Tracking gene expression and oxidative damage of O₂-stressed *Clostridioides difficile* by a multi-omics approach

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Abstract

Clostridioides difficile is the major pathogen causing diarrhea following antibiotic treatment. It is considered to be a strictly anaerobic bacterium, however, previous studies have shown a certain and strain-dependent oxygen tolerance. In this study, the model strain *C. difficile* 630Δ*erm* was shifted to micro-aerobiosis and was found to stay growing to the same extent as anaerobically growing cells with only few changes in the metabolite pattern. However, an extensive change in gene expression was determined by RNA-Seq. The most striking adaptation strategies involve a change in the reductive fermentation pathways of the amino acids proline, glycine and leucine. But also a far-reaching restructuring in the carbohydrate metabolism was detected with changes in the phosphotransferase system (PTS) facilitated uptake of sugars and a repression of enzymes of glycolysis and butyrate fermentation. Furthermore, a temporary induction in the synthesis of cofactor riboflavin was detected possibly due to an increased demand for flavin mononucleotid (FMN) and flavin adenine dinucleotide (FAD) in redox reactions. However, biosynthesis of the cofactors thiamin pyrophosphate and cobalamin were repressed deducing oxidation-prone enzymes and intermediates in these pathways. Micro-aerobically shocked cells were characterized by an increased demand for cysteine and a thiol redox proteomics approach revealed a dramatic increase in the oxidative state of cysteine in more than 800 peptides after 15 min of micro-aerobic shock. This provides not only a catalogue of oxidation-prone cysteine residues in the *C. difficile* proteome but also puts the amino acid cysteine into a key position in the oxidative stress response.

Our study suggests that tolerance of *C. difficile* towards O_2 is based on a complex and far-reaching adjustment of global gene expression which leads to only a slight change in phenotype.

Keywords

Clostridioides difficile; oxidative stress; RNA-Seq; redox proteomics; metabolomics; cysteine modification

Abbreviations

BHI – brain heart infusion, C-metabolism – carbohydrate metabolism, *C. difficile* - *Clostridioides difficile* 630Δ*erm*, CFU – colony forming units, CoA - Coenzyme A, FAD – flavin adenine dinucleotide, FMN – flavin mononucleotide, FDR – false discovery rate, IAM - iodoacetamide, LMW - low molecular weight, MAIT - Mucosal-associated invariant T, MCP - multiple cysteine peptides, OCP - one cysteine peptides, PBS - phosphate buffered saline, PTS – phosphotransferase system, TMT - Tandem Mass Tag

1. Introduction

Clostridioides difficile (formerly *Clostridium difficile* [1]) is the major nosocomial cause of diarrhea in patients with a compromised gut microbiota, often as a result of a treatment with broad-spectrum antibiotics [2,3]. The symptoms of *C. difficile* infections can range from relatively mild diarrhea to pseudomembranous colitis and toxic megacolon [4,5]. According to current knowledge, these symptoms are mainly caused by the production of two toxins, Toxin A and B which glycosylate Rho-family GTPases after uptake into the host cells, leading to the disruption of the cytoskeleton, apoptosis and a strong inflammatory response [6]. The synthesis of toxins is tightly connected to the central physiology of *C. difficile* [7,8]. Understanding the physiology of the pathogen and how it adapts to environmental changes is therefore a prerequisite for the development of novel therapies. In several published studies, omics-technologies have been employed to investigate the response and

adaptation of *C. difficile* to stress and starvation [9–11]. With respect to the strictly anaerobic lifestyle of some human pathogenic bacteria, the presence of oxygen represents a major challenge [12]. The oxidative stress response of *C. difficile* has been the subject of a few studies [11,13], however, detailed knowledge on the signal transduction, regulatory background and exact detoxification pathways as described for *Clostridium acetobutylicum* [14,15] are still missing. It has been appreciated that specific strains of *C. difficile* exhibit a surprisingly high tolerance to oxygen [16] and that the alternative sigma factor SigB seems to play a vital role in this tolerance [17]. One of the cell's most vulnerable components to oxidation are the cysteine residues of proteins. Concurrently, this amino acid adopts several essential functions in a cell, e.g. the complexation of metal ions and formation of disulfide bridges to stabilize the three-dimensional protein structure, the role as catalytic site of enzymes or the action as molecular switch undergoing cycles of oxidation and reduction [18]. Many redox sensors with regulatory functions are based on the reversible oxidation of cysteine, e.g. OxyR [19], OhrR [20] or Spx [21]. Enzymes with active site cysteines can be irreversibly damaged by oxidation of the thiol group to sulfinic or sulfonic acid. To prevent such irreversible oxidation, a protection of oxidation-prone cysteine residues can be achieved by a temporary disulfide bridging with low molecular weight (LMW) thiols as was shown for the glyceraldehyde-3-phosphate dehydrogenase [22].

