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Highlights

- *C. jejuni* molecular response studied after consecutive thermal stresses by RT-qPCR
- Similar gene expression patterns shared between two of the three strains tested
- Up-regulated genes mainly involved in the heat shock response
- Down-regulated genes belonged to metabolic pathways (lipid, amino-acid metabolisms)
- A strain-specific gene expression profile consistent with atypical stress response

Abstract

Broiler meat is considered as the most important source of the foodborne pathogen *Campylobacter jejuni*. Exposure to stress conditions encountered during the slaughtering process may induce bacterial adaptation mechanisms, and enhance or decrease pathogen resistance to subsequent stress. This adaptation may result from changes in bacterial gene expression. This study aims to accurately quantify the expression of selected *C. jejuni* genes after stresses inspired from the poultry slaughtering process. RT-qPCR was used to quantify gene expression of 44 genes in three strains after successive heat and cold stresses. Main results indicated that 26 genes out of 44 were differentially expressed following the successive thermal stresses. Three clusters of genes were differentially expressed according to the strain and the stress condition. Up-regulated genes mainly included genes involved in the heat shock response, whereas down-regulated genes belonged to metabolic pathways (such as lipid, amino-acid metabolisms). However, four genes were similarly overexpressed in the three strains; they might represent indicators of the thermal stress response at the species scale. Advances in the molecular understanding of the stress response of pathogenic bacteria, such as *Campylobacter*, in real-life processing conditions will make it possible to identify technological levers and better mitigate the microbial risk.

Keywords: RT-qPCR, strain variability, food safety, foodborne pathogen, cell history

1 Introduction

Campylobacter has been the main cause of bacterial enteritis worldwide in humans for many years (EFSA and ECDC, 2019). The number of reported confirmed cases of campylobacteriosis is often under-estimated: it was 246,571 in 2018 with an EU notification rate of 64.1 *per* 100,000 population (EFSA and ECDC, 2019). *C. jejuni* infection is usually benign, but may also lead to chronic sequelae such as Guillain-Barré syndrome (GBS) and Miller-Fisher syndrome (MFS) (Altekruse et al., 1999; Silva et al., 2011; WHO, 2013). Consumption of animal products, mainly poultry, is a major cause of infection in humans (Zhong et al., 2016). Although chicken meat is the main source of this pathogen, other foods have been involved in campylobacteriosis outbreaks such as milk (EFSA and ECDC, 2019).

Campylobacter are apparently fragile organisms that cannot grow under aerobiosis conditions; they cannot multiply outside the animal host and are highly susceptible to a number of environmental conditions (Park, 2002). Nevertheless, *Campylobacter* are paradoxically considered as one of the main foodborne pathogens (Solomon and Hoover, 1999). During poultry processing, bacteria are submitted to numerous stressful steps assimilated to heat stress, cold stress, acid stress or oxidative stress. To mitigate stressful effects, the pathogen sets up various defense strategies like production of heat shock proteins (*e.g.* DnaK, GroEL, ClpB) in response to heat or acid stress (Konkel et al., 1998; Reid et al., 2008a; Reid et al., 2008b; Stintzi, 2003; Thies et al., 1998; Thies et al., 1999a; Thies et al., 1999b), but also enzymes (*e.g.* AhpC, KatA, SodB) involved in cell detoxification (Baillon et al., 1999; Grant and Park, 1995; Pesci et al., 1994; van Vliet et al., 1999). Such mechanisms enable *Campylobacter* to adapt to environmental conditions.

Unlike other foodborne pathogens, *Campylobacter* does not grow effectively in the food environment or on food products. However, it can survive under stressful conditions (Duqué et al., 2019; Klančnik et al., 2009; Murphy et al., 2003a, b; Oh et al., 2015; Reid et al., 2008b). Adaptation to stress has been investigated at the transcriptomic level and has determined which genes could be involved. Most studies addressed the acid, oxidative or heat stress responses (Reid et al., 2008a; Reid et al., 2008b; Stintzi, 2003; Varsaki et al., 2015), in which single stresses were applied independently. Many genes play a role in the stress response, such as genes implied in the heat shock response or in the amino acid or lipid metabolisms (Cameron et al., 2012; Guccione et al., 2017; Palyada et al., 2009; Stintzi, 2003). In order to decipher how this pathogen can survive along the food chain, it seemed relevant to evaluate the impact of various food production and preservation conditions on the stress adaptation potential of this foodborne pathogen. Consequently, we transposed consecutive heat and cold stresses inspired from the poultry slaughtering process to the laboratory. The aim was to assess the impact of successive hot and cold temperature stresses on *C. jejuni* gene expression and better understand the adaptation capacity of this pathogen.

To our knowledge, the molecular mechanism underlying the adaptive response of *C. jejuni* resulting from exposure to successive stresses, especially stresses similar to those encountered during the poultry processing steps, has not been studied yet. Exposure to a stress may indeed induce a general stress response enabling bacterial cross-protection from other stress conditions (Kim et al., 2015). In a previous study, we evaluated the response of *C. jejuni* to the application of successive stresses inspired from the poultry slaughtering process at the phenotypic level (Duqué et al., 2019): the inactivation of *C. jejuni* induced by cold storage significantly depended on the previously applied heat stress and differed according to the strain. Our results pointed out the complexity of bacterial behavior and strain variability, and highlighted the need to decipher the molecular mechanisms underlying adaptive responses through gene expression profiling.

The investigation of bacterial adaptive responses requires the use of molecular approaches. In this respect, reverse transcription quantitative PCR (RT-qPCR) is still considered as a standard method for accurate and sensitive measurement of gene expression (Nolan et al., 2006). In addition to the recommendations about the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Bustin et al., 2010; Bustin et al., 2009), stringent quality controls at each critical point throughout the entire RT-qPCR workflow are necessary (Desriac et al., 2017). Besides, to disentangle experimental variation from true biological variation, an internal control to compensate for experimental errors has to be used (Huggett et al., 2005). Housekeeping genes are generally used as internal controls. However, finding appropriate internal control genes whose expression remains stable whatever the experimental conditions is still quite a challenge (Ritz et al., 2009).

In the current study, three *C. jejuni* strains were submitted to successive stresses inspired from those encountered during the poultry slaughtering process. First, an accurate method of RT-qPCR was developed (Rezé et al., 2019). Second, this method was used to study the influence of successive hot and cold temperatures on the expression of selected genes to investigate how bacteria modulate gene expression after two successive stresses.

2 Materials and methods

2.1 Bacterial strains and culture conditions

The culture conditions described in Duqué et al. (2019) were adopted. To sum up, three strains originally isolated from poultry were used: *C. jejuni* C09MJLT205, *C. jejuni* RM1221, and *C. jejuni* C97anses 640 (Fouts et al., 2005; Guyard-Nicodeme et al., 2015; Guyard-Nicodeme et al., 2013). The cultures were stored at -80°C in brain heart infusion (BHI, Biomérieux, Marcy l'Etoile, France) supplemented with 20% (v/v) glycerol. Prior to the experiments, they were cultured on Karmali (Oxoid, Dardilly, France) agar plates at 42°C for 48-72 h under microaerobic conditions. Then, two successive cultures were incubated in Mueller-Hinton broth (MH, Oxoid, Dardilly, France) for 20 h and 18 h, respectively, under microaerobic atmosphere and shaking. Finally, 10-fold dilutions were prepared to obtain the final cultures, which were enumerated.

2.2 Induction of stress conditions

The experimental procedure was as described previously, with slight modifications (Duqué et al., 2019). Briefly, *C. jejuni* strains were submitted to stresses inspired from conditions encountered during the poultry slaughtering process and considered as stressful. These steps are scalding and chilling, and are associated with heat and cold stresses.

The experimental design used "strain" and "hot bath temperature" as variables. For each strain, ten mL of the culture at an initial concentration of 8 $\log_{10}$ CFU.mL^{-1} were transferred into a glass tube and successively submitted to heat and cold. The glass tubes had the following dimensions: 150 mm in length, 18 cm in diameter and 1 mm in thickness. Heat stress consisted in immersing *C. jejuni* cultures in hot water baths at 46, 51 or 54°C for 4 min. After exposure to heat stress, the tubes were immediately cooled for 5 min in a water bath at 22°C. Cold stress was applied immediately after cooling by immersing the cultures into freezing-cold water containing ethylene glycol (-4°C, for 2 h).

In parallel, 10 mL from the same initial culture were kept as a control unstressed culture.

After each step, viable counts of *C. jejuni* were enumerated on Columbia sheep blood agar plates (Biomérieux, Marcy l'Etoile, France) and incubated for 48 h at 42°C under microaerobic conditions using a SPIRAL plater (EasySpiral Interscience, Saint Nom, France) to determine the viability loss, expressed in $\Delta\log_{10}$, resulting from each step. Besides, a volume of culture was kept after each step for RNA extraction. Each experiment was performed in three independent replicates.

2.3 Transcriptomic analysis by RT-qPCR

The molecular response of *C. jejuni* was investigated using RT-qPCR instead of a global approach like RNA-seq because the final objective of this work will be to correlate the phenotypic response of *C. jejuni* (inactivation) with its transcriptomic response, using mathematical modeling to identify biomarkers of stress. The search for biomarkers represents a challenge, and this proof of concept was investigated using RT-qPCR as already performed by Desriac et al (Desriac et al., 2015; Desriac et al., 2012; Desriac et al., 2013). To this end, quantitative data had to be provided using a quantitative method. This was why we chose RT-qPCR rather than RNA-seq, which only provides the relative abundance of reads. Moreover, RT-qPCR is appropriate to study well-known phenotypes and associated genes well described in the literature.

2.3.1 Gene selection

Using RT-qPCR to evaluate the modulation of gene expression requires selecting a limited number of genes to be studied. To compare the transcriptomic response of *C. jejuni* following one stress *versus* two consecutive stresses, we chose forty genes already known to be involved in the *C. jejuni* stress response according to the literature. The selection was based on a decision tree as described in Figure 1. A literature search was first performed on the Web of Science platform by seeking articles with the following keywords in their title and/or their abstract: adaptive AND response AND *Campylobacter*, oxidative AND stress AND *Campylobacter*, transcriptomic AND *Campylobacter*, RT-PCR AND *Campylobacter*, heat AND stress AND *Campylobacter*, cold AND stress AND *Campylobacter*, acid AND stress AND *Campylobacter*, osmotic AND stress AND *Campylobacter*, alkaline AND stress AND *Campylobacter*, gene expression AND stress AND *Campylobacter*, proteomic AND stress AND *Campylobacter*, microarray AND stress AND *Campylobacter*, RNA-seq AND stress AND *Campylobacter*. Based on these criteria, 1, 096 publications were retrieved.

One hundred and twenty studies were selected out of the initial batch of 1,096 publications because they showed differential gene expression following stresses or the involvement of a given gene in the stress response. Around 700 genes were considered in these studies, *i.e.* approximately 43% of the *C. jejuni* whole genome. To avoid selecting marginally studied genes, only genes studied in at least two different laboratories were kept. This represented 430 genes. All the regulators involved in the *C. jejuni* stress response (13 genes) were retained because we assumed that their expression would probably change under stress conditions. Then, we stringently selected 27 genes among the 430 genes studied in at least two different laboratories by trying to capture the variability of the metabolic pathways in which they were involved. To do so, the gene classification according to metabolic pathways was retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Finally, at least one gene from each metabolic pathway was picked up, but genes involved in several metabolic pathways and involved in different stress responses were preferentially selected. Out of 1,600 initially selected genes, 40 were eventually kept for analysis (Table 1).

Only the genes amplified by PCR and therefore present in the three strains were kept. Consequently, two genes – *putA* and *rprB* – were excluded from the analysis because they were not amplified in the

C. jejuni C09MJLT205 strain and in the RM1221 and C94anses640 strains, respectively. The expression levels of the 38 remaining genes were then analyzed.

