Gene expression profiling during heat shock response of *Campylobacter* (*C.*) *jejuni*, *C. coli* and *C. lari*

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Introduction

Although *Campylobacter* spp. is lacking typical stress response mechanisms, e.g. the sigma factor RpoS, it is able to survive in the environment and overcome the barriers along the food chain. The phenotypic response of *Campylobacter* (*C.*) *jejuni* to temperatures above the physiological range is sufficiently characterized. Nevertheless, details about heat shock response mechanisms, metabolic pathways and regulatory factors of *C. jejuni* are still unknown and survival strategies of *C. coli* and *C. lari* at high temperatures are largely unexplored.

First, survival rates at 46 °C were determined to compare the individual fitness of *Campylobacter* species at high temperatures. The aim of our study was to identify genes involved in responses to temperature upshift from 37 °C to 46 °C. It was conducted to examine and compare the transcriptome changes of *C. jejuni* NCTC11168, *C. coli* RM2228 and *C. lari* RM2100 as a consequence of high temperatures.

Material and methods

Bacterial strains, media and growth conditions

C. jejuni NCTC11168, *C. coli* RM2228 and *C. lari* RM2100 were grown on Mueller-Hinton agar containing 5 % sheep blood (MHB; OXOID) for 48 h or in *Brucella* broth (BB; BD) for 24 h at 37 °C in microaerobic conditions.

Survival studies

Bacterial cultures with an OD_{600} of 0.01 (approx. 10^6 to 10^7 CFU/ml) were incubated at 37 °C and 46 °C. Cell numbers were determined by plating the serially diluted culture on MHB agar plates. The survival rates were calculated as percentage of the inoculum.

RNA preparation

Cultures with an OD_{600} of 0.1-0.2 (approx. 10^9 CFU/ml) were exposed to 46 °C for 30 min before RNA extraction. RNA for RT-qPCR was extracted using a peqGOLD Bacterial RNA Kit (Peqlab). RNA for RNA sequencing was extracted using a hot phenol method [1]. RNA integrity and quantity were determined on an Agilent 2100 Bioanalyzer (Agilent Technologies).

RT-qPCR

1 µg of DNase treated RNA were transcribed into cDNA (RevertAid Premium First Strand cDNA synthesis kit and random hexamer primers; Fermentas). Amplification was performed by a CFX96 Real-time system (Bio-Rad) using SYBR-Green assays (SsoFast EvaGreen Supermix; Bio-Rad). Gene-specific primers were designed using the Primer3 input software (http://frodo.wi.mit.edu/) based on the *C. jejuni* NCTC11168, *C. coli* RM2228 and *C. lari* RM2100 genome sequences.

RNA sequencing (RNAseq)

The libraries were generated (Vertis Biotechnologie AG). RNA samples were treated with Terminator exonuclease and poly(A)-tailed. The 5'PPP were removed using tobacco acid pyrophosphatase followed by ligation of the RNA adapter to the 5'-monophosphate of the RNA. First-strand cDNA synthesis was performed with an oligo(dT)-adapter primer and the M-MLV reverse transcriptase. The cDNA was PCR-amplified to reach a concentration of 20-30 ng/µl. The cDNA was purified using the Agencourt AMPure XP kit (Beckman Coulter Genomics) and analyzed by capillary electrophoresis. The primers used for PCR amplification were designed for TruSeq sequencing according to the instructions of Illumina. The combined length of the flanking sequences is 146 bases. The libraries were sequenced with a Illuminua HiSeq machine with 100 cycles in single end mode.

Bioinformatical analysis

The resulting sequence reads were demultiplexed and the adapter sequences removed. The reads (Fastq format) were quality trimmed with a quality cut-off score of 20 and converted to fasta format using fastq_to_fasta [2]. The read processing was performed with the RNA-analysis pipeline RAPL version 0.1 [3] with default parameters which used segemehl version 0.1.3 [4] for the read alignment and DESeq 1.12.0 for differential gene expression analysis [5].

Results

Survival studies

Survival studies at 46 °C revealed differences in the ability to adapt to increased temperature among species (Fig. 1). *C. jejuni* showed a greater ability to survive than the other tested isolates of *C. coli* and *C. lari*. Apart from the 2 h-value cell counts of *C. jejuni* were significantly higher compared to *C. coli* and *C. lari*. While *C. lari* showed a slightly higher decline of cell count no cells were detectable after 8 h in both species.

