Redefining the *Clostridioides difficile* σB regulon: σB activates genes involved in detoxifying radicals that can result from the exposure to antimicrobials and hydrogen peroxide.

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Running Head: The σB regulon of *C. difficile*

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Abstract

In many gram-positive bacteria the general stress response is regulated at the transcriptional level by the alternative sigma factor sigma B (σB). In C. difficile σB has been implicated in protection against stressors such as reactive oxygen species and antimicrobial compounds. Here, we used an anti-σB antibody to demonstrate time-limited overproduction of σB in C. difficile despite its toxicity at higher cellular concentrations. This toxicity eventually led to the loss of the plasmid used for anhydrotetracycline-induced σB gene expression. Inducible σB overproduction uncouples σB expression from its native regulatory network and allowed for the refinement of the previously proposed σB regulon. At least 32% of the regulon was found to consist of genes involved in the response to reactive radicals.

Direct gene activation by C. difficile σB was demonstrated through in vitro run-off transcription of specific target genes (cd0350, cd3614, cd3605, cd2963). Finally, we demonstrated that different antimicrobials and hydrogen peroxide induce these genes in a manner dependent on this sigma factor, using a plate-based luciferase reporter assay. Together, our work suggests that lethal exposure to antimicrobials may result in the formation of toxic radicals that lead to σB-dependent gene activation.

Importance

Sigma B is the alternative sigma factor governing stress response in many gram-positive bacteria. In C. difficile, a sigB mutant shows pleiotropic transcriptional effects. Here, we determine genes that are likely direct targets of σB by evaluating the transcriptional effects of σB overproduction, provide biochemical evidence of direct transcriptional activation by σB, and show that σB-dependent genes can be activated by antimicrobials. Together our data suggest that σB is a key player in dealing with toxic radicals.
Introduction

Disruption of the normal gastrointestinal flora as a result of antimicrobial treatment can lead to a *Clostridiodes (Clostridium) difficile* infection (CDI) (1). *Clostridiodes difficile* is a gram-positive, spore-forming obligate anaerobe and the primary cause for nosocomial infectious diarrhea (2). Its highly resistant endospores are usually transmitted via the oral-fecal route and germinate into vegetative cells in the colon upon contact with primary bile acids and other inducing factors (3). In the gut vegetative *C. difficile* cells are facing many environmental stressors, including variations in oxygen tension, pH, osmolarity, nutrient availability, and the inflammatory responses of the immune system (4). The bacteria are also faced with antimicrobial compounds produced by the host, the resident microbiota, or given externally during medical therapy (5). The physiological response of *C. difficile* to these insults and the inflammatory responses triggered by CDI can result in the production of Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS) and nitric oxide (NO) (2, 6).

Bacteria need to adapt to changing environmental conditions, including stresses, by adapting their physiology in a timely manner. This is achieved by fast transcriptional reprogramming, followed by shortly delayed changes at the translational level (7). The alternative sigma factor sigma B (σ^B, encoded by the *sigB* gene) - which regulates the general stress responses in a variety of gram-positive organisms - is central to the maintenance of the cellular homeostasis during stress adaptation (8, 9).

Sigma factor B activity in Firmicutes is regulated at the protein level by a partner-switching mechanism in which the anti-sigma factor RsbW binds and inhibits σ^B association with the RNA polymerase under non-stressed conditions. When a σ^B-activating stress is sensed the anti-anti-sigma factor RsbV is dephosphorylated by a phosphatase. Subsequently, RsbV is able to bind and sequester the anti-sigma factor RsbW, allowing for the association of free
σ^B with the RNA polymerase core enzyme (8, 10). The factors ultimately involved in sensing and the corresponding dephosphorylation of RsbV are stressor- and species-specific. For instance, Bacillus subtilis and Listeria monocytogenes respond to environmental stresses through a stressosome composed of RsbR/S/T/X, encoded by genes upstream of rsbV, rsbW and sigB in the same operon (8). Ultimately, this leads to activation of the RsbU phosphatase and dephosphorylation of RsbV (8). The C. difficile sigB operon does not contain genes encoding RsbR/S/T/X homologs. Two open reading frames upstream of rsbV (cd0007-cd0008) appear to have no role in σ^B activation (11). Recently a phosphatase, RsbZ, responsible for RsbV dephosphorylation has been characterized in C. difficile, but the gene encoding this protein is not a part of the sigB operon (11). Tight regulation of σ^B by the partner-switching mechanism is required as the energy burden associated with σ^B activity was found to be disadvantageous in several different organisms (12, 13). Nevertheless, σ^B is essential for in-host survival for several pathogenic bacterial species. For example, in L. monocytogenes σ^B is involved in counteracting the effects of the acidic pH encountered in the stomach and upon invasion of intestinal epithelial cells in the lysosome (14, 15).

Antimicrobial resistance in certain pathogenic bacteria is σ^B-dependent. In Staphylococcus aureus σ^B overproduction leads to thickening of the cell wall and increased resistance to beta-lactam antimicrobials (16). The sigB homologue sigF of Mycobacterium tuberculosis is induced by small amounts of rifamycin (17). Analogously, B. subtilis σ^B is involved in resolving a rifampin-induced growth arrest (18). There is also evidence for the involvement of σ^B in C. difficile in the response to antimicrobial substances. Mutants of sigB show increased susceptibility to rifampicin and mitomycin C, and are also more sensitive to hydrogen peroxide, nitroprusside and di-ethylamine NONOate. However, the underlying molecular mechanism remains unknown (19).
genes as the result of a gene-dosage shift has been demonstrated for \textit{C. difficile} exposed to DNA-polymerase inhibitors such as the Phase I drug ibezapostat/ACX-362E (20). In this study, we demonstrate that despite its detrimental cellular effects \(\sigma^B\) overexpression is detectable and tolerated for short periods of time. This allowed for the experimental identification of a set of genes that is most likely directly regulated by \(\sigma^B\). The obtained results show that genes involved in the oxidative and nitrosative stress response form the core of the regulon. Additionally, we show that various antimicrobials and hydrogen peroxide induce the expression of \(\sigma^B\)-regulated genes in a \(\sigma^B\)-dependent manner, suggesting a link between the lethal exposure to antimicrobials and oxidative and nitrosative stresses in \textit{C. difficile}.
Results

C. difficile σB is measurably overproduced upon induction of the sigB gene

Previous investigations of σB in C. difficile have used a sigB mutant and characterized its gene expression in the stationary growth phase in comparison with a wild type strain. Though informative, this method is likely to result in indirect effects of σB due to stationary phase heterogeneity, prolonged incubation and possible positive or negative feedback in the σB regulatory circuit. To circumvent these issues and identify genes likely to be regulated by σB directly, we set out to uncouple sigB expression from its native regulatory circuit by expressing it from an inducible promoter.

