Patterns of global expression changes during heat- and cold-stress in *Vibrio parahaemolyticus*

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Background

Vibrio (V.) parahaemolyticus is a potentially pathogenic bacterium that is widely distributed in marine ecosystems. This species is frequently isolated from sea water, sediment and raw or insufficiently cooked seafood (e.g. crustacean, mussels). Consumption of contaminated seafood or contact to contaminated sea water can cause infections.

The gene expression of *V. parahaemolyticus* is influenced and altered by environmental conditions. For example changing temperatures at storage or during heating of seafood could change the gene expression profile. Non-lethal heat- and cold-stress can induce cellular defense mechanisms like increased production of chaperons as a response to heat-shock. This altered gene expression may improve the survival of *V. parahaemolyticus* in adverse environments.

Heat and cold treatments are applied reducing the numbers of viable bacteria in foods. An induction of a change of gene expression in surviving microorganisms is to be expected. The gene expression of *V. parahaemolyticus* in the border zone between sublethal damage and inactivation due to different temperatures has not been investigated extensively so far.

Therefore we analyzed the impact of altered temperature conditions on the global gene expression of *V. parahaemolyticus*. Different temperatures of relevance were chose to observe its impact thoroughly.

Material and Methods

Incubation and RNA-extraction
Cultures of 10^8 to 10^9 cfu/ml of the completely sequenced strain *V. parahaemolyticus* RIMD 2210633 were grown aerobically and incubated at 4 °C, 15 °C, 20 °C, 37 °C and 42 °C for 30 min. Total RNA of at least three biological replicates per condition was extracted (peqGold Bacterial RNA Kit; Peqlab, Erlangen, Germany).

Quality control
Quality and integrity of RNA samples were checked (Agilent RNA 6000 Nano Kit, 2100 Bioanalyzer; Agilent Technologies, Santa Clara, USA) and samples were only used if the RNA integrity number (RIN) was >9.

Labeling, Hybridisation and signal-generation
RNA-samples were Cy3-labled and hybridized with 8x15k *Vibrio* pan-genome-arrays (Agilent).

RT-qPCR
Aliquots of RNA-samples were transcribed into cDNA (RevertAid™ Premium First Strand cDNA Synthesis Kit and random hexamer primer, Fermentas, Schwerte, Germany) and conducted to RT-qPCR Sybr-Green Assays (SsoFast Eva Green Supermix; BioRad, Hercules, USA). The resulting $\log_2$FC were correlated with microarray results for quality and processing controls.

Data analysis
Software packages R and Bioconductor [1] were used for data analysis. Libraries were generated with Limma [2] and Amap [3]. Heat maps and clustering was performed with Genesis 1.7.6 [4]. The enrichment analyses of gene-sets were created with the web-based Database for Annotation, Visualization and Integrated Discovery (DAVID) [5, 6].

Results and Discussion

For quality control, the expression of selected genes (e.g. *gro*ES, *csp*A) was determined using quantitative real-time PCR (qPCR) at the different test temperatures. Correlation of $\log_2$FC values of qPCR and microarray revealed a R^2-value of 0.7008 (data not shown).

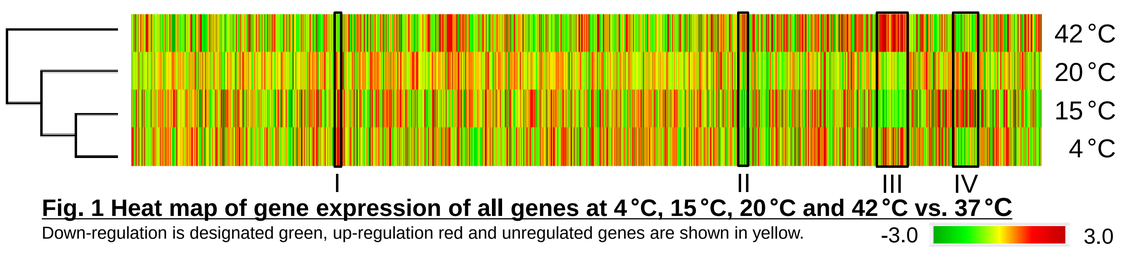

Fig. 1 Heat map of gene expression of all genes at 4 °C, 15 °C, 20 °C and 42 °C vs. 37 °C
Down-regulation is designated green, up-regulation red and unregulated genes are shown in yellow.