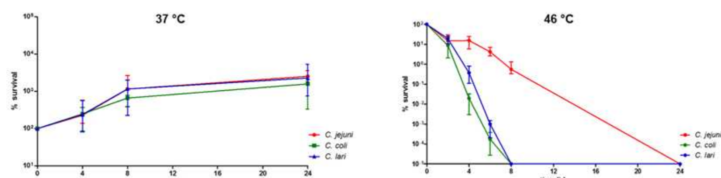

Figure 1: Survival rates (in %) of *C. jejuni* NCTC11168, *C. coli* RM2228 and *C. lari* RM2100 at 37 °C and 46° C over a period of 24 h. Data indicate the medians ± IQR (n = 3)

Gene expression analysis via RT-qPCR

C. jejuni and *C. coli* responded to a temperature upshift from 37 °C to 46 °C with increased expression levels of common heat shock genes e.g. *clp*B, *dna*K, *gro*ES and *grp*E. Apart from down-regulated genes in *C. coli,* different expression levels of the individual genes lead to a pattern that occurs uniformly in *C. jejuni*. This is not evident in *C. lari*. As the expression levels of these heat shock genes are low in *C. lari*, the involvement of these genes as major genes in the heat shock response is questionable (Fig. 2).

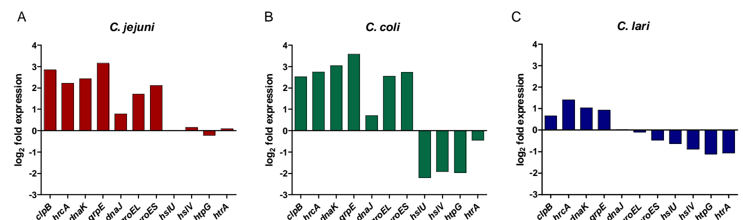

Figure 2: Expression levels of known heat shock genes in *C. jejuni* NCTC 11186 (A), *C. coli* RM2228 (B), and *C. lari* RM2100 (C) analyzed by RT-qPCR following a temperature increase from 37 °C to 46 °C.

RNAseq analysis of heat shock response

Exposure to 46 °C revealed diverse transcriptome changes among *Campylobacter* species (Fig. 3). The highest rate of regulated genes (two-fold change and $p<0.05$) have been observed for *C. coli* (9.11%), followed by *C. lari* (8.97%) and *C. jejuni* (2.70%). In *C. jejuni* 89% of regulated genes showed an increased rate of transcription, whereas in *C. coli* 69% and in *C. lari* 45% are up-regulated. For quality control, the correlation of $\log_2$ fold change values of RT-qPCR and RNAseq results was determined from 0.7687 to 0.8472 for R^2 (data not shown).

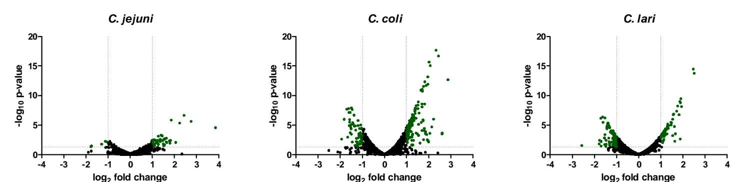

Figure 3: Volcano plots of gene expression pattern of RNAseq results. Green= regulated genes with $p < 0.05$

Heat map analysis visualized the distribution of regulated genes in *C. jejuni*, *C. coli* and *C.lari*. Several genes show in all transcriptomes a temperature dependend regulation (Fig. 4, box II and III), while some genes involved in the heat shock response lack orthologous in the other species (Fig 4, box V). *C. jejuni* shows a higher accordance of the temperature dependent gene expression profile to *C. coli* than to *C. lari* (Fig. 4, box I), which corresponds with RT-qPCR data.

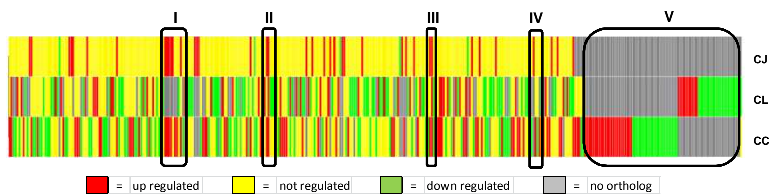

Figure 4: Heat map of gene expression of all regulated genes (with orthologs listed by Fouts et al., 2005) at 46 °C.

Discussion

Strains of *C. jejuni*, *C. coli* and *C. lari* show differences in their ability to adapt to temperature changes. At 46 °C *C. jejuni* has significantly higher survival rates than the other two species. The survival curves of *C. coli* and *C. lari* are almost identical, while the investigated heat shock genes in *C. lari* show a significantly lower expression at this temperature in RT-qPCR. RNAseq studies revealed considerable differences in temperature dependent gene regulation among *Campylobacter* species. At this point the complexity of transcriptome changes in *Campylobacter* is still not fully understood. For that, it is important to structure regulated genes in functional groups and understand mechanisms like co-regulation and the role of other regulatory components.

Acknowledgement
This project was conducted within the scope of the project "Food-Borne Zoonotic Infections of Humans " (FBI Zoo), which is funded by the German Bundesministerium für Bildung und Forschung (BMBF). Parts of this work was performed by the Research Center for Infectious Diseases, University of Würzburg, (Germany).

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