Our study is a starting point to unravel the diverse mechanisms which the aero-tolerant model strain *C. difficile* 630Δ*erm* implements in its resistance to low-level oxygen conditions. For this purpose, RNA-Seq was used to comprehensively track changes in gene expression after the shift to micro-aerobiosis, metabolomics was employed to reveal a possible readjustment in metabolism in the presence of oxygen, and a thiol redox proteomics approach was used to determine the extent of reversible cysteine oxidation after oxygen challenge.

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2. Material and methods

2.1 Bacterial strain and growth conditions

All studies were carried out with *C. difficile* 630Δ*erm* (DSM 28645, [23]) obtained

from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ,

Braunschweig, Germany). Cells were cultured in *Clostridium difficile* minimal medium

(CDMM) and growth conditions were as described earlier [24]. At half-maximal

growth, 220 mL cultures in Afnor bottles were flushed with 0.1 standard liters per

minute of either 100% nitrogen or a mixture of 76.2% nitrogen and 23.8%

compressed air (corresponding to 5% absolute O_2) using the gas mix system of an

Infors Labfors 5 system (Infors GmbH, Einsbach, Germany) equipped with Viton

tubing for either 15 or 60 minutes. A single-use C-Chip Neubauer improved counting

chamber (NanEnTek, Waltham, MA, USA) was anaerobically filled with 10 μL of

culture and sealed. Movies of cells were generated using an Axiostar plus microscop

equipped with an Axiocam 105 color camera and the ZEN lite 2012 software (Zeiss,

Oberkochen, Germany). After the defined time 45 mL of culture was harvested by

centrifugation. Samples for RNA preparation and Coenzyme A (CoA) analysis were

directly frozen into liquid nitrogen. Samples for protein analysis were washed twice

with 10 mL cold, anaerobic phosphate buffered saline (PBS) and frozen at -80 °C

until further processing. Samples for metabolic analysis were quenched and washed

as described previously [25]. To determine the number of viable cells per milliliter

[colony forming units (CFU)/mL], 10-fold serial dilutions were prepared for each

sampling point (15 min and 60 min treatment for both O_2 and N_2) in three biological

replicates, plated on brain heart infusion (BHI) agar plates and incubated

anaerobically at 37 °C for 24 h. Colonies per plate were counted and CFU/mL were

calculated.

2.2 Transcriptomics Analysis

2.2.1 RNA isolation

Total bacterial RNA was isolated from bacterial cell pellets, from three independent experiments, using acid phenol and glass beads as described in Rosinski-Chupin *et al.* [26]. DNA was digested using TURBO DNase (Ambion, Invitrogen) and the DNA-free RNA was purified with phenol:chloroform:isoamylalcohol (25:24:1). Remaining traces of phenol were removed by washing the samples twice with chloroform:isoamylalcohol (24:1). RNA integrity was assessed using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies). To 2 µg of total RNA 1 µL of 1:10 diluted ERCC ExFold RNA Spike-In Mix 1 or 2 (Ambion) was added. rRNA was depleted using the Ribo-Zero rRNA Removal Kit (Gram-Positive Bacteria) as recommended by the manufacturer (Epicentre).

2.2.2 Strand-specific library preparation and Illumina sequencing

Strand-specific barcoded cDNA libraries were prepared using the ScriptSeq v2 RNA-Seq Library Preparation Kit as recommended by the manufacturer (Epicentre). Library quality was validated using Agilent 2100 Bioanalyzer (Agilent Technologies) following the manufacturer's instruction. Cluster generation was performed using the Illumina cluster station. Single-end sequencing on the Illumina HiSeq2500 followed standard protocol. The fluorescent images were processed to sequences and transformed to FastQ format using the Genome Analyzer Pipeline Analysis software 1.8.2 (Illumina). The sequence output was controlled for general quality features, sequencing adapter clipping and demultiplexing using the fastq-mcf and fastq-multxtool of ea-utils [27].