Firstly, in order to confirm overproduction of σB, we measured cellular σB levels using immunoblotting. For this purpose, we heterologously overproduced and purified σB containing a C-terminal His-tag (Figure 1A) and used this protein to raise a polyclonal antiserum. Corresponding polyclonal antibodies were affinity-purified to prevent unspecific immune reactions.

Next, we set out to validate the overproduction of σB in transconjugant C. difficile cells harboring plasmids containing sigB under the control of the anhydrotetracyclin (ATc)-dependent promoter Ptet (21). For this purpose, σB was produced in a sigB mutant background (strain IB58; sigB::CT Ptet-sigB). As a control, we introduced a non-related expression construct in the same strain (IB61; sigB::CT Ptet-slucΔα). We expected a signal at approximately 30 kDa in Western blot experiments for cells grown in the presence of inducer ATc for strain IB58, but not for the uninduced cultures of IB58 or the control strain IB61. Additionally, by growing in the presence or absence of
thiamphenicol, we investigated whether overproduction of σB required selection for the Ptet-sigB expression plasmid.

When strains were grown in BHIY medium supplemented with 20 µg/mL lincomycin and induced for one hour with or without 100 ng/mL ATc in the presence or absence of 20 µg/mL thiamphenicol, we did not detect any signal at the molecular weight expected for σB in the ATc-induced control samples (sigB::CT Ptet-slucopt) or in any of the uninduced samples (Figure 1B). In contrast, after one hour of induction a clear band of the expected molecular weight of σB (~30 kDa) was observed only in the IB58 (sigB::CT Ptet-sigB) samples (Figure 1B). Plasmid selection by inclusion of thiamphenicol in the growth medium did not influence σB overproduction in this time frame.

We conclude that the affinity purified rabbit-α-σB antibody is specific for σB and can be used for its detection in lysates of C. difficile. Furthermore, it is possible to uncouple sigB expression from its tight regulatory network by ATc-inducible overexpression for one hour in trans.

Prolonged overexpression of σB is lethal and leads to loss of plasmids harboring Ptet-sigB

Above we showed that it is possible to overproduce σB in C. difficile and that it is tolerated by the bacterium for one hour. This observation is somewhat at odds with the previously reported toxic nature of overproduced σB (8, 11). To reconcile these two observations, the effect of long-term overexpression of sigB and the stability of the plasmids used for σB overproduction under such conditions were investigated. First, overnight cultures of 630Δerm (wildtype), AP34 (Ptet-slucopt) and JC096 (Ptet-sigB) were adjusted for their OD600nm values and ten-fold serially diluted. Subsequently, 2 µl spots per dilution were made on selective (20 µg/mL thiamphenicol) and non-selective BHIY agar plates, part of which
contained 200 ng/mL ATc to induce Ptet-dependent gene expression. All plates were then incubated anaerobically for 24 hours. On plates without thiamphenicol, regardless of the presence of the inducer ATc, comparable growth was observed for all three strains (Figure 2A). As expected, when selecting for the plasmid using thiamphenicol no growth was observed for the susceptible 630Δerm strain (which lacks the catP gene contained on the expression vector). In the absence of the inducer, no difference in growth was observed for the vector control strain (AP34; Ptet-slucopt) compared to the strain carrying the Ptet-sigB plasmid (JC096). However, upon induction of sigB expression on selective plates a 3–4 log growth defect was observed for the strain carrying Ptet-sigB compared to the vector control strain. We conclude that prolonged induction of sigB expression is toxic when cells are cultured in the presence of thiamphenicol. Our results thus corroborate the finding that σB overproduction is toxic to C. difficile cells in liquid culture (11). The lethality associated with σB overproduction was not seen when cells were grown without thiamphenicol in our experiment (Figure 2A). We considered two possible explanations for this observation. As thiamphenicol is used for ensuring plasmid maintenance, its absence might result in plasmid loss when a toxic protein such as σB is overproduced. The remaining cells, that no longer express σB would consequently be susceptible to thiamphenicol (due to the loss of catP). Alternatively, the combination of σB and thiamphenicol might be toxic to the bacteria. To test whether plasmid loss was the cause of the observed lethality of bacteria overproducing σB in the presence of thiamphenicol, cells from the plates without thiamphenicol (with and without ATc) were resuspended in BHIY medium, adjusted for their OD_{600nm} and ten-fold serially diluted. Ten-µl spots of these dilutions were plated on plasmid-selective (thiamphenicol) and non-selective (no thiamphenicol) plates. Based on the ratio of CFU/mL of the selective and non-selective
plates, the percentage of cells which lost their plasmid was calculated. If $\sigma^B$ overproduction led to the loss of the plasmid under conditions that do not select for its maintenance (no thiamphenicol), we expected significantly reduced growth on plates containing thiamphenicol. Although some plasmid loss was observed under uninduced conditions, as well as for the negative control strain AP34 ($P_{\text{tet}}\text{-slucopt}$)(Figure 2B), all cells originally containing the $P_{\text{tet}}\text{-sigB}$ plasmid (strain JC096) lost this plasmid upon induction of $\sigma^B$ overproduction with ATc. Similar results were obtained for $\text{sigB}$ mutant strains IB58 and IB61 (Figure 2C), indicating the observed effects were solely due to in trans $\sigma^B$ overproduction, and did not result from an interference of the native $\text{sigB}$ regulatory network. Together, these results are consistent with a model in which the vector with the low-copy number pCD6 replicon are rapidly eradicated upon expression of a gene (here $\text{sigB}$) that causes lethal defects (22, 23).