2.3.2 Selection of reference genes

Reference genes are needed for normalized expression. They are usually housekeeping genes, and their expression should remain constant whatever the experimental conditions. Reference genes were selected among a pool of 44 genes composed of i) six genes previously described as potential reference genes for RT-qPCR in *C. jejuni* (Ritz et al., 2009), and ii) the 38 genes selected as potentially involved in the stress response.

Stability of gene expression was assessed as demonstrated by Vandesompele et al. (2002). The gene expression stability measure (M) was calculated using the geNorm module in qBase+ software (version 3.2, Biogazelle). To determine the optimal number of reference genes, the pairwise variation Vn/n+1 was calculated between the two sequential normalization factors (NFn and NFn+1) for all samples. The software program recommended that V should be less than 0.15.

2.3.3 Primer design

For each gene, primers were designed using Primer3Plus, a web interface developed by Untergasser et al. (2007) based on the genome of strain RM1221 – the only sequenced strain in this study. Pairs with the smallest penalty score were retained, and sequences were tested for specificity using NCBI BLAST software, and compared to all available genomes of *C. jejuni* species. Oligonucleotide sequences of the targeted genes with BLAST score values greater than 30 were retained, as suggested by Desriac et al. (2017).

2.3.4 Determination of primer efficiency

DNA was isolated from unstressed *C. jejuni* RM1221 cultures. Briefly, 1 mL was centrifuged at 10,000 x g for 6 min. Then, the supernatant was removed, and DNA was extracted using a Dneasy® Blood and Tissue Kit (Qiagen, Courtaboeuf, France). Combined lysozyme (15 $mg.mL^{-1}$) and proteinase K (20 $mg.mL^{-1}$) digestion was used for lysis. DNA quantity and purity were measured using an Implen NanoPhotometer and stored at -20°C.

After *in silico* design, the PCR efficiency (E) of each primer pair was assessed (Table 2). Briefly, after thawing, each DNA suspension was serially diluted tenfold before amplification and quantification. Then, for each primer pair, Ct values were plotted on a logarithmic scale along with corresponding DNA concentrations. Efficiency was calculated from a linear regression curve through the data points, using the following equation: $E = -1+10^{(-1/slope)}$. All primer pairs showed satisfactory efficiency values ranging between 97 and 109% with an R^2 higher than 0.97.

2.3.5 RNA isolation

RNA was isolated from cultures (i) after the heat stress, and (ii) after the cold stress applied following the heat stress. RNA was also extracted from unstressed cultures that remained under optimal conditions (42°C in microaerobiosis) and considered as the control.

Briefly, 30 mL of stressed or unstressed culture were centrifuged at 10,000 x g at 4°C for 4 min. Thirty mL of culture were necessary to have enough RNA to extract (mostly after stress application). Consequently, the centrifugation step was performed before adding RNA protect to resuspend the pellet in a smaller volume of RNA-protect, as performed by Desriac *et al*. (Desriac et al., 2015; Desriac et al., 2012; Desriac et al., 2013). After removal of the supernatant, 1 mL of RNA protect

(Qiagen, Courtaboeuf, France) was added to suspend the pellet. The suspension was incubated for 5 min at ambient temperature and then centrifuged at 5,000 x g at 4°C for 10 min as recommended by the manufacturer. The supernatant was removed, and the pellet was snap-frozen in liquid nitrogen and stored at -80°C for up to 1 month.

After cell pellets were thawed, RNA extraction was performed using an RNeasy® Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations. Combined lysozyme (15 $mg.ml^{-1}$) and proteinase K (20 $mg.ml^{-1}$) digestion was used for cell lysis. Contaminant genomic DNA was removed from each sample using an Invitrogen Turbo DNA-free ™ Kit (Ambion, Cambridge, USA). A first incubation of 30 min at 37°C was performed, and the supernatant was extracted. A second incubation of 30 min at 37°C was performed to efficiently remove contaminant genomic DNA.

Quality controls of the extracted RNAs were carried out to validate RNA isolation. First, RNA quantity and purity were measured using an Implen NanoPhotometer. Then, after RNA denaturation at 70°C for 9 min, RNA integrity was assessed by electrophoresis on agarose gel (1.2%). Profiles presenting RNA degradation were automatically discarded. The absence of genomic DNA in the RNA samples was checked by PCR. Amplification was performed using primers 341F and 758R (Rodrigues et al., 2016). The thermo-cycling program consisted of 95°C for 5 min, followed by 30 cycles of 30 s at 95°C, 30 s at 59°C and 30 s at 68°C. The absence of DNA was checked using agarose gel (1.2%) electrophoresis.

Each extraction was performed on three independent replicates.

2.3.6 Reverse Transcription (cDNA synthesis)

As recommended by Stahlberg et al. (2004), reverse transcription was performed on 200 ng of the total RNA extract using an iScript cDNA synthesis kit (Bio-Rad, Marnes la Coquette, France). The cDNA synthesis program consisted in initial priming at 25°C for 5 min, followed by reverse transcription at 46°C for 20 min, and inactivation at 95°C for 1 min. cDNA was then stored at -20°C.

2.3.7 Quantitative PCR

Amplification and quantification were performed using a CFX connect device (Bio-Rad, Mitry-Mory, France). For each sample, the reaction mixture contained 10 µL of SoAdvanced SYBR Green Supermix (Bio-Rad, Marnes la Coquette, France), 1 µL of each forward and reverse primer, 4 µL of water and 4 µL of cDNA. The program consisted in initial denaturation at 95°C for 3 min, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C.

A sample maximization method was used, as advised by Hellemans et al. (2007). All samples were assayed in the same run, but genes were analyzed separately. This method avoided the need for an inter-run calibrator (IRC).

2.3.8 Data analysis

Gene expression quantification was performed after each step of the experimental setup (*i.e*. after heat stress and after successive heat and cold stresses). RT-qPCR results were expressed in relative quantity scaled to the expression of the same gene in the control sample (*i.e.* unstressed culture) using the delta Ct transformation with efficiency correction. Then, expression levels were normalized relatively to the expression levels of the reference genes. Reference genes were selected among the 44 genes.

The normalization factor (NF) was calculated as the geometric mean of reference gene expression (Vandesompele et al., 2002). After normalization, relative gene expression was $\log_2$ transformed for further ANOVA analysis (Bengtsson et al., 2005).

For each strain, ANOVA analysis (α=5%) using XLSTAT software (version 2018.5) was performed to assess if the application of successive heat and cold stress had a significant impact on the gene expression compared to the gene expression after the heat stress only. Besides, ANOVA analysis was also performed on genes whose absolute relative expression level (in $\log_2$) was altered more than 2-fold, to assess the impact of the different temperatures of heat stress (Reid et al., 2008a). Fisher's least significant difference test ($P<0.05$) was applied to determine significant differences in mean $\log_2$ fold changes between stresses.

Hierarchical cluster analysis of significantly differentially expressed transcripts was performed using the function heatmap.2 of the gplots package in R. Only genes with an expression level above a 2 $\log_2$ fold change or below a -2 $\log_2$ fold change were considered (Reid et al., 2008a).

3 Results

3.1 Selection of reference genes

As depicted by Desriac et al. (2017), appropriate quality procedures and quality controls should be set up and assessed carefully to reliably interpret RT-qPCR results. The selection of good reference genes is one of the numerous critical points in the procedure. A good reference gene should display constant expression whatever the condition, and the number of reference genes has to be adapted according to their stability.

To select genes with the most stable expression, all 44 genes were considered as candidate reference genes, including those selected as targets. Two criteria were adopted to select the reference genes: (i) the M value, calculated for each gene and for each strain (Supplementary Figure S1), and (ii) pairwise variation – Vn/n+1 –, calculated for each strain. The V2/3 pairwise variation values were < 0.15, meaning that the optimal number of reference genes in this experimental situation was 2 (Supplementary Figure S2). Finally, the two most stable genes were retained for each strain (Table 3). Unfortunately, the reference genes were not the same for all three strains.

3.2 *C. jejuni* inactivation following application of successive stresses

C. jejuni encounters several stresses during the poultry slaughtering process. For example, the scalding and chilling steps can be assimilated to heat and cold stresses, respectively. The inactivation generated by these stresses varied from -0.28 ± 0.10 to 0.54 ± 0.04 according to the strain and the stress applied (Table 4). Negative values mean no inactivation due to the uncertainty from enumeration method.

Since bacteria may respond differently and use different mechanisms of defense according to the stress encountered, their gene expression may also differ from one stress to another. Thus, the gene expression was expected to be modified following the application of the second cold stress in comparison with the application of the only first heat stress. This was evaluated in the next section.

3.3 Differential gene expression of *C. jejuni* between heat and cold stress

Gene expression was evaluated both after heat stress alone and after successive application of heat and cold stresses. Analysis of variance was performed *per* strain to determine if gene expression was

altered in a stress-dependent manner. For C97anses640 strain, gene expression was shown to significantly depend on gene, temperature of the heat stress and the application or not of the second stress, i.e. the cold stress ($P < 0.001$). For the other two strains, the application of the cold stress was shown to affect expression of specific genes only when heat stress had been performed at some temperatures (temperature*coldstress and gene*coldstress significant interactions; $P<0.001$). It appeared that the application of the second stress altered more expression of some genes when strains had been previously submitted to heat stress at 46°C, rather than at 51°C or at 54°C. Genes whose expression was significantly altered at the different temperatures of the heat stress are listed in supplementary materials (Table S1). However, the difference in expression between both stresses remained very low, generally below 2 $\log_2$ fold changes. Among these genes, it is noteworthy that four genes were differentially expressed by all strains after application of 46°C. These are: *katA*, *lysR*, *pebC* and *racR*. Expression of these genes after the heat stress applied at 46°C with or without subsequent application of the cold stress is described in Figure 2. It appears that heat stress followed by cold stress indeed induced a slight change in expression, but in the same direction (up or downregulation) whatever the strain. Three genes, i.e. *katA*, *lysR*, *pebC*, were downregulated following the heat plus cold stresses whereas *racR* was upregulated. The highest change in expression between the heat stress at 46°C and the heat plus cold cold stresses was observed for *pebC*, downregulated from -2 to -3.9 $\log_2$ fold changes depending on the strain.

3.4 Differential *C. jejuni* gene expression following the consecutive stresses

Gene expression following heat + cold stresses slightly differed from gene expression following heat stress.

Only genes differentially expressed by more than 2 $\log_2$ fold change (in absolute value), were considered (Reid et al., 2008a). Overall, among the 44 selected genes, 26 were differentially expressed in at least one of the three heat stress conditions and in one or more strains, with a maximum $\log_2$ fold change of 6.21 and a minimal fold change of -4.17 (Supplementary Table S2). Consequently, these 26 differentially expressed genes were subjected to hierarchical clustering analysis and were grouped into 3 main clusters designated as A, B, and C (Figure 3).

The four genes belonging to cluster A were globally up-regulated in the three strains, and their overexpression varied between 2 and 6 $\log_2$-fold change according to the gene and the strain. However, the expression of all four genes decreased at 54°C in strain C09MJLT205, compared with 46 and 51°C. This cluster mainly included genes associated with bacterial heat shock responses, namely the transcriptional regulator *hrcA* and several chaperones and co-chaperones considered as heat shock proteins (HSPs) (*grpE*, *dnaK* and *clpB*), in agreement with heat stress (Figure 4A).

Clusters B and C were composed of genes differentially expressed according to the strains (Figure 3). Strain C97anses640 had a rather different pattern of gene expression from that of the other two strains.