2.2.3 Bioinformatics and statistics

Quality of the sequencing output was analyzed using FastQC (Babraham Bioinformatics). All sequenced libraries were mapped to the *Clostridioides difficile*

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630Δ*erm* genome [28] using Bowtie2 (Vers. 2.2.3) [29] with default parameters. After read mapping, SAMtools [30] was employed to filter the resulting bam files for uniquely mapped reads (both strands). Reads were classified as uniquely mapped reads with a unique genomic location if and only if they could not be aligned to another location with a higher or equal mapping quality. The resulting bam files constituted the basis for all further downstream analyses.

2.2.4 Feature quantification and detection of differentially expressed genes

Reads aligned to annotated genes were quantified with the *htseq-count* program [31] using the updated *C. difficile* 630Δ*erm* annotation by Dannheim *et al.* [28]. Determined uniquely mapped read counts served as input to *DESeq2* [32] for pairwise detection and quantification of differential gene expression. For *DESeq2* parametrization we used a beta prior and disabled the Cook distance cut off filtering. All other parameters remained the same. The lists of *DESeq2* determined differentially expressed genes which were filtered by a multiple testing corrected p-value of at most 0.05 [33]. The Perseus software platform [34] was used to carry out ANOVA testing and to generate heatmaps on the basis of DESeq2 rlog transformed count data. Voronoi treemaps of *DESeq2* data were constructed using the Paver software (DECODON GmbH, Germany) based on an assignment of corresponding gene products to TIGRFAM roles [35] as described recently [36].

2.3 Proteomics Analysis

2.3.1 Differential isotopic alkylation of cysteine (diaCys) and MS sample preparation

For the diaCys protocol, cell pellets were resuspended in 600 µL lysis buffer (8 M urea, 10 mM EDTA, 1% CHAPS, 200 mM TrisHCl pH 8.0) containing 50 mM iodoacetamide (IAM, Sigma-Aldrich). For a label switch two of the four independent replicates were dissolved in 600 µL lysis buffer containing heavy IAM (Sigma-Aldrich) for this first alkylation step. Cells were lyzed, protein cysteine residues were

differentially labeled according to the diaCys approach and the proteins were then separated via SDS-PAGE according to Sievers *et al*. [37]. Gel lanes were cut into 10 slices, proteins trypsinized in-gel, peptides eluted and desalted as described previously [36].

2.3.2 LC-MS/MS- analysis and raw data processing

Liquid chromatographic separation of peptides was achieved using the EASY-nLC 1000 system (Thermo Scientific) and subsequent peptide detection by an Orbitrap Elite Hybrid mass spectrometer (Thermo Scientific) was performed as described elsewhere [36]. Database searches and quantification were performed within the framework of the MaxQuant software suite (Vers. 1.5.7.0) using the Andromeda algorithm [38]. Only fully tryptic peptides with no more than two missed cleavages were considered. Spectra were searched against the reannotated *Clostridioides difficile* 630Δ*erm* database published by Dannheim *et al*. [28] containing 3781 protein entries and common contaminations. The tolerance for precursor ion mass was 10 ppm and for fragment ion mass 0.5 Da. Samples were searched for twice, firstly as a label experiment of cysteine (light IAM C_2H_4INO and heavy IAM $^{13}C_2H_2D_2INO$) and secondly with a variable IAM modification of cysteine as described elsewhere [37]. A variable oxidation of methionine was allowed in all searches. Information on the abundance of reduced and oxidized species of cysteine peptides was obtained from the MaxQuant intensities of corresponding peptides. A false discovery rate of no more than 1% at peptide and protein level was allowed. Statistical significance of results was assessed by an unpaired Student's t-test.

2.3.3 Western Blotting of iodo Tandem Mass Tag (TMT) zero

C. difficile were grown up to an A_{600} of 0.4 when the cells were flushed with 0.1 standard liters per minute of 100% nitrogen (control). Another four cultures were flushed at the same rate with a synthetic gas of 5% O_2 in N_2 (micro-aerobic stress).