$\sigma^B$ primarily activates genes relating to oxidative/nitrosative stress responses

Above, we have shown that long-term overproduction $\sigma^B$ is detrimental and that this leads to loss of the expression plasmid in the absence of thiamphenicol (Figure 2), but that $\sigma^B$ overproduction nevertheless could clearly be demonstrated when induction is limited to 1 hour (Figure 1C). Therefore we used the time-limited induction to refine the previously proposed regulon (19) in both the presence and absence of thiamphenicol to strike a balance between potential secondary effects due to toxicity associated with $\sigma^B$ overproduction (with thiamphenicol), and loss of the expression plasmid from a subpopulation of cells (without thiamphenicol)(Table 1). We compared transcriptome data from strain IB58 ($\text{sigB::CT P_{\text{tet}}\text{-sigB}}$) to that of strain IB61 ($\text{sigB::CT P_{\text{tet}}\text{-slucopt}}$). IB61 harbors a
vector for the inducible expression of a luciferase gene which does not lead to any toxicity
and growth phenotype (24)
We expected no or a limited number of genes to be differentially expressed (log2
foldchange (log2FC) of ≤ -1.5 or ≥1.5, and adjusted p-value of <0.05) under non-inducing
conditions. Indeed, we found only five differentially expressed genes in the P_{tet-sigB} strain
(IB58) compared to the P_{tet-slu^{opt}} control (IB61) strain (hybridizations 1 and 3) (Dataset S1).
These genes were similarly positively (CD0583, CD0584, both GGDEF domain containing
proteins (25); CD2214, CD2215, both potential transcriptional regulators (26)) and
negatively (CD1616, an EAL domain protein (25)) regulated in all hybridizations, including
those where sigB expression was not induced. These results suggest that the basis for the
observed differential expression of these genes was vector specific, but not dependent on
σ^{B} induction. These genes were therefore not investigated further and are excluded from
the numbers discussed below.
Upon induction of sigB expression 145 genes were differentially expressed when strains
were cultured without thiamphenicol (hybridization 4), and 178 genes were differentially
expressed when thiamphenicol was present during cultivation (hybridization 2) (Figure 3 and
Dataset S1). The majority showed an increase in expression upon induction of sigB
expression (132 in the samples without thiamphenicol, and 163 in the samples with
thiamphenicol), while a minority revealed a decreased expression (13 in the samples
without thiamphenicol, and 16 in the samples with thiamphenicol). Of note, we observed
only a minor difference in the number of differentially expressed genes between the cells
grown in the absence and presence of thiamphenicol (33 genes).
Together, these results demonstrate a high level of consistency in the σ^{B} regulon, despite
potential plasmid-loss (when grown in the absence of thiamphenicol) or toxic effects (when
grown in the presence thiamphenicol). Our results also show that $\sigma^B$ primarily activates gene expression. We focused our further analyses on the data obtained from hybridization 2 (with ATc and thiamphenicol), as this condition provided the broadest dataset (178 differentially expressed genes) for the re-definition of the $\sigma^B$ regulon under our experimental conditions (Dataset S1).

Of the 163 genes up-regulated by $\sigma^B$ the vast majority appeared to be associated with an response to oxidative stress, since they encode various oxidoreductases, peroxidases, and thioredoxin reductases (Table 2). Notably, approximately 51% of the 98 genes previously found to be up-regulated under aerobic stress (7) were also positively regulated by $\sigma^B$ (Table 2). Five additional genes associated with aerobic/nitrosative stress (CD0174/cooS, CD1279/iscS2, CD1280, CD1594/cysK, CD1823 and CD2166/msrAB) were also found to be induced by $\sigma^B$, in agreement with previous findings (19).

Our findings are recapitulated in a volcano plot (27), that clearly shows that genes with lower expression upon sigB induction (in blue) cluster close to the significance threshold whereas those with increased expression (in red) show a larger fold change (Figure 3). We calculated the Manhattan distance for each data point (Dataset S2), and discuss the proteins encoded by the top-10 of the differentially expressed genes below.

CD0051A is a small hypothetical protein of unknown function. It does not contain any recognizable domains and a secondary structure prediction using Phyre2 does not give any clues as to its potential function (28). CD0580 (GapN) is annotated as an glyceraldehyde-3-phosphate dehydrogenase (GADPH), a key glycolytic enzyme, and contains an aldehyde dehydrogenase domain. Interestingly, its activity has been shown to be redox-controlled in other bacteria and has been implicated in the response to reactive oxygen and nitrogen
species (29-31). CD1623 is a putative oxidoreductase with similarity to FAD flavoproteins and rubredoxins. CD1690 (TrxA) and CD1691 (TrxB) are likely encoded in the same operon (32), and form a thioredoxin/thioredoxin-disulfide reductase couple. CD0174 (CooS; IPR010047), CD0175 and CD0176 are likely also encoded in a single operon (32), and function as carbon monoxide dehydrogenase, and two putative oxidoreductases. As mentioned above, CD0174 has been implicated in aerobic/nitrosative stress and it is likely that CD1623, CD1690 and CD1691 also function in this pathway. Finally, CD2115A encodes another small hypothetical protein and as for CD0051A, no function could be assigned on the basis of secondary structure prediction.

As the σ^B regulon that we define here is substantially smaller than that previously reported, the major conclusion is that at least 32% of the σ^B regulon is involved in positively regulating oxidative/nitrosative stress responses. In the previous investigation of the σ^B regulon they were approximately 3.2% (~32/1000) (19). Overall, we conclude that the core functions of the σ^B regulon lie in the regulation of the detoxification response to oxygen- and nitro-radicals.

**In vitro run-off transcriptions demonstrate direction activation of** \( P_{cd0350}, P_{cd2963}, P_{cd3412} \) **and** \( P_{cd3605} \) **by σ^B**

Gene expression can directly or indirectly be influenced by σ^B, and to date no attempts have been made to discriminate these possibilities biochemically (11, 19). Despite the short time of induction and the uncoupling of σ^B from its normal regulatory network, our analyses could possibly also have picked up indirect effects. To determine if the transcription of selected genes is directly activated by σ^B, *in vitro* transcription run-off reactions were performed using purified σ^B\_6xHis and RNA polymerase core enzyme (RNAP\_core) on the
upstream regions of a selection of genes. The genes cd0350 (encoding a putative hydrolase involved in oxidative stress), cd2963 (encoding an L,D-transpeptidase), cd3412 (encoding UvrB, involved in nucleotide excision repair), cd3605 (encoding a ferredoxin) and cd3614 (encoding a hypothetical protein involved in oxidative stress) were selected on the basis of our transcriptome analyses (Dataset S1), previous reporter gene assays (20) and/or the presence of a putative σB recognition site (11). The gene cd0872 (maltose O-acetyltransferase) was not differentially expressed in our transcriptome data and was thus included as a negative control. The promoter of the toxin A gene (tcdA) in combination with purified TcdR was used as a positive control for the assay, as previously described (33).