Cluster B harbored two categories of genes. A first pool was mainly overexpressed in strain C97anses640 when temperature increased, with a maximum of 3.1 $\log_2$ fold change, while they were little or not differentially expressed in the other two strains. This group included genes encoding three regulators – *cbrR*, *hspR* and CJE1780 –, and the chaperone *groEL*, and *nuoL* (Figure 4B). The *hspR* gene encodes a repressor of the previously described operon *clpB-grpE-dnaK,* which encodes HSPs. The second group of genes was mainly under-expressed in strains C09MJLT205 and RM1221, down to -2.88 $\log_2$ fold change, while it remained relatively stable or slightly increased in strain C97anses640. This group was included *pebC*, *rpoB*, *gyrA*, *thiC*, *trpD*, *ilvC* (Figure 4C).

Lastly, cluster C contained eleven genes significantly down-regulated in the three strains from 51°C. Down-regulation varied between -2 and -4 $\log_2$ fold change. It seemed to increase as temperature increased beyond 46°C for strain C09MJLT205, and 54°C for the other two strains, suggesting that the regulation threshold could be strain dependent. This cluster included a two-component sensor histidine kinase CJE1361, a *fliP* gene, and genes belonging to the amino acid metabolism (*argF*, *slyD*), the lipid metabolism (*kdtA*, *yciA*) and the energy metabolism (*atpA*). The regulators *cosR* (Figure 4D) and *csrA*, and the *katA* gene – which are involved in the bacterial oxidative stress response – also belonged to this cluster.

4 Discussion

The objective of the study was to assess the impact of successive hot and cold temperatures on *C. jejuni* gene expression to better understand the adaptation capacity of this pathogen. In addition, we investigated the strain variability response at the molecular level.

Studying gene expression requires an accurate and validated RT-qPCR method to quantify transcripts. Following the recommendations of Desriac et al. (2017), each step was validated (Rezé et al., 2019). Besides, the use of reference genes is mandatory to normalize the expression of target genes. A suitable reference gene should display stable expression under the experimental conditions of the study. Otherwise, the selection of inappropriate reference genes may bias gene expression quantification (Dheda et al., 2005; Hyytiainen et al., 2012; Udvardi et al., 2008). In most cases, reference genes are *a priori* selected among genes commonly found in the literature, such as housekeeping genes. We did not limit our selection to genes previously identified in the literature, but considered a pool of 44 genes to evaluate the stability of their expression following heat and cold stresses. The ΔΔCt method is largely used to quantify gene expression. However, according to this method, only one reference gene is used to normalize gene expression (Livak and Schmittgen (2001)). The use of two reference genes increased quantification accuracy. The pairs of reference genes we chose varied according to the strains (the development of universal reference genes is not always feasible (Bustin et al., 2009). Five different reference genes were selected for the three *C. jejuni* strains: *rrs* – common to all three strains –, and *asd*, *proS*, *thiC* and *dsbI*. *rrs* has been commonly used as a reference gene in several previous works (Bronnec et al., 2016; Hyytiainen et al., 2012; Turonova et al., 2017). *asd*, *proS*, and *thiC* are involved in the amino-acid biosynthesis pathway, and *dsbI* is part of the *C. jejuni* redox system required for disulfide bond formation in some proteins. Surprisingly, *rpoA* and *slyD* were considered as reference genes for *C. jejuni* (Phongsisay et al., 2007; Ritz et al., 2009; Stintzi, 2003), but turned out to be pretty bad reference genes in the current study because their expression changed following stress. This highlights the importance of a robust strategy to select reference genes.

The expression of 44 genes was quantified by RT-qPCR in three *C. jejuni* strains (C09MJLT205, RM1221, and C97anses640) after exposure to stresses inspired by the poultry slaughtering process. In order to mimic conditions that may be encountered by *Campylobacter,* three steps were successively applied, namely a hot stress assimilated to the scalding step, followed by a short resting time at 22°C assimilated to the time poultry is left at ambient temperature between scalding and chilling, and finally a cold step, assimilated to chilling. These conditions were determined after visiting several slaughterhouses in France (Duqué et al., 2019).

In addition to transcriptional analyses, the three strains were enumerated following these stresses. The combined heat and cold stresses barely inactivated all three strains. Despite this low inactivation, these stresses influenced *C. jejuni* gene expression, since 24 genes were differentially expressed under at least one of the temperature conditions and in one or more strains. By selecting genes *a priori*, we found that 59% of them were differentially expressed in at least one of these stressful conditions, showing that such a selection procedure may be suitable when a limited number of genes is targeted. Global transcriptomic approaches highlighted that around 20% of the genes were differentially expressed after a thermal stress (Riedel et al., 2020a; Stintzi, 2003). This represents a higher number of coding sequences than in the present study.

Different profiles of strain-dependent gene expression emerged after thermal stress. Genes expressed in the same manner after stress whatever the strain were mainly involved in the heat shock response. Whereas genes differentially expressed according to the strain were involved in the oxidative stress response or the central metabolism. Most of these genes (14/24) were under-expressed in *C. jejuni* C09MJLT205 and RM1221 strains from 51°C or 54°C, respectively. Besides, strain variability was higher than biological variability (reproducibility of biologically independent replicates) and was consistent with the literature (Aryani et al., 2015; den Besten et al., 2017). This result highlights the reproducibility of the experimental method.

The comparison of the *C. jejuni* transcriptional response following application of one stress *versus* two consecutive stresses revealed that only few genes were differentially expressed following heat stress alone *versus* heat plus cold stresses. Although the transcriptomic response to cold stress was not investigated alone (without pre-adaptation to heat-shock), the comparison between gene expression after heat stress alone (H) and successive heat stress + ambient temperature + cold stress (H+C), seemed to show that the heat stress had a limited pre-adaptation impact on the *C. jejuni* transcriptional response to cold stress (H ~ H+C). The molecular response to the cold stress is poorly documented in *C. jejuni*. No gene encoding cold shock proteins has been identified in genomes available (Parkhill et al., 2000), which suggests that this pathogen may have other tolerance mechanisms to respond to cold stress. Studies so far have shown oxidative stress defense, particularly SodB, played an important role in the cold stress tolerance of *Campylobacter* (Garenaux et al., 2009; Stead and Park, 2000; Stintzi, 2003). In comparison with heat shock, cold stress applied in the current study induced repression of *pebC* gene and overexpression of *racR* in the three *C. jejuni* strains. Some studies have already investigated the implication of RacR in *C. jejuni* thermoregulation under elevated temperatures (Apel et al., 2012; Brás et al., 1999). Our work suggests a potential role of this regulator in a wider range of temperatures. Regarding *pebC* gene, the transition from heat to cold stress led to a dramatically expression decrease by 4 $\log_2$ fold changes, which is different from the results obtained by Shi (2014) (Shi, 2014). The *pebC* gene encodes a component of the amino acid ABC transporter involved in aspartate and glutamate metabolism and required for the microaerobic growth (Stahl et al., 2012). This drop may be linked to a decrease in glutamate utilization during cold stress, because of its rapid metabolization during stationary phase before stress induction (Stahl et al., 2012).

Following the successive heat and cold stresses, the gene expression profiles of each strain were compared by hierarchical clustering analysis. Two strains – C09MJLT205 and RM1221 – had a rather similar profile compared with the third one (C97anses640). The 24 genes differentially expressed following the two stresses were mainly categorized into three different groups: i) genes involved in the general stress response, ii) regulators, and iii) genes involved in the central metabolism.

Among the 24 differentially expressed genes, four genes – the transcriptional regulator *hrcA* and the chaperone-encoding genes *clpB, dnaK, grpE* – were up-regulated by thermal stress whatever the strain. This suggests that these genes can be considered as biomarkers of sublethal thermal stress in *C. jejuni*. The up-regulation of these genes was consistent with several results demonstrating the involvement of these HSPs in response to heat, acid or oxidative stresses in *C. jejuni* but also in *C. coli* and *C. lari* (Andersen et al., 2005; Cameron et al., 2012; Flint et al., 2014; Holmes et al., 2010; Konkel et al., 1998; Palyada et al., 2009; Reid et al., 2008a; Reid et al., 2008b; Riedel et al., 2020a; Riedel et al., 2020b; Stintzi, 2003; Sulaeman et al., 2012). They act by repairing and preventing damage caused by the accumulation of unfolded proteins (Stintzi, 2003). Several heat shock proteins (*e.g.*, DnaK) also play a crucial role under optimal physiological conditions by assisting in the proper folding of newly synthesized proteins (Stintzi, 2003). However, other genes were expected to be differentially expressed following thermal stress. For example, *hspR* – encoding a transcriptional regulator – and the chaperone-encoding gene *groEL*, both involved in the *C. jejuni* heat stress response, were not significantly over-expressed in any of the three strains.

The heatmap analysis also highlighted that three transcripts were significantly less abundant in the three strains at the highest temperature. These genes included the two regulators *cosR* and *csrA,* which are involved in the *C. jejuni* oxidative stress response (Fields and Thompson, 2008; Garénaux et al., 2008; Hwang et al., 2011b). The differential expression of genes involved in the oxidative stress response was no surprise because *C. jejuni* were exposed to atmospheric oxygen in addition to heat stress. Furthermore, oxidative stress is related to the *C. jejuni* response to temperature stress. This pathogen is indeed more susceptible to oxidative stress at high temperatures (42°C) than at low temperatures (4°C), suggesting that temperature affects oxidative stress resistance in *C. jejuni* (Garenaux et al., 2008). In *Escherichia coli*, increasing heat increases the generation of reactive oxygen species (ROS), which leads to an oxidative stress response (Marcén et al., 2017). Interestingly, the expression levels of genes involved in the oxidative stress response, namely *cosR*, *csrA* and *katA*, were lower with increased temperature. This result can be linked to oxygen solubility in water, which decreases as temperature increases. This may have resulted in a lower oxygen concentration in contact with *C. jejuni* cells, and in turn lower expression of these genes at 54°C than at 46°C.

CosR (*Campylobacter* oxidative stress regulator) is an OmpR-type response regulator involved in the control of oxidative stress resistance in *C. jejuni* (Hwang et al., 2011a; Hwang et al., 2011b; Hwang et al., 2012). Autoregulation of CosR could also be influenced by the post-transcriptional regulator CsrA (Fields et al., 2016). CosR may respond selectively to superoxide stress (compared to peroxide stress), as superoxide is the first toxic by-product of the oxygen reduction cycle (Hwang et al., 2011b). Resistance of *C. jejuni* to superoxide stress (and to aerobiosis conditions) may ensue from reduced CosR protein levels, which would derepress *sodB*. SodB is the only enzyme able to detoxify superoxide ions in *C. jejuni*. In addition, decreased *cosR* expression may lead to reduced *katA* and *ahpC* gene expression, which cannot contribute to superoxide detoxification (Hwang et al., 2012). In the present study, *cosR* and *katA* gene expression decreased at the highest temperature in the three strains, in line with the literature (Hwang et al., 2011a; Hwang et al., 2011b; Hwang et al., 2012). However, *sodB* and *ahpC* expression was not significantly affected. It should be recalled that CosR is not the only regulator of ROS detoxification genes. Regulators PerR and Fur also play a role (Butcher et al., 2015; Kim et al., 2011; Palyada et al., 2009; van Vliet et al., 1999). Therefore, these findings underline the complexity of the regulation of gene expression and an interaction between co-regulators and the environment.

Eleven genes implied in the lipid, amino acid and energy metabolisms were down-regulated in the two C09MJLT205 and RM1221 strains from 51°C and at 54°C, respectively. Among them, four genes were involved in amino-acid transport and metabolism (*slyD*, *argF*, *trpD*, *ilvC*), in line with previous studies dealing with the effect of cold or heat stresses on *Campylobacter* (Guccione et al., 2017; Shi, 2014). Furthermore, the down-regulation of *csrA* in the three strains at the highest temperature could explain the low level of transcripts involved in the amino acid metabolism (*ilvC*, *argF*), the energy metabolism (*atpA*) and the stress response (*katA*), as shown by Fields et al. (2016) in *csrA* mutants. CsrA appears to regulate a number of cellular processes, including transcription and translation, the nucleotide / amino acid metabolism, DNA repair, energy homeostasis, etc. (Fields et al., 2016).