After 15 min, cells were harvested by centrifugation and cell pellets were washed three times with ice cold, oxygen-free PBS. Cells were lyzed by ultrasonication in UHE buffer (8 M urea, 200 mM HEPES, 10 mM EDTA, pH 8.0) containing 50 mM IAM, cell lysates were left for 30 min in the dark at 30 °C to allow for blocking of all reduced protein cysteine residues. Cell debris were removed by centrifugation and proteins were precipitated with acetone at -20 °C. Dry protein pellets were dissolved in UHE buffer and protein concentration was determined. 20 µg of proteins of each sample were treated with 5 mM Tris-(2-carboxyethyl)-phosphin (TCEP) for 15 min at 30°C to reduce all reversibly modified cysteines to thiols. Originated thiols of each sample were alkylated with 1/10 of the content of one iodoTMTzero (Thermo Scientific) vial and incubated at 30 °C for 1 h. Proteins and a protein ladder (Precision Plus Protein Kaleidoscope Prestained Protein Standard, Biorad) were separated via SDS-PAGE (gradient 4-15%, Biorad) for 1 h at constant voltage of 120 V, and blotted onto a PVDF membrane for 1 h at 100 V. The membrane was incubated for 90 min in PBS buffer containing 0.05% Tween (PBST) and 5% skimmed milk powder. After three washing steps in PBST, the 1st antibody (1:1,000 of TMT Monoclonal Antibody for Western Blot, Invitrogen, in 150 mM NaCl, 5% bovine serum albumin, 0.02% Azide, 50 mM TrisHCl pH 7.6) was bound to the membrane overnight at 4 °C. The membrane was washed three times in PBST, before incubation with the 2nd antibody (goat anti-Mouse IgG, Alexa Fluor Plus 680, Invitrogen) diluted 1:15,000 in PBST, 5% skimmed milk powder, 0.02% SDS at room temperature for 1 h. The membrane was washed three times in PBST. Signals reflecting the oxidative state of cysteine were detected on an Odyssey CLx (LI-COR) at 700 nm and quantitated by Image Studio Vers. 2.0.

2.4 Metabolomics Analysis

For analysis of the metabolome, cell lysis and metabolite extraction were performed for five independent experiments as described previously [39]. Extracellular samples were prepared and volatile and non-volatile compounds in the culture supernatants and cell extracts were analyzed as described by Neumann-Schaal *et al.* [24]. Raw data obtained from GC/MS measurements were processed by applying Vers. 2.2N-2013-01-15 of the in-house developed software MetaboliteDetector [40]. The peak identification was performed with a non-targeted and combined compound library for each GC column applied. After processing, non-biological peaks and artefacts were eliminated by the aid of blanks. Peak areas were normalized to the corresponding internal standards (o-cresol or ribitol) and derivatives were summarized. For CoA-ester analysis, cells were lysed using a Precellys 24 homogenisator (Peqlab, Germany) at -10 °C and the CoA-esters were extracted on a Strata XL-AW solid phase extraction column (Phenomenex, Germany). Analysis and data processing were performed as described earlier [41]. Statistical significance of differences between metabolite levels was evaluated by non-parametric Wilcoxon-Mann-Whitney test using the Benjamini–Hochberg correction to control the false discovery rate [33,42].

3. Results and Discussion

3.1 General observations

Surprisingly, we did not observe any growth impairment after 15 min of O_2 challenge compared to the reference condition (ΔA_{600nm} O_2 = 0.105 ± 0.017 and ΔA_{600nm} N_2 0.098 ± 0.017). In samples subjected to 1 h of oxygen stress, growth was slightly impaired (ΔA_{600nm} O_2 = 0.135 ± 0.012 and 0.161 ± 0.025 for N_2). Distorted optical densities caused by morphological differences were excluded by analyzing cells under the microscope and by counting the number of viable cells at each sampling time point

(Video S1, Fig. S1). However, both cultures were apparently stressed by the increased agitation due to the gas flow. The influence of agitation on *C. difficile* has been observed before and may be attributed to a disruption of the fragile exopolysaccharide matrix [39]. Oxygen stressed cultures turned darker compared to reference cultures which may be attributed to increased iron sulfide precipitation.

3.2 Transcriptomic adaptations

In order to obtain a comprehensive picture on the adaptation of gene expression under micro-aerobiosis, a transcriptomic analysis was performed applying RNA-Seq technology. The complete and unfiltered raw data of the analysis is deposited in the NCBI database under GEO accession GSE114463 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114463) and can be viewed by using token abgfgsmerlyxval. Due to the restrained effect on growth caused by 5% O_2, the observed and tremendous change in gene expression was not expected. Of the approximately 2,000 RNAs, which could be statistically reliably quantified (Table S1), 49 RNAs significantly changed in abundance after 15 min of O_2 exposure (adjusted p-value < 0.05), (Fig. S2A). After one hour of micro-aerobiosis more than 20% of the quantified RNAs (adjusted p-value < 0.05) were found to be altered in amount (Fig. S2B). For a global overview an ANOVA test requiring a permutation based false discovery rate (FDR) of 0.01 was performed, resulting in 89 differentially expressed RNAs which were presented in a heatmap (Fig. 1). When applying a FDR of 0.05 a total of 316 RNAs were identified to be differentially expressed (Fig. S3). Additionally, relative quantification data for the 15 min and 60 min time point were visualized in Voronoi treemaps with allocation of RNAs to TIGRFAM roles (Fig. S4).