As expected, no in vitro transcript was observed for a linear DNA fragment containing P_{cd0872} incubated with purified σB\(_{6xHis}\) and RNAP\(_{core}\) under our experimental conditions, whereas a specific product was obtained for the positive control P_{tcdA} in the presence of TcdR and RNAP\(_{core}\) (Figure 4). An RNAP\(_{core}\)- and σB\(_{6xHis}\)-specific signal was observed for fragments containing the putative promoter regions of the genes cd0350, cd2963, cd3412 and cd3605, demonstrating that expression of these genes was directed by σB. For the fragments containing the putative promoter of cd3614 we did not get a consistent product in the in vitro transcription experiments, though some smearing is visible in the lane with RNAP\(_{core}\) and σB\(_{6xHis}\). As cd3614 demonstrates clear differential expression in the DNA array experiments and it upstream region harbors the σB consensus sequence WGWTT-N\(_{13-17}\)-(G/T)GGTWA (19), we consider it likely that this gene is directly regulated by σB and our failure to obtain a discrete signal is due to our experimental conditions or the lack of an auxiliary factor in our in vitro assays.

Overall, we provide the first biochemical evidence for direct σB-dependent activation of several genes identified via transcriptome analyses as part of the σB regulon in C. difficile.
Antimicrobials and hydrogen peroxide activate σB-directed gene transcription

The redefined σB regulon pointed towards a substantial role for σB in coordinating the oxidative- and nitrosative stress response, which could result from antimicrobial treatment. In order to test for the activation of σB-dependent promoters by antimicrobials, we set up a plate-based luciferase reporter assay. In this assay cells harboring σB-dependent luciferase reporter constructs were plated on BHIY agar to give confluent growth and exposed to antimicrobials either through an epsilometer test (E-test) or through a filter disc.

Subsequently, luciferase activity was imaged (for details, see Materials and Methods). A strain harboring Ptet-slucopt (AP34) served as negative control, as this promoter is not expected to respond in a σB-dependent manner (Figure 5A).

First, the σB-dependent response to metronidazole was investigated. Metronidazole, formerly used as a first line treatment for CDI, is believed to cause DNA damage through the formation of nitro-radicals although its exact mode of action remains unclear (6, 34). To survey a full spectrum of metronidazole concentrations, we evaluated luminescence after 24h of incubation of an metronidazole E-test. If metronidazole treatment results in σB-dependent activation of gene transcription, we expect to see a luciferase signal in the wild type, but not in a σB knockout background. In agreement with this, activation of Pcd0350 was observed at the edge of the halo resulting from the metronidazole E-test in the wild type background, but not in the σB knockout strain (Figure 5B). No signal was observed for the negative control Ptet-slucopt (Figure 5A). The observed activation σB-dependent activation of gene expression at the edge of the halo, but not further into the plate suggests that the metronidazole induced, σB-dependent activation of Pcd0350 occurred close to the Minimal Inhibitory Concentration (MIC). Expression of the luciferase from Pcd2963 was found to be
strictly dependent on $\sigma^B$ as no luciferase activity was observed in the sigB knockout strain. However, there was limited to no increase in reporter gene expression in the presence of metronidazole. Metronidazole strongly activated transcription from $P_{cd3412}$ at MIC levels of metronidazole, but this appeared to be independent of $\sigma^B$ in this assay since in the sigB mutant a similar induction was observed. Finally, in a manner comparable to $P_{cd0350}$, the activation of $P_{CD3614}$ was strongly induced by metronidazole at values close to MIC in a $\sigma^B$-dependent manner, but residual activity was observed in the $\sigma^B$ knockout strain independent of metronidazole levels. We noted that metronidazole-induced promoter activation appeared to occur on the inside of the E-test halo, which might be attributed to the secretion of the luciferase reporter.

The observed diverse regulatory responses at different tested promoters during the treatment of C. difficile with metronidazole pointed towards a more complex regulatory network with the participation of $\sigma^B$. Antimicrobial-driven (and $\sigma^B$-dependent) activation of $\sigma^B$ target genes could be specific to metronidazole or represent a more general response to cellular (toxic) stresses. Therefore, we evaluated the effects of different antimicrobial compounds and the radical producer H$_2$O$_2$ as a positive control (19), using the $P_{cd0350}$ reporter construct, as this promoter demonstrated the clearest $\sigma^B$ dependent activation in the presence of metronidazole (Figure 5B). We tested the cell wall biosynthesis inhibitor vancomycin, the protein synthesis inhibitor lincomycin and the DNA polymerase inhibitor ibezapolstat (formerly known as ACX-362E) (35). We observed clear activation of $P_{cd0350}$ in the presence of all added stressors, but not for a negative control containing water (Figure 5C).
We conclude that, at least for the σ^B dependent promoter of *cd0350*, activation does not only occur upon exposure to lethal levels of metronidazole, but also with unrelated antimicrobials and toxic stressors such as hydrogen peroxide.
Discussion

In this work we have demonstrated by Western blotting using an affinity purified anti-σ^B^ antibody that σ^B^ can be overproduced for a limited period of time, sufficient for transcriptome analyses. The induced production of σ^B^ in a sigB mutant background yielded highly consistent results, despite potential toxicity and plasmid-loss (Figure 2), and the results were used to redefine the surprisingly large σ^B^ regulon previously proposed (19). As our approach more accurately measures changes in transcription directly related to σ^B^ production, the refined regulon described here is much smaller (Dataset S1). Its size is fully in line with that of the σ^B^ regulon of other gram-positive bacteria such as L. monocytogenes (~130 genes), B. subtilis (~150 genes) and S. aureus (~200 genes) (8). The redefined regulon underscores the importance of σ^B^ in responding to oxidative/nitrosative stresses as genes implicated in such processes are significantly enriched in the smaller regulon.