Genes encoding cell surface components were also down-regulated, such as *yciA* involved in the lipid metabolism, *fliP* in flagellum biosynthesis, and *kdtA* encoding for lipid A of the lipooligosaccharide (LOS). *C. jejuni* LOS consists of a lipid A moiety, an inner core composed of a conserved trisaccharide, and a strain-variable outer core consisting of various sugars (Karlyshev et al., 2005). This structure is important for many pathogens to bypass the host immune defenses. The effect of temperature on LOS length is strain-dependent (Semchenko et al., 2010), suggesting a role in the pathogen's adaptive mechanisms or stress response. *kdtA* can also be down-regulated under acid or oxidative stress conditions (Guccione et al., 2017; Kaakoush et al., 2009; Palyada et al., 2009; Reid et al., 2008a), but not under heat stress conditions (Stintzi, 2003). In addition, cell envelope proteins can be down-regulated following cold shock (Shi, 2014; Stintzi and Whitworth, 2003). These findings could suggest a remodeling of the membrane and cell wall structures in response to stress. The cell membrane composition is generally modified by bacteria under unfavorable environments such as heat or cold stress in order to maintain membrane integrity against stressful conditions (Hughes et al., 2009; Siliakus et al., 2017). The rapid chilling of *C. jejuni* cultures, analogous to poultry carcass chilling, would result in a rapid and large-scale shutdown of cellular processes (Hughes et al., 2009).

Interestingly, two strains, C09MJLT205 and RM1221, had rather similar transcriptomic profiles compared with the third one, C97anses640, in agreement with the results of a previous study. Indeed, *C. jejuni* C97anses640 previously submitted to successive heat and cold stresses was less resistant than strains C09MJLT205 and RM1221 to a subsequent stressing step of refrigerated storage under a modified atmosphere (Duqué et al., 2019). These results suggest a possible correlation between stress-induced gene expression and future bacterial behavior.

Conclusion

Consecutive thermal stresses inspired from the broiler slaughtering process induced a limited inactivation of *C. jejuni* populations, but altered the molecular response of the pathogen at the transcriptomic level. Among the 44 genes under study, 24 were differentially expressed following the two stresses and belonged to three different groups: i) genes involved in the general stress response, ii) regulators, and iii) genes involved in the central metabolism. Our results pointed out transcriptomic variability of *C. jejuni* strains exposed to stresses based on the poultry slaughtering process. Indeed, thermal stress application induced strain-dependent gene expression patterns, with one strain exhibiting a profile significantly different from the two other strains. Genes involved in the heat shock response were mainly overexpressed after stress application in the same manner no matter the strain. In contrast, genes implicated in the oxidative stress response or the central metabolism were differentially expressed according to the strain. For example, it appeared that among the differentially expressed genes, some genes involved in oxidative response like *CbrR, CJE1780* and *nuoL*, were mainly more expressed in the C97anses640 than in the two other strains whereas genes

505 involved in amino acid metabolism, lipid metabolism and energy metabolism were less down
506 regulated in the atypical strain. These results suggest that the adaptative stress response in bacteria is
507 strain-dependent. Since the atypical C97anses640 strain was also shown to behave differently
508 according to stress, these results pave the way for further investigations on bacterial adaptation.

509 Further work is indeed needed to consider the possible correlation between the altered gene
510 expression patterns highlighted in the current work and enhanced *C. jejuni* resistance to a subsequent
511 stress. If this is confirmed, these genes could be used as biomarkers to improve the prediction of
512 bacterial fitness and adaptation because biomarkers are a promising avenue for next-generation
513 predictive models.

514

515 **5 Conflict of Interest**

516 The authors declare that the research was conducted in the absence of any commercial or financial
517 relationships that could be construed as a potential conflict of interest.

518 **6 Author Contributions**

519 J-M.M., S.G. and N.H. conceived the original idea. B.D., J-M.M., S.G. and N.H. conceived and
520 planned the experiments. B.D., S.R., and A.R. carried out the experiments. B.D., S.R., S.G. and N.H.
521 contributed to the interpretation of the results. B.J., S.G. and N.H. took the lead in writing the
522 manuscript. All authors provided critical feedback and helped shape the research, analysis and
523 manuscript.

524 **7 Acknowledgments**

525 We especially thank the Regions Pays de La Loire and Bretagne for their financial support in the
526 framework of the Biomics project. BD obtained a PhD-fellowship grant from the Région Pays de la
527 Loire.

References

Altekruse, S.F., Stern, N.J., Fields, P.I., L, S.D., 1999. *Campylobacter jejuni* - An emerging foodborne pathogen. Emerging Infectious Diseases 5, 28-35. DOI: 10.3201/eid0501.990104.

Andersen, M.T., Brondsted, L., Pearson, B.M., Mulholland, F., Parker, M., Pin, C., Wells, J.M., Ingmer, H., 2005. Diverse roles for HspR in *Campylobacter jejuni* revealed by the proteome, transcriptome and phenotypic characterization of an *hspR* mutant. Microbiology 151, 905-915. DOI: 10.1099/mic.0.27513-0.

Apel, D., Ellermeier, J., Pryjma, M., DiRita, V.J., Gaynor, E.C., 2012. Characterization of *Campylobacter jejuni* RacRS reveals roles in the heat shock response, motility, and maintenance of cell length homogeneity. Journal of Bacteriology 194, 2342-2354. DOI: 10.1128/jb.06041-11.

Aryani, D.C., den Besten, H., Hazeleger, W.C., Zwietering, M., 2015. Quantifying variability on thermal resistance of *Listeria monocytogenes*. International Journal of Food Microbiology 193, 130-138. DOI: 10.1016/j.ijfoodmicro.2014.10.021.

Askoura, M., Sarvan, S., Couture, J.F., Stintzi, A., 2016. The *Campylobacter jejuni* ferric uptake regulator promotes acid survival and cross-protection against oxidative stress. Infection and Immunity 84, 1287-1300. DOI: 10.1128/IAI.01377-15.

Askoura, M., Youns, M., Halim Hegazy, W.A., 2020. Investigating the influence of iron on *Campylobacter jejuni* transcriptome in response to acid stress. Microbial Pathogenesis 138, 103777. DOI: 10.1016/j.micpath.2019.103777.

Baek, K.T., Vegge, C.S., Skorko-Glonek, J., Brondsted, L., 2011. Different contributions of HtrA protease and chaperone activities to *Campylobacter jejuni* stress tolerance and physiology. Applied and Environmental Microbiology 77, 57-66. DOI: 10.1128/AEM.01603-10.

Baillon, M.L.A., van Vliet, A.H.M., Ketley, J.M., Constantinidou, C., Penn, C.W., 1999. An iron-regulated alkyl hydroperoxide reductase (AhpC) confers aerotolerance and oxidative stress resistance to the microaerophilic pathogen *Campylobacter jejuni*. Journal of Bacteriology 181, 4798-4804. DOI:

Bengtsson, M., Ståhlberg, A., Rorsman, P., Kubista, M., 2005. Gene expression profiling in single cells from the pancreatic islets of Langerhans reveals lognormal distribution of mRNA levels. Genome Research 15, 1388-1392. DOI: 10.1101/gr.3820805.

Birk, T., 2012. Acid stress response and protein induction in *Campylobacter jejuni* isolates with different acid tolerance. BMC Microbiology 12, 174. DOI: 10.1186/1471-2180-12-174.

Boehm, M., Lind, J., Backert, S., Tegtmeyer, N., 2015. *Campylobacter jejuni* serine protease HtrA plays an important role in heat tolerance, oxygen resistance, host cell adhesion, invasion, and transmigration. European Journal of Microbiology and Immunology 5, 68-80. DOI: 10.1556/EuJMI-D-15-00003.

Brás, A.M., Chatterjee, S., Wren, B.W., Newell, D.G., Ketley, J.M., 1999. A novel *Campylobacter jejuni* two-component regulatory system important for temperature-dependent growth and colonization. Journal of Bacteriology 181, 3298-3302. DOI: 10.1128/JB.181.10.3298-3302.1999.

Brondsted, L., Andersen, M.T., Parker, M., Jorgensen, K., Ingmer, H., 2005. The HtrA protease of *Campylobacter jejuni* is required for heat and oxygen tolerance and for optimal interaction with human epithelial cells. Applied and Environmental Microbiology 71, 3205-3212. DOI: 10.1128/AEM.71.6.3205-3212.2005.

570 Bronnec, V., Turonova, H., Bouju, A., Cruveiller, S., Rodrigues, R., Demnerova, K., Tresse, O.,
571 Haddad, N., Zagorec, M., 2016. Adhesion, biofilm formation, and genomic features of
572 *Campylobacter jejuni* Bf, an atypical strain able to grow under aerobic conditions. Frontiers in
573 Microbiology 7, 1-14. DOI: 10.3389/fmicb.2016.01002.

574 Burgess, C.M., Gianotti, A., Gruzdev, N., Holah, J., Knochel, S., Lehner, A., Margas, E., Esser, S.S.,
575 Sela Saldinger, S., Tresse, O., 2016. The response of foodborne pathogens to osmotic and desiccation
576 stresses in the food chain. International Journal of Food Microbiology 221, 37-53. DOI:
577 10.1016/j.ijfoodmicro.2015.12.014.

578 Bustin, S.A., Beaulieu, J.-F., Huggett, J., Jaggi, R., Kibenge, F.S., Olsvik, P.A., Penning, L., C.,
579 Toegel, S., 2010. MIQE précis: Practical implementation of minimum standard guidelines for
580 fluorescence-based quantitative real-time PCR experiments. BMC Molecular Biology 11, 74. DOI:
581 http://www.biomedcentral.com/1471-2199/11/74.

582 Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T.,
583 Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines:
584 minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry
585 55, 611-622. DOI: 10.1373/clinchem.2008.112797.

586 Butcher, J., Handley, R.A., van Vliet, A.H., Stintzi, A., 2015. Refined analysis of the *Campylobacter*
587 *jejuni* iron-dependent/independent Fur- and PerR-transcriptomes. BMC Genomics 16, 498. DOI:
588 10.1186/s12864-015-1661-7.

589 Cameron, A., Frirdich, E., Huynh, S., Parker, C.T., Gaynor, E.C., 2012. Hyperosmotic stress
590 response of *Campylobacter jejuni*. Journal of Bacteriology 194, 6116-6130. DOI: 10.1128/JB.01409-
591 12.

592 Cohn, M.T., Ingmer, H., Mulholland, F., Jorgensen, K., Wells, J.M., Brondsted, L., 2007.
593 Contribution of conserved ATP-dependent proteases of *Campylobacter jejuni* to stress tolerance and
594 virulence. Applied and Environmental Microbiology 73, 7803-7813. DOI: 10.1128/AEM.00698-07.

595 den Besten, H., Aryani, D.C., Metselaar, K.I., Zwietering, M., 2017. Microbial variability in growth
596 and heat resistance of a pathogen and a spoiler: All variabilities are equal but some are more equal
597 than others. International Journal of Food Microbiology 240, 24-31. DOI:
598 10.1016/j.ijfoodmicro.2016.04.025.

599 Desriac, N., Coroller, L., Jannic, F., Postollec, F., Sohier, D., 2015. mRNA biomarkers selection
600 based on Partial Least Square algorithm in order to further predict *Bacillus weihenstephanensis* acid
601 resistance. Food Microbiology 45, 111-118. DOI: 10.1016/j.fm.2014.01.004.

602 Desriac, N., Coroller, L., Sohier, D., Postollec, F., 2012. An integrative approach to identify *Bacillus*
603 *weihenstephanensis* resistance biomarkers using gene expression quantification throughout acid
604 inactivation. Food Microbiology 32, 172-178. DOI: 10.1016/j.fm.2012.05.008.