3.3 Oxidation of protein thiols

Aim of the proteomics analysis was to identify protein damage in terms of reversible cysteine oxidation. With respect to the observed oxygen tolerance of *C. difficile* 630Δ*erm* [16], it was not expected to see extensive oxidative damage within the thiol proteome, but rather a specific oxidation of exceedingly sensitive cysteine residues. Whereas most of the ~1,800 redox-quantified peptides that contain one cysteine residue (OCPs for <u>o</u>ne <u>c</u>ysteine <u>p</u>eptides) exist in a highly reduced state (< 30% oxidation), a dramatic shift towards higher oxidation rates, with the majority of peptides being oxidized by 40-60%, was observed (Fig. 2). Peptides containing multiple cysteine residues (MCPs for <u>m</u>ultiple <u>c</u>ysteine <u>p</u>eptides) have also been detected in this analysis. However, redox-quantification of the cysteine residues in such MCPs proved to be a challenging task [37] and was left out from the primary evaluation in this study.

The average of the oxidized proportion of all redox-quantified OCPs at control conditions (N_2) was 27.7%, whereas the average of the oxidized fraction of OCPs after 15 min O_2 challenge was 49.7% (Table S2). This is an increase of 22% of the overall oxidation status of the thiol proteome. To validate this finding in the overall oxidation state of protein cysteines after O_2 challenge, we performed an experiment, in which reduced cysteine residues were blocked with iodoacetamide in the first step, and all free thiols of cysteines, that arose after reduction with TCEP, were alkylated with iodoTMTzero. Protein extracts were separated via SDS-PAGE and iodoTMTzero labeled proteins visualized on a Western Blot employing an iodoTMTzero specific antibody (Fig. S5). Comparing the total signal intensity of Western Blots from control samples and O_2 stressed samples results in a calculated increase of 35% in cysteine oxidation in the O_2 stress samples, higher than the 22% determined by the diaCys approach, and also validates the finding of a comprehensive oxidation of the thiol proteome of *C. difficile* 630Δ*erm* after 15 min of micro-aerobiosis. A question that

remains is the nature of the thiol oxidation and how *C. difficile* can proceed to grow with a large part of its thiol proteome being oxidized or modified. The diaCys assay that was employed in this study is capable of determining reversible cysteine oxidation, e. g. the oxidation to sulfenic acid or the formation of a disulfide bridge. Knowing about the dimension of reversible cysteine modifications one could speculate on an intentional and reversible large-scale modification of protein cysteine residues in order to protect them from irreversible oxidation and damage. Such transient reversible modifications of cysteine residues by LMW thiols were described several times in other bacteria. The most studied LMW thiol is certainly glutathione which can form mixed disulfide bonds with cysteine residues and reduce them back to thiols [43]. However, not all bacteria possess glutathione but feature other LMW thiols that can fulfill this redox-balancing function. In several Gram-positive bacteria with low GC content the LMW thiol bacillithiol [44] is involved in the redox signal transduction [45] and can adopt a cysteine-protecting function [46]. A similar role is taken by the LMW thiol mycothiol in *Actinobacteria* [47]. There are no homologs for the synthesis of glutathione or bacillithiol encoded in the *C. difficile* 630Δ*erm* genome. The identification of a LMW thiol which could possibly protect cysteine in proteins, for instance, during micro-aerobic shock is a focus of our current research. The difference of the redox state of a specific cysteine residue under stress and control conditions represents the change in redox state this cysteine undergoes when *C. difficile* is shifted to micro-aerobiosis. Doing this for all OCPs that could be redox-quantified in at least three out of four replicates (1484 peptides, Tab. S2) once more reveals the sweeping oxidation of *C. difficile´*s thiol proteome with 780 peptides showing an increase in oxidation of up to 50% (p-value < 0.01), (Fig. 3), which is even more prominent than the affect the disulfide instigator diamide has on the thiol proteome of *C. difficile* [37]. This observation gives rise to the question how the

pathogen manages to keep growing after such a dramatic modification of its thiol proteome and once more leads to the hypothesis that the modification is of a protective nature rather than caused by oxidative damage.

3.4 Metabolic changes

Compared to the changes observed in the transcriptome and proteome, changes in the metabolic level were unexpectedly low (Table S3). In the non-targeted approach 101 intracellular and 43 extracellular medium metabolites were determined. The few metabolomic changes, which are more precisely discussed in section 3.5, are dominated by alterations in fermentation associated pathways and the cysteine balance (Fig. 4). However, none of these findings severely affected growth which shows the absolute adaptation of the strain to physiologic oxygen concentrations observed in the human gut epithelium [12].