The majority of the genes in our regulon were found to be induced, rather than repressed, by σ^B^. This is in line with sigma factors acting as specificity determinants for transcription initiation (36). Similar observations have been made for the σ^B^ regulon of L. monocytogenes (37, 38). For the first time, direct evidence of C. difficile σ^B^-dependent gene activation is provided by the results of the in vitro run-off transcriptions (Figure 4), which demonstrate that RNAP^core^ and σ^B^ are sufficient to generate transcripts from Pcd0350, Pcd2963, Pcd3412 and Pcd3605. Notably, these experiments pave the way for a further in vitro characterization of this sigma factor in C. difficile including validation of the σ^B^ binding sequence and the interplay with other regulators.

Although the promoters of cd3412 (uvrB) and cd3614 were reported to have a σ^B^ consensus sequence and are differentially expressed upon σ^B^ overexpression (19, 35), our results clearly demonstrate that they can also be expressed in a σ^B^-independent manner (Figure
This is most notable for Pcd3412, which is still activated by metronidazole in the absence of σ^B, in line with results obtained with ibezapolstat in a different study (20). Both metronidazole and ibezapolstat treatment can cause DNA damage, and DNA-damage dependent induction of cd3412 therefore likely depends on a sigB-independent pathway. The observed σ^B-dependent gene repression could be indirect (σ^B induces the transcription of a repressor gene), or the result of competition (σ^B competes with other sigma factors for RNAP). We consider the second scenario more likely for the following reasons. First, little overproduction of σ^B was detected after 30 minutes of induction. This leaves only a limited time for indirect effects to occur in our setup. Second, the majority of genes down-regulated upon overexpression of σ^B fall into a single functional group (flagellar motility). These genes are known to be regulated by the dedicated sigma factor, σ^D (39), supporting the model of sigma factor competition. Strikingly, in L. monocytogenes σ^B activity (indirectly) also results in down-regulation of flagellar gene expression, but this is mediated by the repressor MogR (40). Protein BLAST analyses revealed that C. difficile does not possess a MogR homologue. Nevertheless, the conserved inverse correlation between the σ^B-dependent general stress response and bacterial motility could represent a cost-saving strategy for bacterial cells (41). The indirect mechanism underlying the observed σ^B-dependent down-regulation in C. difficile remains to be determined.

There appears to be an intriguing link between σ^B and the response to toxic compounds, as a sigB mutant was more susceptible to rifampicin and mitomycin C (19) and exposure to antimicrobials (metronidazole, vancomycin, lincomycin and ibezapolstat) or hydrogen peroxide leads to σ^B-dependent promoter activation (Figure 5C). The mechanism behind the latter is unclear. It has been suggested that antimicrobials at toxic concentrations can influence metabolism and respiration (42, 43), potentially resulting in the formation of
bactericidal concentrations of radical species (44-46). A strong connection between σ^B and oxidative (and/or nitrosative) stress in *C. difficile* (**Table 2**) and other bacteria (7, 18, 19), as well as a recently described radical scavenging strategy that increases tolerance to antimicrobials (47) are consistent with such a model. However, additional research is necessary to determine exactly how these processes occur and are influenced by antimicrobials in anaerobic organisms under anoxic conditions.

In conclusion, we have demonstrated that σ^B is directly involved in metabolic and oxidative stress responses and that lethal stresses may influence these processes, resulting in activation of σ^B targeted genes.
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Materials and Methods

Construction of σ^8 expression- and luciferase-reporter vectors

All oligonucleotides used in this study can be found in table 2. Plasmids and strains can be found in table 3. The P_{TT^7} sigB_{6α} expression vector pIB14 was created by restriction-ligation using restriction enzymes Ndel and Xhol. Using primers oIB-1 and oIB-2 on C. difficile 630Δerm chromosomal DNA, the sigB CDS was amplified by PCR. All PCR products used for sequencing or plasmid synthesis were performed with Q5 high-fidelity polymerase (NEB). The resulting DNA fragment was digested and ligated into Ndel-xhol digested pET21b(-) vector, generating expression vector pIB14. Plasmids pIB27, pIB68, pIB69 and pIB74 have been described previously (20). The CD0872 promoter area was amplified using primers oIB-14 and oIB15 and the P_{cd0872} luciferase-reporter plasmid was created by restriction-ligation using restriction enzymes KpnI and Sacl in digested pAP24 backbone, generating plasmid pIB21. Using Gibson assembly as described in (20) the P_{cd3605} luciferase-reporter plasmid was generated using primers oIB-90 and oIB-99, yielding plasmid pIB73. A plasmid containing P_{tet}-sigB was generated by cloning the 630Δerm sigB CDS amplified with oWKS-1498 and oWKS-1499 in pMiniT (NEB E1202) per manufacturer’s instructions. Using restriction enzymes Sacl and BamHI this PCR fragment was cloned into pRPF185 generating pWKS1760. All plasmids were verified by Sanger sequencing.

Bacterial strains and growth conditions

Strains of E. coli were grown aerobically at 37°C in Luria-Bertani broth (Affymetrix) supplemented with ampicillin (50 ug/mL), kanamycin (50 ug/mL) and/or chloramphenicol (20 ug/mL) when required. Plasmids were maintained in E. coli strains DH5α or MDS42.
(Scarab Genomics) under appropriate antimicrobial selection and cells were transformed using standard procedures (48). For plasmid conjugation into recipient *C. difficile* 630Δerm, *E. coli* strain CA434 was used as a donor strain as previously described (49). *C. difficile* strains were cultured anaerobically at 37°C in either a Don Whitley VA-1000 or A55 workstation. Cells were cultured in Brain Heart Infusion (BHI, Oxoid) broth supplemented with 0.5% (w/v) yeast-extract (BHIY) and 20 µg/ml thiamphenicol when appropriate. Unless additional antimicrobials/stressors were added (metronidazole E-test and sterile pads supplemented with different stressors), medium was supplemented with *C. difficile* Selective Supplements (CDSS, Oxoid).