605 Desriac, N., Postollec, F., Coroller, L., Pavan, S., Combrisson, J., Hallier-Soulier, S., Sohier, D.,
606 2017. Trustworthy Identification of Resistance Biomarkers of *Bacillus weihenstephanensis*:
607 Workflow of the Quality Assurance Procedure. Food Analytical Methods 11, 921-932. DOI:
608 10.1007/s12161-017-1058-0.

609 Desriac, N., Postollec, F., Coroller, L., Sohier, D., Abee, T., den Besten, H.M., 2013. Prediction of
610 *Bacillus weihenstephanensis* acid resistance: the use of gene expression patterns to select potential
611 biomarkers. International Journal of Food Microbiology 167, 80-86. DOI:
612 10.1016/j.ijfoodmicro.2013.03.014.

613 Dheda, K., Huggett, J., Chang, J., Kim, L., Bustin, S.A., Johnson, M., Rook, G., Zumla, A., 2005.
614 The implications of using an inappropriate reference gene for real-time reverse transcription PCR
615 data normalization. Analytical Biochemistry 344, 141-143. DOI: 10.1016/j.ab.2005.05.022.

616 Dufour, V., Li, J., Flint, A., Rosenfeld, E., Rivoal, K., Georgeault, S., Alazzam, B., Ermel, G.,
617 Stintzi, A., Bonnaure-Mallet, M., Baysse, C., 2013. Inactivation of the LysR regulator Cj1000 of
618 *Campylobacter jejuni* affects host colonization and respiration. Microbiology 159, 1165-1178. DOI:
619 10.1099/mic.0.062992-0.

620 Duqué, B., Haddad, N., Rossero, A., Membré, J.-M., Guillou, S., 2019. Influence of cell history on
621 the subsequent inactivation of *Campylobacter jejuni* during cold storage under modified atmosphere.
622 Food Microbiology 84. DOI: 10.1016/j.fm.2019.103263.

623 EFSA, ECDC, 2019. The European Union One Health 2018 zoonoses report. EFSA Journal 17,
624 e05926. DOI: https://doi.org/10.2903/j.efsa.2019.5926.

625 Fields, J.A., Li, J., Gulbronson, C.J., Hendrixson, D.R., Thompson, S.A., 2016. *Campylobacter jejuni*
626 CsrA regulates metabolic and virulence associated proteins and is necessary for mouse colonization.
627 PLoS One 11, e0156932. DOI: 10.1371/journal.pone.0156932.

628 Fields, J.A., Thompson, S.A., 2008. *Campylobacter jejuni* CsrA mediates oxidative stress responses,
629 biofilm formation, and host cell invasion. Journal of Bacteriology 190, 3411-3416. DOI:
630 10.1128/JB.01928-07.

631 Flint, A., Sun, Y.-Q., Butcher, J., Stahl, M., Huang, H., Stintzi, A., 2014. Phenotypic screening of a
632 targeted mutant library reveals *Campylobacter jejuni* defenses against oxidative stress. Infection and
633 Immunity 82, 2266-2275. DOI: 10.1128/IAI.01528-13.

634 Fouts, D.E., Mongodin, E.F., Mandrell, R.E., Miller, W.G., Rasko, D.A., Ravel, J., Brinkac, L.M.,
635 DeBoy, R.T., Parker, C.T., Daugherty, S.C., Dodson, R.J., Durkin, A.S., Madupu, R., Sullivan, S.A.,
636 Shetty, J.U., Ayodeji, M.A., Shvartsbeyn, A., Schatz, M.C., Badger, J.H., Fraser, C.M., Nelson, K.E.,
637 2005. Major structural differences and novel potential virulence mechanisms from the genomes of
638 multiple *Campylobacter* species. PLoS Biology 3, e15. DOI: 10.1371/journal.pbio.0030015.

639 Garénaux, A., Guillou, S., Ermel, G., Wren, B., Federighi, M., Ritz, M., 2008. Role of the Cj1371
640 periplasmic protein and the Cj0355c two-component regulator in the *Campylobacter jejuni* NCTC
641 11168 response to oxidative stress caused by paraquat. Research in Microbiology 159, 718-726. DOI:
642 10.1016/j.resmic.2008.08.001.

643 Garenaux, A., Jugiau, F., Rama, F., de Jonge, R., Denis, M., Federighi, M., Ritz, M., 2008. Survival
644 of *Campylobacter jejuni* strains from different origins under oxidative stress conditions: effect of
645 temperature. Current Microbiology 56, 293-297. DOI: 10.1007/s00284-007-9082-8.

646 Garenaux, A., Ritz, M., Jugiau, F., Rama, F., Federighi, M., de Jonge, R., 2009. Role of oxidative
647 stress in *C. jejuni* inactivation during freeze-thaw treatment. Current Microbiology 58, 134-138. DOI:
648 10.1007/s00284-008-9289-3.

649 Grant, K.A., Park, S.F., 1995. Molecular characterization of katA from *Campylobacter jejuni* and
650 generation of a catalase-deficient mutant of *Campylobacter coli* by interspecific allelic exchange.
651 Microbiology 141 (Pt 6), 1369-1376. DOI: 10.1099/13500872-141-6-1369.

652 Guccione, E.J., Kendall, J.J., Hitchcock, A., Garg, N., White, M.A., Mulholland, F., Poole, R.K.,
653 Kelly, D.J., 2017. Transcriptome and proteome dynamics in chemostat culture reveal how
654 *Campylobacter jejuni* modulates metabolism, stress responses and virulence factors upon changes in
655 oxygen availability. Environmental Microbiology 19, 4326-4348. DOI: 10.1111/1462-2920.13930.

656 Gundogdu, O., da Silva, D.T., Mohammad, B., Elmi, A., Mills, D.C., Wren, B.W., Dorrell, N., 2015.
657 The *Campylobacter jejuni* MarR-like transcriptional regulators RrpA and RrpB both influence
658 bacterial responses to oxidative and aerobic stresses. Frontiers in Microbiology 6. DOI:
659 10.3389/fmicb.2015.00724.

660 Guyard-Nicodeme, M., Rivoal, K., Houard, E., Rose, V., Quesne, S., Mourand, G., Rouxel, S.,
661 Kempf, I., Guillier, L., Gauchard, F., Chemaly, M., 2015. Prevalence and characterization of
662 *Campylobacter jejuni* from chicken meat sold in French retail outlets. International Journal of Food
663 Microbiology 203, 8-14. DOI: 10.1016/j.ijfoodmicro.2015.02.013.

664 Guyard-Nicodeme, M., Tresse, O., Houard, E., Jugiau, F., Courtillon, C., El Manaa, K., Laisney,
665 M.J., Chemaly, M., 2013. Characterization of *Campylobacter* spp. transferred from naturally
666 contaminated chicken legs to cooked chicken slices via a cutting board. International Journal of Food
667 Microbiology 164, 7-14. DOI: 10.1016/j.ijfoodmicro.2013.03.009.

668 Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative
669 quantification framework and software for management and automated analysis of real-time
670 quantitative PCR data. Genome Biology 8, R19. DOI: 10.1186/gb-2007-8-2-r19.

671 Holmes, C.W., Penn, C.W., Lund, P.A., 2010. The hrcA and hspR regulons of *Campylobacter jejuni*.
672 Microbiology 156, 158-166. DOI: 10.1099/mic.0.031708-0.

673 Huggett, J., dheda, K., Bustin, S.A., Zumla, A., 2005. Real-time RT-PCR normalisation; strategies
674 and considerations. Genes & Immunity 6, 279-284. DOI: 10.1038/sj.gene.6364190

675 Hughes, R., Hallett, K., Cogan, T., Enser, M., Humphrey, T., 2009. The response of *Campylobacter*
676 *jejuni* to low temperature differs from that of *Escherichia coli*. Applied and Environmental
677 Microbiology 75, 6292. DOI: 10.1128/AEM.00993-09.

678 Hwang, S., Jeon, B., Yun, J., Ryu, S., 2011a. Roles of RpoN in the resistance of *Campylobacter*
679 *jejuni* under various stress conditions. BMC Microbiology 11, 207. DOI: 10.1186/1471-2180-11-207.

680 Hwang, S., Kim, M., Ryu, S., Jeon, B., 2011b. Regulation of oxidative stress response by CosR, an
681 essential response regulator in *Campylobacter jejuni*. PLoS One 6, e22300. DOI:
682 10.1371/journal.pone.0022300.

683 Hwang, S., Zhang, Q., Ryu, S., Jeon, B., 2012. Transcriptional regulation of the CmeABC multidrug
684 efflux pump and the KatA catalase by CosR in *Campylobacter jejuni*. Journal of Bacteriology 194,
685 6883-6891. DOI: 10.1128/JB.01636-12.

686 Hyytiainen, H., Juntunen, P., Akrenius, N., Hanninen, M.L., 2012. Importance of RNA stabilization:
687 evaluation of *ansB*, *ggt*, and *rpoA* transcripts in microaerophilic *Campylobacter jejuni* 81-176.
688 Archives of Microbiology 194, 803-808. DOI: 10.1007/s00203-012-0820-3.

689 Kaakoush, N.O., Baar, C., Mackichan, J., Schmidt, P., Fox, E.M., Schuster, S.C., Mendz, G.L., 2009.
690 Insights into the molecular basis of the microaerophily of three Campylobacterales: a comparative
691 study. Antonie van Leeuwenhoek 96, 545-557. DOI: 10.1007/s10482-009-9370-3.

692 Karlyshev, A.V., Ketley, J.M., Wren, B.W., 2005. The *Campylobacter jejuni* glycome. FEMS
693 Microbiology Reviews 29, 377-390. DOI: 10.1016/j.femsre.2005.01.003.

694 Kim, J.-C., Oh, E., Kim, J., Jeon, B., 2015. Regulation of oxidative stress resistance in
695 *Campylobacter jejuni,* a microaerophilic foodborne pathogen. Frontiers in Microbiology 6, 751-751.
696 DOI: 10.3389/fmicb.2015.00751.

697 Kim, M., Hwang, S., Ryu, S., Jeon, B., 2011. Regulation of perR Expression by Iron and PerR in
698 Campylobacter jejuni. Journal of Bacteriology 193, 6171-6178. DOI: 10.1128/jb.05493-11.

699 Klančnik, A., Guzej, B., Jamnik, P., Vuckovic, D., Abram, M., Mozina, S.S., 2009. Stress response
700 and pathogenic potential of *Campylobacter jejuni* cells exposed to starvation. Research in
701 Microbiology 160, 345-352. DOI: 10.1016/j.resmic.2009.05.002.

702 Konkel, M.E., Kim, B.J., Klena, J.D., Young, C.R., Ziprin, R., 1998. Characterization of the thermal
703 stress response of *Campylobacter jejuni*. Infection and Immunity 66, 36666-33672. DOI:

704 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time
705 quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408. DOI:
706 10.1006/meth.2001.1262.

707 Marcén, M., Ruiz, V., Serrano, M.J., Condón, S., Mañas, P., 2017. Oxidative stress in *E. coli* cells
708 upon exposure to heat treatments. International Journal of Food Microbiology 241, 198-205. DOI:
709 10.1016/j.ijfoodmicro.2016.10.023.

710 Murphy, C., Carroll, C., Jordan, K.N., 2003a. Identification of a novel stress resistance mechanism in
711 *Campylobacter jejuni*. Journal of Applied Microbiology 95, 704-708. DOI: 10.1046/j.1365-
712 2672.2003.02029.x

713 Murphy, C., Carroll, C., Jordan, K.N., 2003b. Induction of an adaptive tolerance response in the
714 foodborne pathogen, *Campylobacter jejuni*. FEMS Microbiology Letters 223, 89-93. DOI:
715 10.1016/S0378-1097(03)00348-3.