3.5 Physiological rearrangement during micro-aerobiosis

Despite the moderate effect 5% O_2 challenge had on the growth of *C. difficile* 630Δ*erm*, an extensive modulation in gene expression was revealed by the comprehensive analysis in this study. The affected genes are distributed over a wide range of functional classes (Table 1). The most prominent ones represent genes involved in the metabolism of the amino acids proline, glycine, cysteine and leucine, and genes that are involved in carbohydrate metabolism, predominantly enzymes of fermentation and sugar import via phosphotransferase-systems (PTS). Figure 5 gives an overview of those central metabolic rearrangements. Further clusters of genes that are characterized by a change in expression are involved in the synthesis of the cofactors riboflavin, cobalamin and thiamin or associated with cell surface and sporulation processes. Also, genes for DNA replication and repair as well as enzymes for purine synthesis show a modified expression, just as gene products anticipated to play a central role in the oxidative stress response (e.g. thioredoxins,

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chaperones and proteases). Three functional clusters of genes will be discussed in more detail at this point.

3.5.1 Amino acid metabolism

One of the most striking observations in the metabolomics analysis was the accumulation of proline in the extracellular medium in the O_2 challenged cultures by a factor of 17 compared to control cultures (Fig. 4, Table S3). Intracellularly, proline was below the detection limit in the control sample indicating an efficient turnover of the amino acid. However, proline could be measured in the cytosol of O_2 stressed cells suggesting its hampered metabolism. Strikingly, a strong repression of the genes encoding the proline-t-RNA ligase (*proS1* and *proS2*) after 60 min at 5% O_2 compared to control conditions was determined (Table 1). One could assume that the higher expression of t-RNA ligase at control conditions might compensate for the very low concentration of proline and ensure sufficiency of charged proline-t-RNAs for protein synthesis.

On the contrary to proline, the concentration of cysteine dropped dramatically in the medium after 60 min of micro-aerobiosis indicating a stronger uptake of this amino acid. Simultaneously, the genes for the de-novo synthesis of cysteine (*cysK, cysE*) from serine were strongly induced, according to the RNA-Seq data (Fig. 5, Table 1). The higher demand of cysteine during times of oxidative stress could be explained by the increased synthesis of oxidative stress proteins, whose reducing power relies on the redox-cycling of cysteine. But also, the novel synthesis of proteins that underwent oxidative damage on cysteine residues or the need for cysteine to produce a still unknown LMW thiol, which could protect vulnerable cysteine residues from oxidation by formation of reversible disulfide bridges, could be an explanation for the higher demand of cysteine.

Another amino acid that differed in amount between O_2 and N_2 samples is glycine, which is more abundant intracellularly in the O_2 stressed cells (Fig. 4, Table S3). The entire operon of the glycine reductase complex *grd* including a thioredoxin and a thioredoxin reductase (genes: *grdDCBAE, trxA2, trxB3, grdX*) was consistently downregulated at micro-aerobic conditions (Table 1). An oxidative inactivation of the Grd enzyme complex is conceivable, since the catalytic mechanism was described to involve a reactive cysteine that forms a thiol ester [49]. In the diaCys analysis one cysteine residue (Cys26) of the selenoprotein A (GrdA) of the Glycine reductase complex was detected with an increased oxidation of 20% (Table S2).

Whereas catabolism of glycine and proline decreased, an elevated reductive fermentation of leucine was indicated by induction of the *had* operon (*hadAIBC*), [50] and enzymes involved in the transformation of 2-isocaprenoyl-CoA to isocaproyl-CoA (*acdB*, *etfA* and *etfB*), (Fig. 6A). Furthermore fermentation intermediates as 2-hydroxyisocaproate, 2-hydroxyisocaproyl-CoA and Isocaproyl-CoA were found in higher abundance in the intracellular metabolome and the concentration of leucine in the growth medium was decreased. Higher levels of 2-hydoxybutanoate may also reflect the higher activity of this pathway with 2-oxobutanoate/threonine as a substrate.