**Overproduction, purification and affinity purification of σ6xHis for synthesis of a polyclonal anti-σB antibody**

**Overproduction and purification of σ6xHis**

Overexpression of σ6xHis was performed by using Escherichia coli (*E. coli*) Rosetta (DE3) pLysS cells (Novagen) harboring the *E. coli* expression plasmid pIB14. These cells were cultured in Luria-Bertani (LB) broth and induced with 0.5 mM IPTG for one hour starting at an optical density = 0.6. Cells were collected by centrifugation at 4°C and pellets were resuspended in lysis buffer (pH=8.0, 50 mM NaH2PO4, 300 mM NaCl, 5 mM β-mercaptoethanol, 0.1% NP-40 and Complete protease inhibitor cocktail (CPIC, Roche Applied Science). Through the addition of 1 mg/mL lysozyme and sonication (6 times 20 seconds) cells were lysed. The lysate was drawn through a blunt 1.2mm needle and was clarified by centrifugation at 13000 xg at 4°C for 25 minutes. The supernatant containing recombinant σ6xHis was purified on TALON Superflow resin (GE healthcare) per manufacturer’s instructions. Proteins were dialyzed and stored in buffer (pH=8.0) containing...
50 mM NaH2PO4, 300 mM NaCl and 12% glycerol. Protein concentrations were determined using a Bradford assay (Biorad) and 2 mL of σB6x-His protein solution containing 2 mg/mL measured protein was sent to BioGenes GmbH (Berlin) for generation of a polyclonal rabbit-anti-σB antibody.

Affinity purification of the polyclonal anti-σB antibody

Affinity purification of the antibody was performed to increase specificity. Approximately 350µg of purified σB6xHis protein was loaded on an SDS-PAGE gel. After transferring proteins to a PVDF membrane using standard blotting procedures, purified σB6xHis protein was visualized by Ponceau S stain and the membrane containing the protein was cut as small as possible whilst retaining the region with the protein. The membrane was destained and washed with TBST buffer (500 mM NaCl, 20 mM Tris-base, 0.05% v/v Tween-20, pH=7.4) twice for 5 minutes at room temperature. The membrane was then pre-eluted by soaking in acidic glycine solution (100 mM, pH=2.5) for 5 minutes prior to washing with TBST twice for five minutes at room temperature. Subsequently the membrane was blocked in 5% non-fat milk powder solution (Campina Elk, dissolved in TBST buffer) for one hour at room temperature after again washing twice with TBST for 5 minutes. Serum containing anti-σB antibody was incubated on the membrane overnight at 4°C. After three 5-minute washes with TBST, the membrane was washed twice for 5 minutes in PBS. Affinity purified antibody was eluted from the membrane by adding acidic glycine solution and incubating for 10 minutes at room temperature. The pH of the eluate was adjusted to 7.0 through the addition of 1M Tris-HCl (pH=8.0). This step was repeated twice more and the eluates were pooled and centrifuged (1 min maximum speed) to remove precipitated protein and membrane particles. Bovine serum albumin (BSA) and sodium azide were added to the
480 affinity purified anti-σ^B antibody to end concentrations of 1 mg/mL and 5mM respectively,
481 and the affinity purified antibody was stored at -80°C.

482 Characterization of the σ^B regulon
483 σ^B overproduction in C. difficile
484 Exponentially growing starter cultures of IB58 and IB61 were diluted to an OD_{600}=0.05 in
485 BHIY supplemented with 20 μg/mL lincomycin with thiamphenicol (20 μg/mL) where
486 appropriate. Cells were grown until OD_{600}=0.3 after which 1 mL sample was taken for
487 control by Western blot and cells were induced with 100 ng/mL ATc for one hour. After one
488 hour of induction, 1 mL of sample was taken for control by Western Blot and 50 mL was
489 collected by centrifugation and stored at -20°C until RNA extraction. Non-induced samples
490 were treated and collected identically, except that no ATc was added at OD_{600}=0.3. All
491 samples were corrected for OD_{600} prior to analysis by Western Blot.
492 RNA extraction
493 Bacterial RNA was extracted and analyzed as previously described (50). Briefly, cell pellets
494 were lysed in enzymatic lysis buffer consisting of 15 mg/mL lysozyme and TE buffer. Further
495 disruption of cells was performed by vigorous mechanical lysis for 3 minutes in RLT buffer to
496 which one spatula of glass beads was added. After samples were centrifuged (3 minutes at
497 10.000 rpm at 4°C) and 100% ethanol was added to the supernatant, RNA was purified using
498 the Qiagen RNeasy Kit protocol according to manufacturer’s instructions. DNA
499 contamination was removed by using RNAse-free DNase I (Qiagen) twice prior to elution of
500 the RNA samples in H₂O. RNA quality and integrity numbers (RINs) were assessed with a
501 Bioanalyzer 2100 (Agilent) and RNA 6000 Nano Reagents (Agilent). Only samples with an RIN
502 of ≥ 7 were used for analysis.
DNA microarray and data analysis

A customized whole-genome DNA microarray of 630Δerm was used (8x15K format, Agilent) (50). Quadruplicate samples were analyzed for the DNA microarray. Using the ULS fluorescent labelling kit for Agilent arrays (Kreatech), 1 µg of total RNA was used for labelling with either Cy3 (P_{tet-sigB}) or Cy5 (P_{tet-slucopt}). After pooling and fragmentation, 300 ng of labelled RNA per sample was hybridized according to the two-color microarray protocol from Agilent. DNA microarrays were scanned with an Agilent C scanner and analyzed as described (50). A gene was considered differentially expressed if the log2 foldchange (log2 FC) was ≤ -1.5 or ≥1.5 and the p-value was <0.05. Results were visualized in VolcanoseR (27), and are available as an interactive graph via the URL contained in Text S1.

The data used in the visualization has been deposited at Zenodo for this purpose (doi: 10.5281/zenodo.3945936). Full transcriptome data has been deposited in the GEO database, and can be accessed through the identifier GSE152515.