716 Nolan, T., Hands, R.E., Bustin, S.A., 2006. Quantification of mRNA using real-time RT-PCR.
717 Nature Protocols 1, 1559-1582. DOI: 10.1038/nprot.2006.236

718 Oh, E., McMullen, L., Jeon, B., 2015. Impact of oxidative stress defense on bacterial survival and
719 morphological change in *Campylobacter jejuni* under aerobic conditions. Frontiers in Microbiology
720 6, 295. DOI: 10.3389/fmicb.2015.00295.

721 Palyada, K., Sun, Y.-Q., Flint, A., Butcher, J., Naikare, H., Stintzi, A., 2009. Characterization of the
722 oxidative stress stimulon and PerR regulon of *Campylobacter jejuni*. BMC Genomics 10, 481. DOI:
723 10.1186/1471-2164-10-481.

724 Park, S.F., 2002. The physiology of *Campylobacter* species and its relevance to their role as
725 foodborne pathogens. International Journal of Food Microbiology 74, 177-188. DOI: 10.1016/s0168-
726 1605(01)00678-x

727 Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., Chillingworth, T.,
728 Davies, R.M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A.V., Moule, S., Pallen, M.J., Penn,
729 C.W., Quail, M.A., Rajandream, M.A., Rutherford, K.M., van Vliet, A.H., Whitehead, S., Barrell,
730 B.G., 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals
731 hypervariable sequences. Nature 403, 665-668. DOI:

732 Pesci, E.C., Cottle, D.L., Pickett, C.L., 1994. Genetic, enzymatic, and pathogenic studies of the iron
733 superoxide dismutase of *Campylobacter jejuni*. Infection and Immunity 62, 2687-2694. DOI:
734 10.1128/IAI.62.7.2687-2694.1994.

735 Phongsisay, V., Perera, V.N., Fry, B.N., 2007. Expression of the htrB gene is essential for
736 responsiveness of Salmonella typhimurium and *Campylobacter jejuni* to harsh environments.
737 Microbiology 153, 254-262. DOI: 10.1099/mic.0.29230-0.

738 Reid, A.N., Pandey, R., Palyada, K., Naikare, H., Stintzi, A., 2008a. Identification of *Campylobacter*
739 *jejuni* genes involved in the response to acidic pH and stomach transit. Applied and Environmental
740 Microbiology 74, 1583-1597. DOI: 10.1128/AEM.01507-07.

741 Reid, A.N., Pandey, R., Palyada, K., Whitworth, L., Doukhanine, E., Stintzi, A., 2008b.
742 Identification of *Campylobacter jejuni* genes contributing to acid adaptation by transcriptional
743 profiling and genome-wide mutagenesis. Applied and Environmental Microbiology 74, 1598-1612.
744 DOI: 10.1128/AEM.01508-07.

745 Rezé, S., Duqué, B., Rossero, A., Membré, J.-M., Guillou, S., Haddad, N., 2019. Validation de la
746 RT-qPCR en vue d'évaluer l'effet de stress sur l'expression génique de *Campylobacter jejuni*, SFM,
747 Paris.

748 Riedel, C., Förstner, K.U., Püning, C., Alter, T., Sharma, C.M., Golz, G., 2020a. Differences in the
749 transcriptomic response of *Campylobacter coli* and *Campylobacter lari* to heat stress. Frontiers in
750 Microbiology 11, 523. DOI: 10.3389/fmicb.2020.00523.

751 Riedel, C., Förstner, K.U., Püning, C., Alter, T., Sharma, C.M., Gölz, G., 2020b. Differences in the
752 transcriptomic response of *Campylobacter coli* and C*ampylobacter lari* to heat stress. Frontiers in
753 Microbiology 11. DOI: 10.3389/fmicb.2020.00523.

754 Ritz, M., Garenaux, A., Berge, M., Federighi, M., 2009. Determination of *rpoA* as the most suitable
755 internal control to study stress response in *C. jejuni* by RT-qPCR and application to oxidative stress.
756 Journal of Microbiological Methods 76, 196-200. DOI: 10.1016/j.mimet.2008.10.014.

757 Rodrigues, R.C., Haddad, N., Chevret, D., Cappelier, J.M., Tresse, O., 2016. Comparison of
758 proteomics profiles of *Campylobacter jejuni* strain Bf under microaerobic and aerobic conditions.
759 Frontiers in Microbiology 7, 1596. DOI: 10.3389/fmicb.2016.01596.

760 Semchenko, E.A., Day, C.J., Wilson, J.C., Grice, I.D., Moran, A.P., Korolik, V., 2010. Temperature-
761 dependent phenotypic variation of *Campylobacter jejuni* lipooligosaccharides. BMC Microbiology
762 10, 305. DOI: 10.1186/1471-2180-10-305.

763 Shi, Z., 2014. Proteomic analysis of the cold stress response in *Campylobacter jejuni*. Lincoln
764 University, Canterbury, New Zealand, p. 134.

765 Siliakus, M.F., van der Oost, J., Kengen, S.W.M., 2017. Adaptations of archaeal and bacterial
766 membranes to variations in temperature, pH and pressure. Extremophiles 21, 651-670. DOI:
767 10.1007/s00792-017-0939-x.

768 Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P.A., Teixeira, P., 2011. *Campylobacter* spp. as
769 a foodborne pathogen: A review. Frontiers in Microbiology 2, 200. DOI: 10.3389/fmicb.2011.00200.

770 Solomon, E.B., Hoover, D.G., 1999. *Campylobacter jejuni*: a bacterial paradox. Journal of Food
771 Safety 19, 121-136. DOI: 10.1111/j.1745-4565.1999.tb00239.x.

772 Stahl, M., Butcher, J., Stintzi, A., 2012. Nutrient acquisition and metabolism by *Campylobacter*
773 *jejuni*. Frontiers in Cellular and Infection Microbiology 2, 5-5. DOI: 10.3389/fcimb.2012.00005.

774 Stahlberg, A., Hakansson, J., Xian, X., Semb, H., Kubista, M., 2004. Properties of the reverse
775 transcription reaction in mRNA quantification. Clinical Chemistry 50, 509-515. DOI:
776 10.1373/clinchem.2003.026161.

777 Stead, D., Park, S.F., 2000. Roles of Fe superoxide dismutase and catalase in resistance of
778 *Campylobacter coli* to freeze-thaw stress. Applied and Environmental Microbiology 66, 3110. DOI:
779 10.1128/AEM.66.7.3110-3112.2000.

780 Stintzi, A., 2003. Gene expression profile of *Campylobacter jejuni* in response to growth temperature
781 variation. Journal of Bacteriology 185, 2009-2016. DOI: 10.1128/JB.185.6.2009-2016.2003.

782 Stintzi, A., Whitworth, L., 2003. Investigation of the *Campylobacter jejuni* cold-shock response by
783 global transcript profiling. Genome Letters 2, 18-27. DOI: 10.1166/gl.2003.000.

784 Sulaeman, S., Hernould, M., Schaumann, A., Coquet, L., Bolla, J.-M., Dé, E., Tresse, O., 2012.
785 Enhanced adhesion of *Campylobacter jejuni* to abiotic surfaces is mediated by membrane proteins in
786 oxygen-enriched conditions. PLoS One 7, 1-14. DOI: 10.1371/journal.pone.0046402.t001.

787 Thies, F.L., Hartung, H.P., Giegerich, G., 1998. Cloning and expression of the *Campylobacter jejuni*
788 lon gene detected by RNA arbitrarily primed PCR. FEMS Microbiology Letters 165, 329-334. DOI:
789 10.1111/j.1574-6968.1998.tb13165.x.

790 Thies, F.L., Karch, H., Hartung, H.P., Giegerich, G., 1999a. Cloning and expression of the dnaK
791 gene of *Campylobacter jejuni* and antigenicity of heat shock protein 70. Infection and Immunity 67,
792 1194-1200. DOI: 10.1128/IAI.67.3.1194-1200.1999

793 Thies, F.L., Weishaupt, A., Karch, H., Hartung, H.P., Giegerich, G., 1999b. Cloning, sequencing and
794 molecular analysis of the *Campylobacter jejuni* groESL bicistronic operon. Microbiology 145 (Pt 1),
795 89-98. DOI: 10.1099/13500872-145-1-89.

796 Trigui, H., Lee, K., Thibodeau, A., Lévesque, S., Mendis, N., Fravalo, P., Letellier, A., Faucher, S.P.,
797 2017. Phenotypic and transcriptomic responses of *Campylobacter jejuni* suspended in an artificial
798 freshwater medium. Frontiers in Microbiology 8. DOI: 10.3389/fmicb.2017.01781.

799 Turonova, H., Haddad, N., Hernould, M., Chevret, D., Pazlarova, J., Tresse, O., 2017. Profiling of
800 *Campylobacter jejuni* proteome in exponential and stationary phase of growth. Frontiers in
801 Microbiology 8. DOI: 10.3389/fmicb.2017.00913.

802 Udvardi, M.K., Czechowski, T., Scheible, W.-R., 2008. Eleven golden rules of quantitative RT-PCR.
803 The Plant Cell 20, 1736-1737. DOI: 10.1105/tpc.108.061143.

804 Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., Leunissen, J.A.M., 2007.
805 Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Research 35, W71-W74. DOI:
806 10.1093/nar/gkm306.

807 van Vliet, A.H., Baillon, M.L., Penn, C.W., Ketley, J.M., 1999. *Campylobacter jejuni* contains two
808 fur homologs: characterization of iron-responsive regulation of peroxide stress defense genes by the
809 PerR repressor. Journal of Bacteriology 181, 6371-6376. DOI:

810 Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F.,
811 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of
812 multiple internal control genes. Genome Biology 3, 1-12. DOI: research0034.1–0034.11.

813 Varsaki, A., Murphy, C., Barczynska, A., Jordan, K., Carroll, C., 2015. The acid adaptive tolerance
814 response in *Campylobacter jejuni* induces a global response, as suggested by proteomics and
815 microarrays. Microbial Biotechnology 8, 974-988. DOI: 10.1111/1751-7915.12302.

816 WHO, 2013. The global view of Campylobacteriosis. World Health Organization.

817 Zhong, X., Wu, Q.P., Zhang, J.M., Shen, S.X., 2016. Prevalence, genetic diversity and antimicrobial
818 susceptibility of *Campylobacter jejuni* isolated from retail food in China. Food Control 62, 10-15.
819 DOI: 10.1016/j.foodcont.2015.09.032.

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821 **Table 1.** Function of the forty target genes of *C. jejuni* used in this study.