Heat maps of biological replicates revealed good reproducibility of experiments (data not shown). Microarray slide signals were normalized using 37 °C sets and a combination of replicates. Heat maps were created to assess hybridization quality and reproducibility (Fig. 1). Expression at 4 °C and 15 °C were most similar, whereas 42 °C showed differently expression. Most genes were found unregulated at 20 °C.

After normalization to 37 °C cluster analysis was performed (Fig. 2). Normalized expression of genes at 37 °C was set to $\log_2 0$. The analysis revealed different patterns of gene expression of which some were identifiable in the heat maps (Fig. 1). In Cluster I, genes (n=627) showed up regulation at 4 °C and 15 °C, intermediate regulation at 20 °C and down regulation at 42 °C. Genes of cluster II (n=274) possessed an antagonistic pattern. All genes located in cluster III (n=412) showed increased expression at 4 °C and 42 °C and down regulation at 15 °C and 20 °C. Antagonistic expression was identified in genes of cluster IV (n=538) with down regulation at extreme temperatures of 4 °C and 42 °C and up regulation at 15 °C and 20 °C.

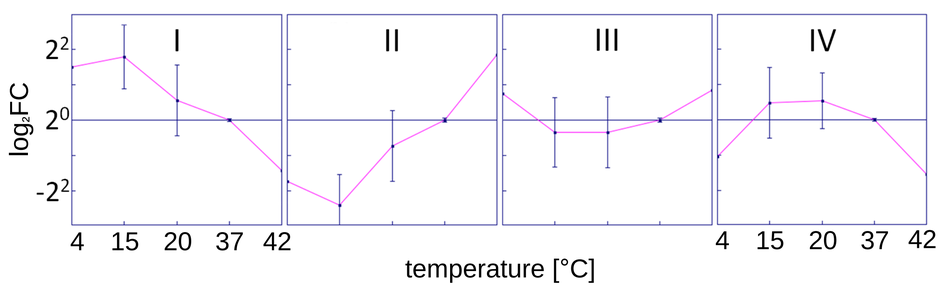

Fig. 2 Cluster analysis of all temperature settings distinct gene expression patterns
Log_2 fold change = $\log_2$FC

Volcano plot analysis visualized the distribution of all 4820 data points (Fig. 3). Genes with an at least $\log_2$2-fold changed expression (compared to expression at 37 °C) and a *p*-value ≤ 0,05 were plotted in red. Unregulated genes were plotted in blue and were not analyzed in detail. In total 3 % (n=144) of genes at 20 °C, 7.4 % (n=358) at 4 °C, 10.8 % (n=522) at 15 °C and 13.3 % (n=639) at 42 °C were expressed differentially.