3.5.2 Carbohydrate metabolism

Not only was the amino acid metabolism restructured during micro-aerobiosis, but also the usage of carbohydrates was significantly altered (Fig. 5, Table 1). Giordano *et al*. [13] analyzed the alterations in *C. difficile* that was grown under 2% O_2 for 4.5 h in complex medium. They reported a repression of glucose import, with induced transportation of alternative carbon sources such as ribose, fructose, mannose, and lactose instead. This partially corresponds to the data of this study. Many components of PTS-systems for sugar import were found to be repressed after 60

min of O_2 exposition including the ones for glucose and beta-glucosides. Only component IIB of a fructose-type PTS-system and a PTS-system mannose-type IIA component were statistically significantly induced according to the transcriptomics analysis (Table 1). The decreased usage of glucose under micro-aerobiosis can also be deduced from the downregulation of the enzymes of glycolysis, a higher concentration of glucose in the growth medium and slightly also intracellularly, but a lower concentration of glycolysis intermediate metabolites (Fig. 6B). Remarkably, oxidized cysteine peptides could be identified for all proteins encoded by the hexacistronic operon of glycolytic enzymes (Table 2) indicating their possible oxidative inactivation.

The alteration in carbon metabolism is furthermore reflected by the induction of *ccpA*, a global regulator of carbon metabolism, repression of the 6-phospho-β-glucosidase *bglA3*, repression of succinate-semialdehyde dehydrogenase *sucD* by a factor of 12 (60 min value) and the decreased expression of an operon involved in butanoate fermentation (*bcd, etfB3, etfA3, crt2, hbd, thlA1*) (Fig. 5, Table 1). The latter observation is supported by metabolomics data which revealed an intracellular decrease of coenzyme A-derivatives of the butanoate fermentation pathway and its side products (3-hydroxybutanoyl-CoA, 3-hydroxypentanoyl-CoA, pentenoyl-CoA) with only 50% of butanoate detectable extracellularly after 60 min of oxygen exposition (Fig. 4, Table S3). Furthermore, the diaCys approach revealed cysteines of the proteins Bcd (butyryl-CoA dehydrogenase catalytic subunit), EtfB3 (butyryl-CoA dehydrogenase electron transfer flavoprotein beta subunit), EtfA3 (butyryl-CoA dehydrogenase electron transfer flavoprotein alpha subunit) and Hbd (3-hydroxybutyryl-CoA dehydrogenase) to be significantly oxidized after O_2 treatment (Table S2).

3.5.3 Cofactor synthesis and transport

The last cluster of affected genes to be discussed in detail in this work, are the gene products involved in the synthesis or uptake of cofactors.

Most striking is the strong upregulation of the uptake and *de novo* synthesis of riboflavin after 15 min of O_2 challenge (*ribU, ribH, ribBA, ribD*). The period of this induction is transient, as it cannot be detected after 60 min of micro-aerobiosis (Table 1). An increase in riboflavin as a first line of defense against oxidative stress is conceivable as many electron-transfer proteins are flavoproteins and thus rely on riboflavin as a cofactor. Due to the low redox potential of flavins, also a fast oxidation of FMN and FAD and hence an oxidative damage of enzymes featuring such cofactors is conceivable [51]. In either case, the concentration of reduced FMN and FAD cofactors in the cells drops explaining the higher demand for riboflavin. Although there was no riboflavin provided in the growth medium, expression of transporter *ribU* is induced most likely because of a post-transcriptional regulation via a FMN riboswitch [52]. The fast but short-lived induction of riboflavin is also interesting from a patho-immunological point of view. In 2012 Kjer-Nielsen *et al.* reported Mucosal-associated invariant T (MAIT) cells to be activated by intermediates of the riboflavin synthesis that are presented by the major histocompatibility complex (MHC) class I-like molecule MR1 [53]. *C. difficile* cells that extensively synthesize riboflavin after an oxidative shock could therefore be recognized and eliminated by the host´s MAIT cells. In *Streptococcus pneumoniae* a variation in riboflavin metabolism was recently shown to play a significant role for the activation of MAIT cells and the outcome of infection [54].

Riboflavin was not the only effected cofactor under micro-aerobiosis. The operon for the synthesis of thiamine pyrophosphate (*thiCSFGH1*) is repressed in the presence of O_2 (Table 1). Thiamine pyrophosphate is a cofactor of enzymes catalyzing the transfer of carboxy-groups. The mechanism of such reactions involves radical

intermediates. Reactive oxygen species that originate from O_2 in the stressed samples possibly interfere with the radical-dependent enzyme functions.