Categories according to the tree decision	Gene	Accession number of *C. jejuni* RM1221	Annotations	Involvement in stress response						References
				oxidative	heat	cold	acid	basic	osmotic	
Regulation/regulator	*hrcA*	CJE0848	heat-inducible transcriptional repressor	X	X		X		X	(Cameron et al., 2012; Flint et al., 2014; Holmes et al., 2010; Palyada et al., 2009; Reid et al., 2008b; Stintzi, 2003)
	perR	CJE0367	peroxide stress response regulator	X			X			(Burgess et al., 2016; Palyada et al., 2009; Reid et al., 2008b)
	cbrR	*CJE0746*	two-component response regulator	X					X	(Cameron et al., 2012; Kaakoush et al., 2009)
	Fur	*CJE0449*	fur ferric uptake regulator	X						(Askoura et al., 2016; Burgess et al., 2016; Butcher et al., 2015; Palyada et al., 2009; van Vliet et al., 1999)
	hspR	*CJE1365*	heat shock transcriptional regulator		X		X		X	(Askoura et al., 2020; Cameron et al., 2012; Holmes et al., 2010; Stintzi, 2003)
	lysR	*CJE1080*	transcriptional regulator	X						(Dufour et al., 2013)
	CJE1780	*CJE1780*	two-component regulator	X			X			(Guccione et al., 2017; Reid et al., 2008b)
	cmeR (tetR)	*CJE0417*	transcriptional regulator	X			X			(Butcher et al., 2015; Guccione et al., 2017; Reid et al., 2008b)
	cosR	CJE0404	two-component regulator	X			X			(Garenaux et al., 2009; Hwang et al., 2011b; Varsaki et al., 2015)
	racR	*CJE1397*	two-component regulator	X	X				X	(Apel et al., 2012; Brás et al., 1999)
	rprA	*Cj1546*	transcriptional regulator	X						(Gundogdu et al., 2015)
	rprB	*Cj1556*	transcriptional regulator	X						(Gundogdu et al., 2015)
	csrA	CJE1246	carbon storage regulator	X						(Fields and Thompson, 2008)
Involvement in one stress response	*clpX*	*CJE0324*	ATP-dependent protease ATP-binding subunit	X						(Cohn et al., 2007)
Involvement in two stress responses	*atpA*	*CJE0100*	Synthase subunit alpha	X					X	(Cameron et al., 2012; Kaakoush et al., 2009)
	fliP	*CJE0907*	flagellar biosynthesis protein					X	X	(Butcher et al., 2015; Palyada et al., 2009)
	CJE1361	*CJE1361*	two-component sensor histidine kinase				X		X	(Butcher et al., 2015; Kaakoush et al., 2009; Palyada et al., 2009; Varsaki et al., 2015)
	putA	*CJE1676*	proline dehydrogenase /delta-1-pyrroline-5-carboxylate dehydrogenase	X			X			(Guccione et al., 2017; Reid et al., 2008b)
	dsbl	*CJE0017*	disulfite bond formation protein	X			X			(Butcher et al., 2015; Guccione et al., 2017; Reid et al., 2008b)
	pebC	*CJE1000*	amino acid ABC transporter ATP-binding	X			X			(Butcher et al., 2015; Guccione et al., 2017; Reid et al., 2008b)

Categories according to the tree decision	Gene	Accession number of *C. jejuni* RM1221	Annotations	Involvement in stress response						References
				oxidative	heat	cold	acid	basic	osmotic	
			protein							
	ahpC	CJE0379	alkyl hydroperoxide reductase	X			X			(Baillon et al., 1999; Birk, 2012; Kaakoush et al., 2009; Oh et al., 2015; Palyada et al., 2009; Reid et al., 2008b; Varsaki et al., 2015)
	trpD	*CJE0395*	anthranilate synthase subunit II	X			X			(Guccione et al., 2017; Reid et al., 2008b)
	yidC	*CJE1038*	membrane protein insertase	X			X			(Guccione et al., 2017; Reid et al., 2008b)
	hupB	*CJE0991*	NA-binding protein	X		X				(Guccione et al., 2017; Shi, 2014)
	proS	*CJE0647*	proline--tRNA ligase			X	X			(Shi, 2014; Varsaki et al., 2015)
	asd	*CJE1167*	aspartate-semialdehyde dehydrogenase	X		X				(Butcher et al., 2015; Guccione et al., 2017; Shi, 2014)
Involvement in ≥ 3 stress responses	*grpE*	CJE0849	heat shock protein	X	X		X		X	(Askoura et al., 2020; Cameron et al., 2012; Flint et al., 2014; Palyada et al., 2009; Reid et al., 2008b)
	groEL	CJE1356	Chaperone protein	X	X		X			(Askoura et al., 2020; Cameron et al., 2012; Reid et al., 2008b; Stintzi, 2003; Sulaeman et al., 2012)
	dnaK	CJE0850	Chaperone protein	X	X		X		X	(Cameron et al., 2012; Flint et al., 2014; Reid et al., 2008b; Stintzi, 2003; Sulaeman et al., 2012)
	katA	CJE1576	catalase A	X			X			(Cameron et al., 2012; Oh et al., 2015; Palyada et al., 2009; Reid et al., 2008b; Varsaki et al., 2015)
	argF	*CJE1074*	delta-aminolevulinic acid dehydratase	X		X	X			(Guccione et al., 2017; Shi, 2014; Varsaki et al., 2015)
	rpoB	CJE0528	DNA-directed RNA polymerase subunit beta	X		X	X			(Guccione et al., 2017; Reid et al., 2008b; Shi, 2014)
	yciA	*CJE0993*	acyl-CoA thioesterase	X			X	X		(Butcher et al., 2015; Guccione et al., 2017; Reid et al., 2008b)
	kdtA	*CJE0807*	3-deoxy-D-manno-octulosonic acid transferase	X	X		X			(Kaakoush et al., 2009; Palyada et al., 2009; Reid et al., 2008b; Stintzi, 2003)
	clpB	CJE0616	Chaperone protein		X		X		X	(Cameron et al., 2012; Reid et al., 2008b; Stintzi, 2003)
	sodB	CJE0164	superoxide dismutase							(Cameron et al., 2012; Kaakoush et al., 2009; Oh et al., 2015; Stintzi, 2003)
	htrA	*CJE1363*	serine protease	X	X		X		X	(Baek et al., 2011; Boehm et al., 2015; Brondsted et al., 2005; Cameron et al., 2012; Reid et al., 2008b; Sulaeman et al., 2012)
	sdhA	*CJE0488*	succinate dehydrogenase	X	X		X			(Palyada et al., 2009; Reid et al., 2008b; Stintzi, 2003)

Categories according to the tree decision	Gene	Accession number of *C. jejuni* RM1221	Annotations	Involvement in stress response						References
				oxidative	heat	cold	acid	basic	osmotic	
			flavoprotein subunit							
	htrB	CJE1276	lipid A biosynthesis lauroyl acyltransferase	X	X		X		X	(Phongsisay et al., 2007 ; Trigui et al., 2017)
	nuoL	*CJE1739*	NADH-quinone oxidoreductase subunit L	X			X		X	(Cameron et al., 2012; Kaakoush et al., 2009; Varsaki et al., 2015)

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824 **Table 2.** Primer pairs sequences for *C. jejuni* genes used in this study

Genes	Forward	Reverse	Amplicon size (pb)	Efficiency (%)	R^2
Control of RNA samples					
341F/758R	CCTACGGGAGGCAGCAG	CTACCAGGGTATCTAATCC	439	/	/
Potential reference genes					
rpoA	CGAGCTTGCTTTGATGAGTG	AGTTCCCACAGGAAAACCTA	80	100	1.00
rrs	AAGGGCCATGATGACTTGAC	AGCGCAACCCACGTATTTAG	107	100	1.00
ilvC	GCATGCAGAACGCAAAAATA	TGATCCAAGGCATCATAGCA	109	92	1.00
gyrA	GTTATTATAGGTCGTGCTTT	CTATGAGGTGGGATGTTTGT	92	89	1.00
slyD	TACGATGAAAATGCCGTTCA	TTCGCCAAAAAGCTCCATAC	84	95	1.00
thiC	TTATCTTTGGGCGATGCTTT	CATCCCAAGCCCTTTGAGTA	109	93	1.00
Target genes					
hrcA	GGGCTTAAGGTAGATGCGCA	TCATGCCGCCTCCTTTATGT	111	93	0.97
groEL	AGTAATCGGTGGTGGTGCAG	CTTTCAACGATAGCTGCGCC	96	109	1.00
atpA	CGTGAAGCTTATCCAGGCGA	GCAATGCCGTCAAAGAACCA	103	98	1.00
clpX	AGCTGCAGATGGAGATGTGC	AAGCTTGTTGCACACCCTCT	131	94	1.00
dnaK	CACGCGGTATGCCACAAATC	CCTCGCTAAGTCCGCTTGAA	132	97	1.00
sodB	CAAAACTTCAAATGGGGGCGT	ACAGCCACAGCCTGTACTTG	97	94	1.00
katA	CAGGCGCAAAAGGACCTTTG	TTCCCTTGGCATGAACGGTT	105	93	1.00
perR	CCTTCAATCTCTTTAGCGACGG	CCACATTTGGTGCAAACAACA	146	90	1.00
cbrR	CTTGCCAGATGCCCCAAATG	GCCTTATCACCACTAGCCGT	90	98	1.00
CJE1780	TCCAACAAGCAAAGCAGTGC	AGAGATTGTTTTCTGTGGGAAGT	136	94	1.00
cmeR (tetR)	TCCAATTGGCAAGATGTCTATCT	TCAATCAACCAGAAGCTGTAGC	81	93	1.00
cosR	GGGAAGCAGAAACGGTATCCT	TGCCATCTGGGAAGAACCTG	141	99	1.00
fur	CCATTTCTTTTGGTTCAGCAGGT	GCAATCAAGGCTTGCTGTCT	136	92	1.00
hspR	AGAGCCAAGTAGAACCGATGG	ACTCCAGCAAGATTGATTCCCA	111	93	1.00
lysR	CGCTCTCAAACGCAGAATTCA	AGCTGGATCAAGTTCTGCTTCA	93	93	1.00
putA	GCTAGTGGTGCAGCTATGGT	TTCGCACCTAAGGGCATAGG	83	106	1.00
racR	TTGTGGGGCTTCAAATCGGT	TCAACTCTTTTTGTGCGACGA	106	93	1.00
rprA	CGCTCAAATTCCACCCAAGG	TGCCCCATTTGCTCATAGCT	89	99	0..98
rprB	CGAGATTTGTTGCAAGGGACTAA	CTTTGCGTTTGATCAGTTTTGC	139	93	1.00
fliP	TTTGCGTCCCCATAGCTTGT	TGCGCTCGCTCCAAGTATAG	92	92	1.00
dsbl	AAGCCCTACTGCTGTCATGA	GCTGGGGACAAAGAGAAGCA	116	90	1.00
pebC	GGTTTCTGGATCAAGGGCTGA	AGCAACCCTTTCAGGTGGAC	112	91	1.00
htrA	TGCCGTTATTTCCACCACCA	CAGGTGGAGCTTTGGTGGAT	84	103	1.00
ahpC	GAACAGTTCGCCATGCTGTG	TCGCCCTTATTCCATCCTGC	133	97	0.99
grpE	TGAAAAACATGGGGTGGCTCT	AGCACTTGAACCACTTCACCA	117	98	1.00
hupB	TCTTTTTGCCGCTTGCTTTTG	GCTAGAGTACCAAGCACAGGA	112	97	1.00
proS	GGCTATTGGTGGAAGTGGCT	GCAGCTTCAACATTAGCCGC	108	94	1.00
asd	GCATTCAGGAACCACCAAAGG	GCAGGTGGAAGTGTGAGTGA	120	91	1.00
sdhA	GGTAGATCAAGACGCGGCTT	AAACGCTGAGCTACTCCACC	117	92	1.00

Genes	Forward	Reverse	Amplicon size (pb)	Efficiency (%)	R^2
Control of RNA samples					
htrB	TGCGGCTAAATATGGTGCGA	AAATTGGGTGCGACTTTGGC	91	95	1.00
yciA	TCCTGAACGCGTTGTAACCA	CGCTGATTGAAGTGTTGCCC	119	93	1.00
argF	ATCCTTTACAGGCTCGCCAC	CCATAACAGAGCTTGGCGGA	80	100	1.00
rpoB	GGCTAATGGCGTTGATGCAG	TCACCTGGGCGCATAACTTT	141	90	1.00
CJE1361	TCGCATTGCAATTTCTTTGGC	GCAAATTACTTCAGGGGCTGC	88	94	1.00
YidC	TCACAACGATAGCCCAACCC	ATCCACGCCTTGAAGCAGTT	120	92	1.00
trpD	AAGCGGTAGAATGCAAGGGG	GCCACTAAAGGCGAACTTGC	96	95	0.99
kdtA	TGCCAAACCTAAGGAAAAGCT	TAAAACGCTCAGGGTGTCGT	131	97	0.99
csrA	GAAAGTATAATTATCGGAGAAGG	CAACAGAATGCAAATTTTCATC	151	87	1.00
nuoL	ACCAAATAAACCCCCGCTGT	CACACTTGGCTTGCTGATGC	92	87	1.00
clpB	GGCGCACCTAAGAGTCTTGA	GGCCAACAGGGGTAGGAAAA	136	87	1.00

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827 **Table 3.** Reference genes selected and associated M value for each *C. jejuni* strain. In bold, the two
828 genes selected as internal reference for each strain, according to their M-value (< 0.5).

