complemented these studies by exploring the ability of the *sigB* mutant to colonize the intestinal tract of mice. Taken together, the data derived from these studies provide solid support for the notion that SigB and members of the general stress regulon are critically involved, in a multi-factorial manner, in the colonization of the intestinal tract by *C. difficile* and the management of different types of stresses the entero-pathogen might encounter in this environment (Kint *et al*., 2017). Importantly, Kint *et al*. (2017) found that SigB does not affect the expression of the genes for the TcdA and TcdB toxins present in the strain they studied. Hence, SigB influences pathophysiology and gut colonization by *C. difficile* in ways that are different from those afforded by the TcdA and TcdB virulence factors (Aktories, 2015; Abt *et al*., 2016; Janoir, 2016; Martin-Verstraete *et al*., 2016).

As observed for other firmicutes (Hecker *et al*., 2007; Guldimann *et al*., 2016), SigB is dispensable for growth in standard laboratory rich media [e.g. tryptone-yeast extract (TY)] used to propagate *C. difficile*. However, the survival rate of the *sigB* mutant in TY medium was much more strongly impaired then its isogenic *sigB*$^+$ parent strain (Kint *et al*., 2017), consistent with the function of SigB as a stationary phase sigma factor. Kint *et al*. (2017) then went on to conduct a transcriptional profiling study under these growth conditions and found that approximately 25% of all *C. difficile* genes were differentially affected in the *sigB*$^+$/*sigB* pair of strains; 595 genes were up-regulated and 410 genes were down-regulated in a SigB-dependent manner (Kint *et al*., 2017). This is a rather large fraction of the gene content of the *C. difficile* strain studied by the authors given that, for instance, the SigB-regulon of *B. subtilis*

comprises only about 200 genes (Nannapaneni *et al*., 2012; Nicolas *et al*., 2012). Kint *et al*. (2017) used bioinformatics to extract a consensus sequence for 410 *C. difficile* genes positively affected by SigB. While such a consensus sequence could be derived, not all genes affected in their transcriptional profile in the *sigB* mutant apparently seemed to possess a promoter adhering to this SigB-consensus sequence (Kint *et al*., 2017). This raises questions about indirect effects in the data set derived from the *sigB*$^+$/*sigB* array experiment, which might plague the author's efforts to precisely define the genetic boundaries of the *C. difficile* SigB-regulon. Some of these indirect effects might stem from the existence of sub-regulons whose expression might be indirectly affected by SigB. Clarity in these issues might come from follow-up studies using growth conditions that will lead to a very strong induction of the SigB response (e.g. treatment with ethanol or severe salt shocks), an approach that was highly rewarding in the precise definition of the genetic boundaries of the SigB-regulon from *B. subtilis* (Nannapaneni *et al*., 2012; Nicolas *et al*., 2012).

If one takes the reported data from the transcriptional profiling study at face value, the global and multi-factorial involvement of the general stress response regulator SigB in stress management and intestinal colonization by *C. difficile* becomes apparent. These processes include cell envelope and cell wall homeostasis, central metabolism, DNA repair and resistance against bile salts, defense against acid stress, resistance against reactive oxygen species and oxidative stress, thiol homeostasis and assembly of Fe-S clusters. I do not want to rehash here in detail all the interesting findings from the comprehensive array study relevant for these processes, but I would like to highlight three issues that in my view command particular attention when one reads the paper by Kint *et al*. (2017).

(i) *C. difficile* is regarded as a strict anaerobe. Kint *et al*. (2017) found that it can tolerate O_2 concentrations below 1% and they found that SigB contributes directly to oxygen-mediated stress tolerance by regulating genes that are known to be involved in O_2 detoxification in other anaerobes. This is, from a physiological point of view, a rather notable finding since the SigB-mediated general stress response system has so far only been studied in any level of detail in aerobic microbes (Hecker *et al*., 2007; Guldimann *et al*., 2016).