In vitro transcription

DNA oligonucleotides oWKS-1506 and oWKS-15136 (64- and 82 bp, respectively) were end-labelled with γ-32P-ATP using T4 Polynucleotide Kinase (PNK) and used as a size indicator for the in vitro transcription reactions. For the end labelling reaction, 1 µl γ-32P-ATP was incubated together with 200 pmol oligonucleotide and 1 µl PNK in Forward Reaction buffer (70 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 100 mM KCl, 1 mM 2-mercaptoethanol) at 37°C for 30 minutes. For the in vitro run-off transcriptions sigma factors and RNA polymerase core enzyme were preincubated with PCR amplified promoter areas (for P_{CD0350}, P_{CD0872}, P_{CD2963}, P_{CD3412}, P_{CD3605} and P_{CD3614}) or XbaI linearised pCD22 (P_{tcda}) for 30 minutes at 37°C prior to start of the reaction. PCR products of the promoter areas as used for the in vitro
transcription reactions were loaded on and excised from agarose gels and purified using a NucleoSpin™ Gel and PCR Clean-up kit (Machery-Nagel). In vitro transcription reactions mixtures contained 1 µl E. coli RNAP core (NEB M0550S), 16 pmol sigma factor, 0.5 pmol DNA, 10 mM NTP mix and 0.3 µl α-32P-ATP in reaction buffer (40 mM Tris-HCl, 150 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.01% triton X-100, pH=7.5) and were incubated for 15 minutes at 37°C. Transcripts and labelled oligonucleotides to be used as a size indication were purified using P-30 Bio-Gel spin columns (Biorad). All reactions were stopped in gel loading buffer II (Invitrogen) containing 95% Formamide, 18 mM EDTA and 0.025% each of SDS, xylene cyanol, and bromophenol blue at 95°C for 5 minutes and loaded on 8% monomeric UreaGel (SequaGel, National Diagnostics). Gels were dried and exposed to PhosphorImager screens overnight (approximately 17 hours) and imaged with a Typhoon-9410 scanner (GE Healthcare).

Spot assay for viability upon σ⁰ overproduction in *C. difficile* and vector stability assay

*C. difficile* overnight pre-cultures were corrected for OD₆₀₀ and subsequently tenfold serially diluted in BHI medium. Two-µl spots of each dilution were plated on selective (20 µg/mL thiamphenicol) and unselective square (90x15 mm, VWR international) BHI plates with or without 200 ng/mL anhydrotetracycline (ATc). Growth was evaluated after 24 hours and subsequently swabs were taken from all strains grown on unselective BHI agar plates with- and without 200 ng/mL ATc for the vector stability assay. These swabs used for the vector stability assay were resuspended in BHI medium, adjusted for OD₆₀₀-values and tenfold serially diluted in unselective BHI medium. Of these OD-corrected, serially diluted suspensions, 10-µl spots were then plated on selective (20 µg/mL thiamphenicol + CDSS) and non-selective (BHI +CDSS) plates and CFU/mL was counted after 24-48 hours of growth.
Percentage plasmid maintained was calculated as $[\text{CFU/mL}]_{\text{selective}}/\text{CFU/mL}_{\text{non-selective}} \times 100%$. If no growth was detected on selective plates percentage of plasmid maintained was set as 0%. To calculate statistical significance between % plasmid maintained in strains induced- or not induced by ATc, an unpaired student’s t-test was used.

Plate based luciferase assay with metronidazole E-test and disk diffusion

Strains harboring luciferase-reporter plasmids were grown on prerduced, selective BHI plates for 24 hours. Subsequently bacterial suspensions corresponding to 1.0 McFarland turbidity were applied on BHI agar supplemented with 0.5% yeast-extract after which a metronidazole E-test or plain disks were applied. Disks were spotted with 10 µl each of sterile H$_2$O, 1M H$_2$O$_2$, 3000 µg/mL lincomycin, 200 µg/mL metronidazole, 400 µg/mL ubezapolstat and 200 µg/mL vancomycin. After 24 hours of growth luciferase activity was visualized by spraying one spray of 1:100 reconstituted NanoGlo Luciferase substrate (Promega N1110) per agar plate using a disposable spray flask. One spray corresponded to approx. 250 µl reconstituted NanoGlo Luciferase substrate. Luminescence was recorded using a Uvitec Alliance Q9 Advanced imager (BioSPX) after 10 seconds exposure time per plate. Reporter constructs were generated in a $\text{sigB}$ knockout made by Allelic Coupled Exchange (51) as opposed to the ClosTron mutant background used for the DNA arrays. However, no differences between these backgrounds has ever been observed in our assays.
Figure 1. Recombinant σ^B was used to generate a *C. difficile* specific antibody for intracellular detection. A) Coomassie Blue-stained 12.5% SDS-PAGE gel of purified recombinant σ^B-6xHis. B) Western Blot using affinity purified σ^B antibody (1:500) on strains IB58 (*sigB::CT P_{tet}-sigB*) and IB61 (*sigB::CT P_{tet}-sluc^opt*). Cells were grown in lincomycin (20 µg/mL) and in the presence or absence of thiamphenicol (20 µg/mL) until OD_{600nm}=0.3, after which the indicated samples were induced with 100 ng/mL ATc. Samples were collected directly after the addition of ATc (or at the time ATc would have been added in the uninduced controls) at T=0h and after 1 hour of induction (T=1). 


A

MW (kDa)

Coomassie

σB-6xHis

B

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MW (kDa)

affinity purified anti-σB
**Figure 2. Overexpression of σ^B is toxic for *C. difficile* and leads to plasmid loss. A**) Tenfold serial dilutions on BHIY agar plates of 630Δerm (wildtype), AP34 (P_{tet}-sluc^{opt}) and JC096 (P_{tet}-sigB). Similar results were obtained for strains IB58 and IB61 (data not shown). **B**) Percentage of cells retaining the plasmid in AP34 (P_{tet}-sluc^{opt}) and JC096 (P_{tet}-sigB). **C**) Percentage of cells retaining the plasmid in strains IB61 (sigB::CT P_{tet}-sluc^{opt}) and IB58 (sigB::CT P_{tet}-sigB). Percentages were calculated based on the ratio of CFU/mL of the paired selective (with thiamphenicol)- and non-selective (without thiamphenicol) plates. *** p≤0.001 as determined by an unpaired student’s t-test, n=3.
A