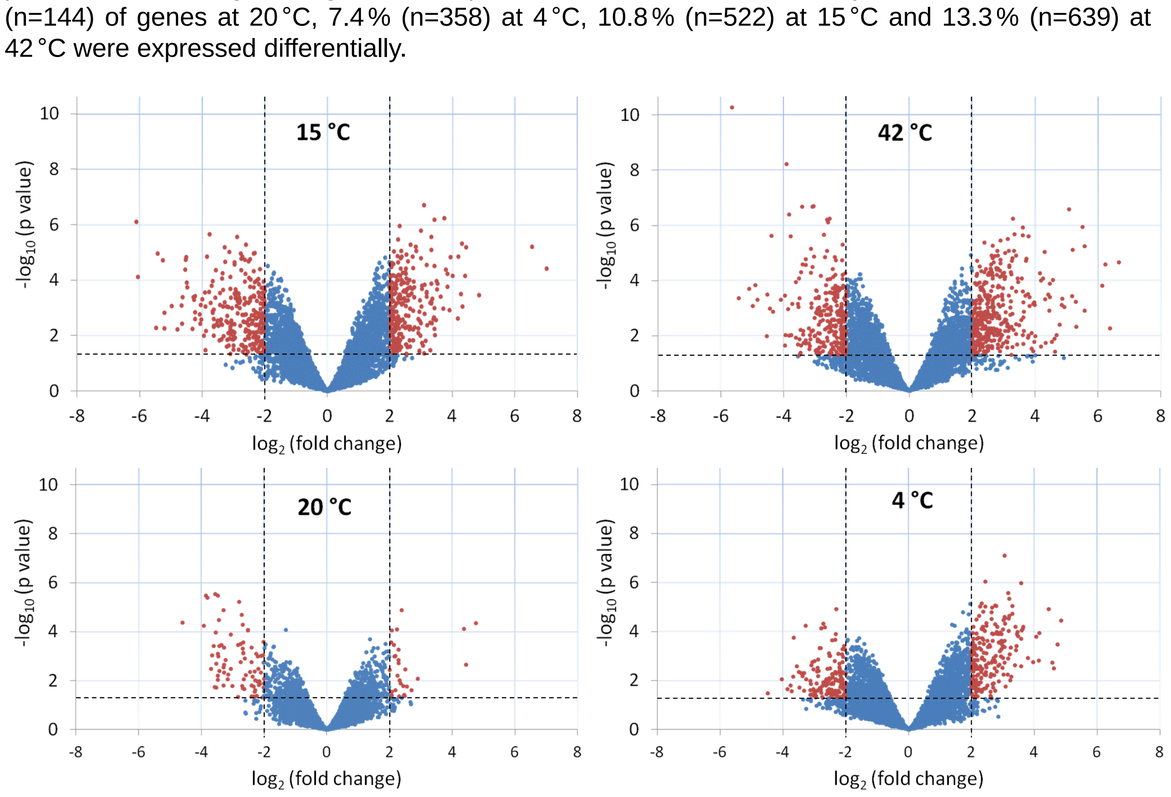

Fig. 3 Volcano plots of normalized gene expression

The patterns of gene expression at 15 °C and 42 °C were similar. However, an antagonistic expression appeared for instance in cell metabolism genes (Fig. 4). A total of 83 genes were up regulated under one condition (15 °C) while being down regulated under the other (42 °C); 26 genes showed homologous expression. Both temperatures, 15 °C and 42 °C, deviate 5 °C from the native temperature range (20-37 °C). This could suggest a similar forming of gene regulation. At 20 °C more genes showed down regulation, whereas at 4 °C more genes were up regulated.