Also, synthesis of cobalamin is downregulated during micro-aerobiosis (Table 1). Transcription of multiple genes from the gene locus CDIF630erm_03727 to CDIF630erm_03748 encoding for enzymes required for cobalamin synthesis are repressed in the O_2 samples with enzymes HemB (delta-aminolevulinic acid dehydratase), CobA-HemD (bifunctional uroporphyrinogen-III methyltransferase/uroporphyrinogen-III synthase), CbiKP (sirohydrochlorin cobaltochelatase) and CobT (nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase) complying with an adjusted p-value < 0.05. Two different pathways of cobalamin synthesis are known - an aerobic and an anaerobic pathway. The latter involves intermediates that are not stable in the presence of oxygen [55]. In this light, the decline of cobalamin synthesis appears reasonable.

Due to the high number of genes which showed alterations in expression when *C. difficile* was stressed with 5% O_2 not all affected genes could be discussed in detail at this point. A complete list of differentially expressed genes is provided in the supplementary data (Table S1).

4. Conclusions

The multi-omics approach of this study gave rise to a comprehensive picture on the stress response of the strictly anaerobic pathogen *C. difficile* to O_2 at the level of RNA expression, protein damage and metabolic profiles. By choosing two different sampling time points, a direct and very fast reaction involving possible signal transduction pathways could be detected, but also an extensive adaptation process after one hour of O_2 challenge could be determined. This study not only validates gene expression results from previous studies [11,13], but also significantly extends

the knowledge on the oxidative stress response of *C. difficile* 630∆*erm* and is a starting point to explain the O_2 tolerance of this pathogen. Because *C. difficile* is exposed to traces of O_2 and to high concentrations of reactive oxygen species during inflammation, the mechanistic elucidation of its stress response is of major importance, and identification of specific elements mediating the observed oxidative tolerance could turn into promising targets for the development of novel therapies.

Appendix A. Supplementary data

Supplementary data related to this article can be found at

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Elucidation of the anaerobic pathway for the corrin component of cobalamin (vitamin
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Figure legends

Figure 1: Differential gene expression according to RNA-seq analysis. A

heatmap was generated on the basis of ANOVA testing of RLOG data of the RNA

analysis. Induction of gene expression is displayed in red and repression in blue. A

number of 89 RNAs met the criteria of a permutation based FDR of 0.01. Results of

three independent experiments are visualized.

Figure 2: **Oxidation of OCPs after 15 min of 5% O_2 exposure.** Redox-quantified

OCPs were sorted into 10% steps according to their redox state. The average of at

least three replicates was plotted for both, control condition (N_2) and O_2 challenge.

Figure 3: Changes in redox state of OCPs. Volcano plots show the difference in

redox state of single OCPs after challenge with 5% O_2 for 15 min compared to control

conditions. Each spot represents one peptide, and peptides to the right of the y-axis

were oxidized whereas peptides to the left were reduced. The higher the value on the

y-axis (-log10 of p-value), the more significant is the change in redox state. A line

corresponding to p-value 0.05 is indicated.

Figure 4: Metabolic alterations. Changes in the intracellular (A) and extracellular (B) metabolome after challenge with 5% O_2 for 15 and 60 min compared to control cells. Data was obtained from five replicates. Changes in metabolite concentration are depicted as log2 fold change. Metabolites to the right of the y-axis were increased in abundance, while metabolites to the left were less abundant. The higher the value on the y-axis, the more significant the data (-log10 of adjusted p-value). Metabolites discussed in this paper are labeled.

Figure 5: Overview of changes in carbon and amino acid metabolism after 60 min of 5% O_2-challenge. Gene names of the upregulated enzymes are shown in dark grey and marked with ↗, downregulated enzymes are shown in light grey and marked with ↘. Figure was partially adapted from Dubois *et al*. [48].

Figure 6: **Modulation of specific metabolic pathways**. The reduction of leucine to isocaproate via Stickland reaction was induced (**A**) and glucose utilization via glycolysis was repressed (**B**) after 60 min of 5% O_2 challenge. Gene names of enzymes, their determined log2 fold change and corresponding p-value are shown in green. If determined, log2 fold changes of pathway intermediates (boxed) are given in blue. Positive values indicate an increase and negative values a decrease in gene expression and metabolite concentration, respectively.

Table legends

Table 1: Functional classes of differentially expressed genes during micro-aerobiosis.

Selection of gene products that belong to specific functional classes of genes which are characterized by an altered expression after *C. difficile* was stressed with 5% O_2.

Table 2: **Thiol oxidation of glycolytic proteins.** Peptides of several glycolytic enzymes were determined to be oxidized on cysteine residues after 15 min O_2

exposure. Proteins, amino acid sequences of oxidized peptides and the specific

increase in oxidation are given.