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B

% containing plasmid

C

% containing plasmid
Figure 3. Volcano plot of the transcriptome analysis of the σB regulon. Graphical representation of differential gene regulation upon overproduction of σB. Dashed lines indicated the significance threshold: |log2FC| > 1.5 and adjusted p-value < 0.05. Genes significantly up-regulated by σB are indicated in red, and genes downregulated are indicated in blue. The top-10 of up-regulated genes and 5 selected down-regulated genes are annotated in the figure. An interactive version of the graph is available for exploration via the URL provided in the Text S1.
Figure 4. *In vitro* run-off transcription of selected promoter regions. Samples were run on an 8% urea gel. The two bands corresponding to 82 bp and 64 bp are end-labelled oligonucleotides. Reactions without sigma factor (σB or TcdR, respectively) or RNAP\textsuperscript{core} were analyzed as controls. Asterisks indicate the presence of distinct transcripts. RNAP\textsuperscript{core} = *E. coli* RNA polymerase core enzyme (NEB). A) Controls for the *in vitro* run-off transcriptions. Purified TcdR, a sigma factor demonstrated to activate *tcdA* transcription *in vitro* (33), was used with P\textsubscript{tcdA} (pCD22) as a positive control for the assay. P\textsubscript{cad0872} (derived from pIB21) shows no altered transcription in the DNA array analysis and was taken along as negative control. B) *In vitro* run-off transcriptions for selected genes induced by σB overproduction.
Figure 5. Plate-based luciferase assay shows $\sigma^B$-dependant promoter activity from antimicrobial and hydrogen peroxide exposure. A) Setup of the assay. E-test halos (left panel) were sprayed with luciferase substrate and imaged (right panel). The dotted line indicates the location of the halo based on the left panel. Strain AP34 ($\text{P}_{\text{tet}}$-sluc$^{\text{opt}}$) shows no signal due to the absence of inducer. B) Luciferase reporters of different $\sigma^B$ regulated promoters were tested for luciferase signal after a metronidazole E-test. Halos are indicated by the dashed lines as in panel A. C) Luciferase activity of the $\text{P}_{\text{cd0350}}$ luciferase reporter was imaged in the presence of discs containing 10 µL of various compounds: sterile H$_2$O (mQ), Lincomycin (3000 µg/ml; Lin), metronidazole (200 µg/ml; Met), vancomycin (200 µg/ml; Van), Ibezapolstat (400 µg/ml; Ib) and hydrogen peroxide (1M; H$_2$O$_2$). Halos and the location of the different stressors are indicated by red circles (disc) and black dashed circles (halo).
A  

E-test  

luminescence  

\[ \text{P}_{\text{tet}}-\text{sluc}^{\text{opt}} \]

B  

\[ \text{P}_{\text{cd0350}}-\text{sluc}^{\text{opt}} \]  

\[ \text{P}_{\text{cd2963}}-\text{sluc}^{\text{opt}} \]  

\[ \text{P}_{\text{cd3412}}-\text{sluc}^{\text{opt}} \]  

\[ \text{P}_{\text{cd3614}}-\text{sluc}^{\text{opt}} \]  

wild type  

\[ \Delta\text{sigB} \]

C  

compounds  

disc diffusion  

luminescence  

- mQ  
- Lin  
- H_2O_2  
- Met  
- Ib  
- Van
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<td>4</td>
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<td>4</td>
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<td>136 (132)</td>
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**Table 1.** Setup of the DNA array and number of differentially expressed (DE) genes, including number of positively (POS) and negatively (NEG) regulated genes. Numbers inbetween brackets correspond to the number of differentially expressed genes after the 5 genes from hybridisations 1 and 3 are excluded.
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<td>P-value</td>
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<td>CD630DERM_34090</td>
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**Other genes involved in oxidative/nitrosative stress positively regulated by σB**

<table>
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<td>Carbon monoxide dehydrogenase</td>
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<td>CD630DERM_14740</td>
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<td>Putative rubrerythrin (Rr)</td>
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<td>CD630DERM_15940</td>
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<td>4.44E-03</td>
<td>O-acetyl-serine thiol-lyase A (O-acetyl-sulphhydrase)(OAS-TL)</td>
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<tr>
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<td>CD630DERM_21660</td>
<td>4.7</td>
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<td>Peptide methionine sulfoxide reductase MsrA/MsrB</td>
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**Table 2. Selected genes differentially expressed upon overexpression of σB.** Genes positively regulated by σB aerobic stress and other genes involved in oxidative/nitrosative stress (7, 19) are highlighted here. CD numbers corresponding to the published annotation of strain CD630 (52) can be derived by removing “630DERM_” and removing the last digit (in case of a 0) or replacing it with an A (in the case of a 1).
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<th>Name</th>
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<td>Forward primer for sigB cloning in pET21b, containing a NdeI restriction site</td>
<td>This study</td>
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<td>Reverse primer for sigB cloning in pET21b, containing an XhoI restriction site</td>
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<td>oIB-15</td>
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<td>Reverse primer for Pcd0872, Sacl restriction site</td>
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<td>oIB-26</td>
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<tr>
<td>oIB-27</td>
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<td>Reverse primer for Pcd3412, containing a Sacl restriction site (20)</td>
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**Table 3. Oligonucleotides used in this study.** Restriction sites are underlined, 30 bp overlapping regions as used in Gibson Assembly are indicated in lowercase letters.
Table 4. Plasmids and strains used in this study.

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References


The multidrug-resistant human pathogen Clostridium difficile has a highly mobile, mosaic genome. Nat Genet 38: 779-86.


Complete genome sequence of the Clostridium difficile laboratory strain 630Deltaerm reveals differences from strain 630, including translocation of the mobile element CTn5. BMC Genomics 16:31.

Legends for Supplemental Material

 Dataset S1. Genes differentially expressed in σB overproducing cells compared to controls. Cells were harvested from cultures with (induced) or without (uninduced) ATc, and with (+Tm) or without (-Tm) thiamphenicol. Gene name = generic gene name (or locus tag if not available). log2FC is the log2 of the fold change in gene expression. Four different comparisons are shown, genes are aligned between comparisons. Up-regulated genes are indicated in green. Down-regulated genes are indicated in red. Genes not considered not part of the σB regulon are highlighted in yellow.

 Dataset S2. Manhattan distance for differentially regulated genes from hybridization 2 (cells harvested from cultures grown in the presence of both ATc and thiamphenicol). Change indicates whether expression is higher (increased) or lower (decreased) upon overproduction of σB. log2FC = log2 of the fold change in gene expression. Significance = -10log of the adjusted p-value (see Dataset S1). Manhattan distance was calculated by and exported using the online tool VolcanoseR.

 Text S1. Link to access an interactive version of the volcano plot based on hybridization 2 (cells harvested from cultures grown in the presence of both anhydrotetracyclin and thiamphenicol).