(ii) *C. difficile* can form highly stress- and desiccation-resistant endospores (Fimlaid and Shen, 2015; Bhattacharjee *et al*., 2016) and the fecal/oral route of these metabolically dormant entities is an important way for the dissemination of this pathogen and for the overall infection and intestine colonization process (Shen, 2015; Abt *et al*., 2016; Warriner *et al*., 2017). Kint *et al*. (2017) found that more than 200 genes encoding proteins involved in different stages of the sporulation process were differentially expressed in the *sigB*$^+$/*sigB* pair of *C. difficile* strains. Remarkably, SigB served as a negative control element for these sporulation-specific genes, and, in line with the obtained transcriptome data, the *sigB* mutant produced about 10-fold more spores then the isogenic *sigB*$^+$ *C. difficile* parent strain. The data reported by Kint *et al*. (2017) indicate that SigB influences sporulation of *C. difficile* by modulating the phosphorylation status of Spo0A, the master regulation of this cellular differentiation program in many Gram-positive bacteria (de Hoon *et al*., 2010; Higgins and Dworkin, 2012). There is precedent for such a suggestion from data reported by M. Hecker and co-workers on an interconnection between the general stress response system and the sporulation process in *B. subtilis* (Reder *et al*., 2012a,b). In this bacterium, sporulation is influenced by a SigB-dependent induction of *spo0E* that encodes a phosphatase involved in fine-tuning the phosphorylation status of Spo0A (de Hoon *et al*., 2010; Higgins and Dworkin, 2012). In this way, the influence of the master regulator of the general stress system is injected into the decision-making process by the master regulator of sporulation (Reder *et al*., 2012a,b).

(iii) To compare the ability of the *sigB* mutant and the *C. difficile* wild-type strain to colonize the intestinal tract, Kint *et al*. (2017) used a *C. difficile* dixenic mouse model for colonization studies. This particular model system allows for moderate host inflammation and immune responses. Since differences in germination efficiencies could severely affect the outcome of such studies, the authors tested spore germination and found that SigB was not involved in this process, at least not when the cells were grown in TY medium. In their infection mouse model system, *C. difficile* wild-type cell readily proliferated in the intestine of the mice and reached 5×10^8 bacteria per gram faeces two days after post-infection. Conversely, the *sigB* mutant led to a 3-log fold decrease in the burden of *C. difficile* bacteria within this time frame and decreased further (down to a 5-log fold) after 15 days of infection while the wild-type strain persisted at sustained high cell numbers. Similar data were obtained when *C. difficile* cells were enumerated in caecal-lumen content and when the caecal mucosa was tested for adherence of *sigB*$^+$ and *sigB* mutant cells, (Kint *et al*., 2017). Collectively, these data strongly suggest that colonization of the mouse intestine by *C. difficile* was severely impaired when the master regulator of the

general stress response was not performing its job. However, the details why this might be caused by a defect in SigB still need to be worked out and several hypotheses (e.g. differences in adhesion, metabolic changes, differences in the ability to resist host defenses) were put forward to possibly explain the striking difference between the *sigB*$^+$/*sigB* mutant pair of strains to successfully colonize and persist in the mouse intestine (Kint *et al.*, 2017).

Overall, the study by Kint *et al.* (2017) conclusively showed that the master regulator of the general stress response performs a crucial function for a diverse set of cellular processes at the onset of stationary phase and during the infection and colonization of the intestine by *C. difficile*. The fact that the transcription of about 25% of all *C. difficile* genes are affected in a *sigB* mutant suggests that SigB affects stress management and pathophysiology of this enteropathogen in a multi-factorial fashion as highlighted by the interconnection between the general stress response system and sporulation, a process crucial for the dissemination of *C. difficile* between patients and re-occurring infections. The types of stresses encountered by *C. difficile* during the primary infection and the subsequent colonization process of the intestine are not yet clearly enough defined, nor are the environmental and cellular cues that will lead to SigB activation under these conditions and during the horrific *C. difficile*-triggered inflammation of the gut. The study reported by Kint *et al.* (2017) in a recent issue of *Environmental Microbiology* provides a facet-rich blueprint to inform and direct these urgently needed studies to more fully understand the biology of this formidable pathogen (Elliott *et al.*, 2017).

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