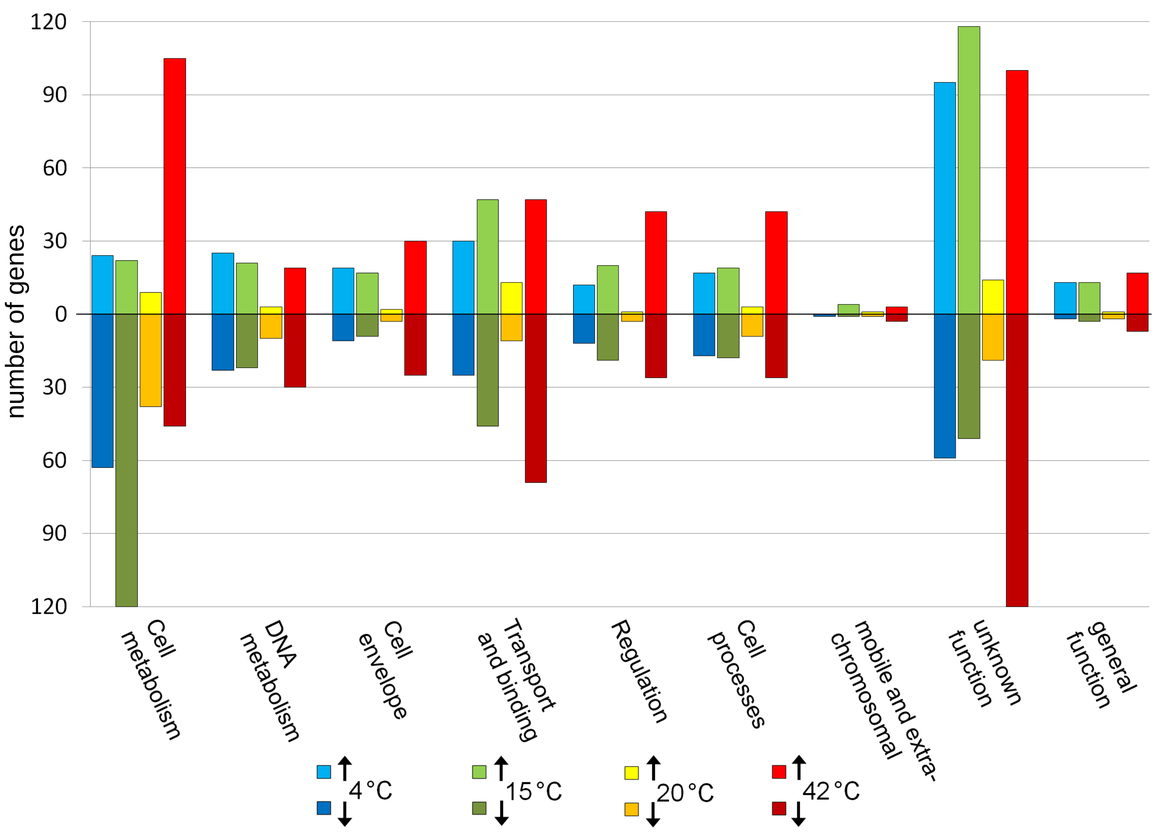

Fig. 4 significant, normalized gene expression sorted by gene function

Analysis of differential gene expression (Fig. 4) showed especially at 15 °C and 42 °C a regulation of numerous genes. Especially genes classified as ‘unknown function’ were up or down regulated. Furthermore genes participating in ‘cell processes‘, ‘binding and transport‘ and ‘cell metabolism‘ were considerably regulated.
Of the 83 antagonistically regulated genes at 15 °C, approx. half were up (n=41) and half down (n=42) regulated. Approx. 69 % (n=18) of genes with homologous regulation were up regulated at both temperatures.

With only 3 % of all genes showing significant differences regarding their expression the temperature range between 20 °C and 37 °C could be considered as normal conditions.

At 4 °C considerably less genes showed significantly altered expression compared to 15 °C and 42 °C. However, at such low temperatures the metabolic activity of the cell is reduced which could result in an overall diminishment of gene expression. Nonetheless trends were visible as known cold-shock genes like *csp*A showed the expected up regulation.

Conclusion

Analysis of global gene expression showed considerable differences in the temperature dependent gene regulation of *Vibrio parahaemolyticus* RIMD 2210633.

Only 3 % of the genes had a significantly altered expression at 20 °C compared to 37 °C, with more genes down regulated. In contrast, at 4 °C 7.4% genes showed an up regulation.

At temperatures 15 °C and 42 °C, 10.8% and 13.3% of genes, were differentially expressed (compared to 37 °C). Especially genes belonging to the categories ‘cell processes‘, ‘binding and transport‘ and ‘cell metabolism‘ showed high regulation rates. Comparing 15 °C and 42 °C, 83 genes were regulated antagonistically. At 15 °C and 42 °C, 26 genes had a homologous expression, of which 69 % were up regulated.

Currently web-based databases like DAVID are used. Clusters of similar regulated gene sets obtained by DAVID analysis are under investigation.

Acknowledgement
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[1] Gentleman RC, Carey VJ, Bates BM, et al. Bioconductor: open software development for computational biology and bioinformatics, Genome Biology, vol. 5, artic.num. R80, 2004. [2] Smyth, GK. Limma: linear models for microarray data. In: 'Bioinformatics and Computational Biology Solutions using R and Bioconductor'. Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W (eds), Springer, New York, pp 397-420, 2005.
[3] Antoine Lucas, Amap: Another Multidimensional Analysis Package, R package version 0.8-7, http://CRAN.R-project.org/package=amap, 2011. [4] Sturn, A. Quackenbusch, Trajanoski, Z:. Genesis: cluster analysis of microarray data, Bioinformatics, vol. 18(1), pp 207-208, 2002.
[5] Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nature Protoc., vol. 4(1), pp. 44-57, 2009. [6] Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res., vol. 37(1), pp. 1-13, 2009

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