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***C. jejuni* strain**	**Gene**	**M value**
C09MJLT205	***asd***	**0.16**
	dsbI	0.475
	proS	**0.16**
	thiC	0.255
	rrs	0,485
RM1221	***asd***	**0.41**
	dsbI	0.735
	proS	0.790
	thiC	0.865
	rrs	**0,410**
C97anses640	*asd*	0,525
	dsbI	**0.30**
	proS	0.375
	thiC	**0.30**
	rrs	0,565

830

831 **Table 4.** Mean inactivation (expressed in Δlog CFU.mL^{-1}) induced by heat stress only, cold stress
832 only and cumulated stresses, with respective standard deviation, of the three strains of *C. jejuni*
833 (C97anses40, C09MJLT205 and RM121) as a function of the heat stress temperature (54, 51 and
834 46°C).

835

Heat stress temperature (°C)	Strain	Δlog (CFU.mL^{-1})		
		Heat stress	Cold stress	Cumulated stress
54	C97anses640	0.37 ± 0.11	-0.03 ± 0.13	0.34 ± 0.01
	C09MJLT205	0.35 ± 0.22	0.15 ± 0.19	0.51 ± 0.04
	RM1221	0.16 ± 0.03	0.26 ± 0.10	0.44 ± 0.07
51	C97anses640	0.03 ± 0.05	0.19 ± 0.19	0.23 ± 0.11
	C09MJLT205	-0.06 ± 0.14	0.01 ± 0.22	-0.05 ± 0.25
	RM1221	-0.02 ± 0.10	0.33 ± 0.04	0.31 ± 0.08
46	C97anses640	-0.06 ± 0.07	-0.04 ± 0.06	-0.10 ± 0.06
	C09MJLT205	-0.13 ± 0.11	-0.15 ± 0.20	-0.28 ± 0.10
	RM1221	0.01 ± 0.02	0.16 ± 0.04	0.17 ± 0.03

836 Negative values mean no inactivation due to the uncertainty from enumeration method.

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840 **List of figures**

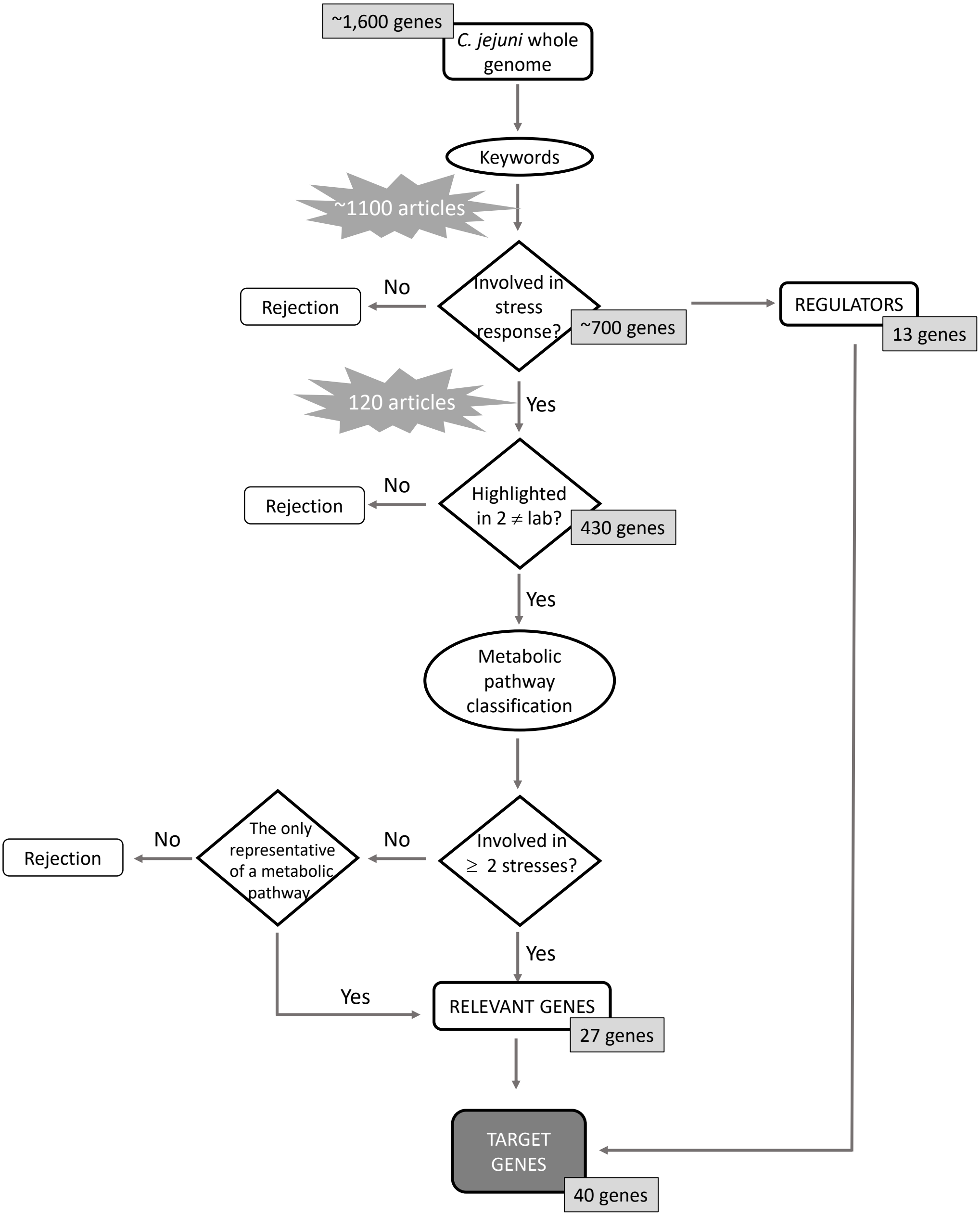
841 **Figure 1.** Decision tree for the selection of target genes according to the literature analysis.

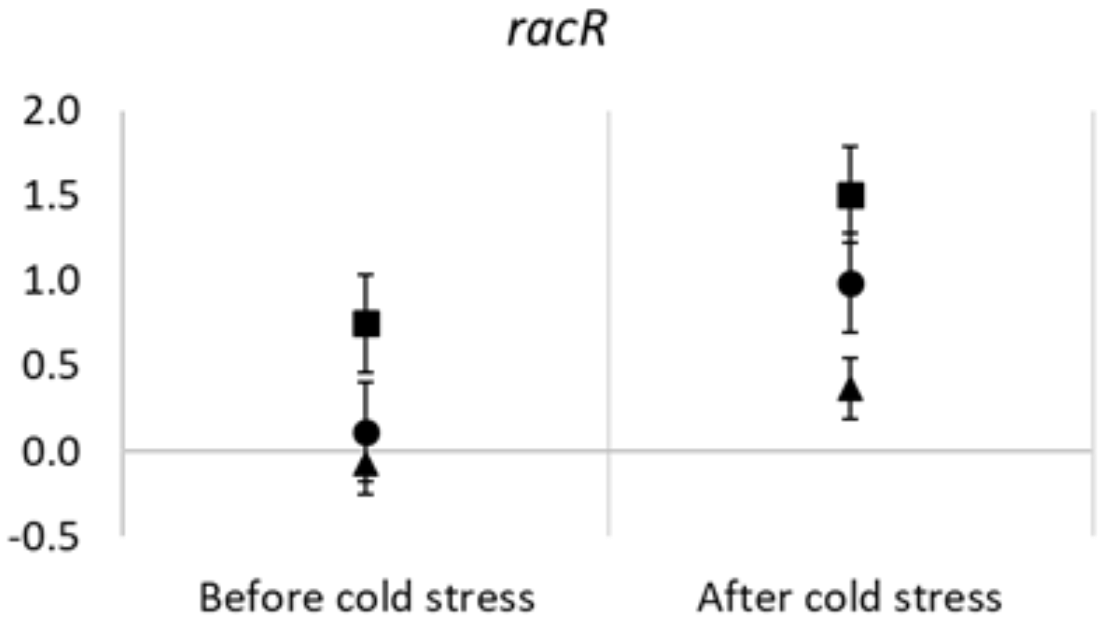
842 **Figure 2.** Expression of *katA*, *lysR*, *pebC* and *racR* following heat stress at 46°C with or without
843 subsequent application of the cold stress. Symbols correspond to mean $\log_2$ fold change (●: RM1221
844 ; ▲: C09MJLT205 ; ■: C97anses640) and bars to 95% confidence intervals.

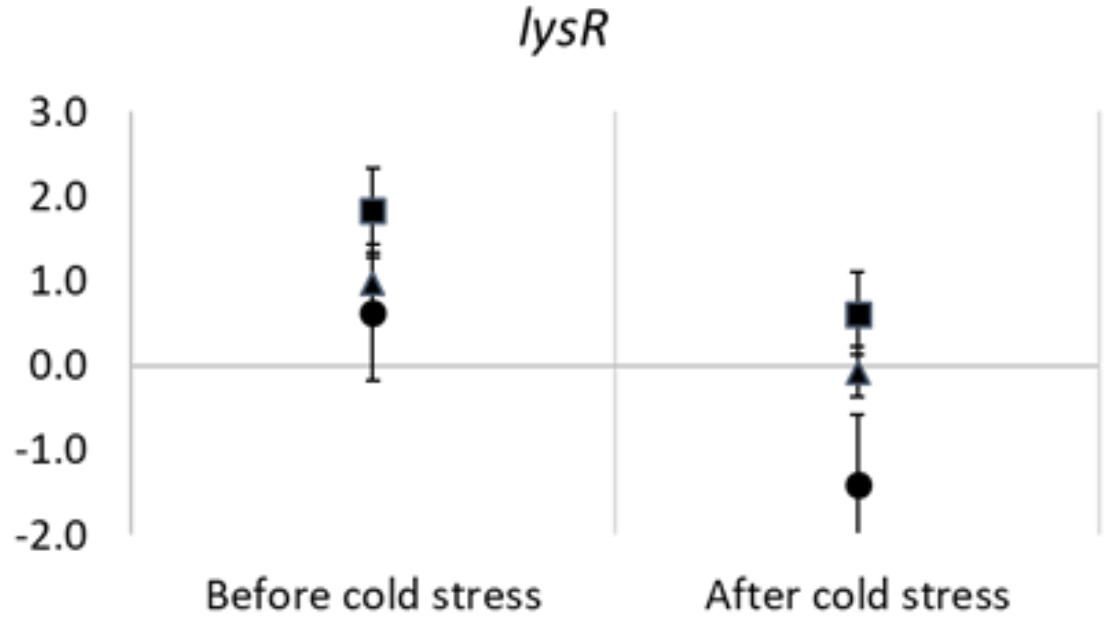
845 **Figure 3.** Hierarchical clustering analysis of gene expression for three *C. jejuni* strains
846 (C09MJLT205, C97anses640, RM121) after application of successive stresses: heat (46°C, 51°C and
847 54°C during 3 min) and cold temperature (-4°C during 2 h). A threshold $\log_2$ value of 2 was used in
848 this figure. The intensity of the color is proportional to expression change. Data were normalized
849 with the control condition, which was the unstressed culture. The color variation indicates level of
850 gene expression as compared to the normalizing condition, *i.e.* green, downregulation; red,
851 upregulation. Each capital letter corresponds to a cluster after the hierarchical clustering was
852 performed.

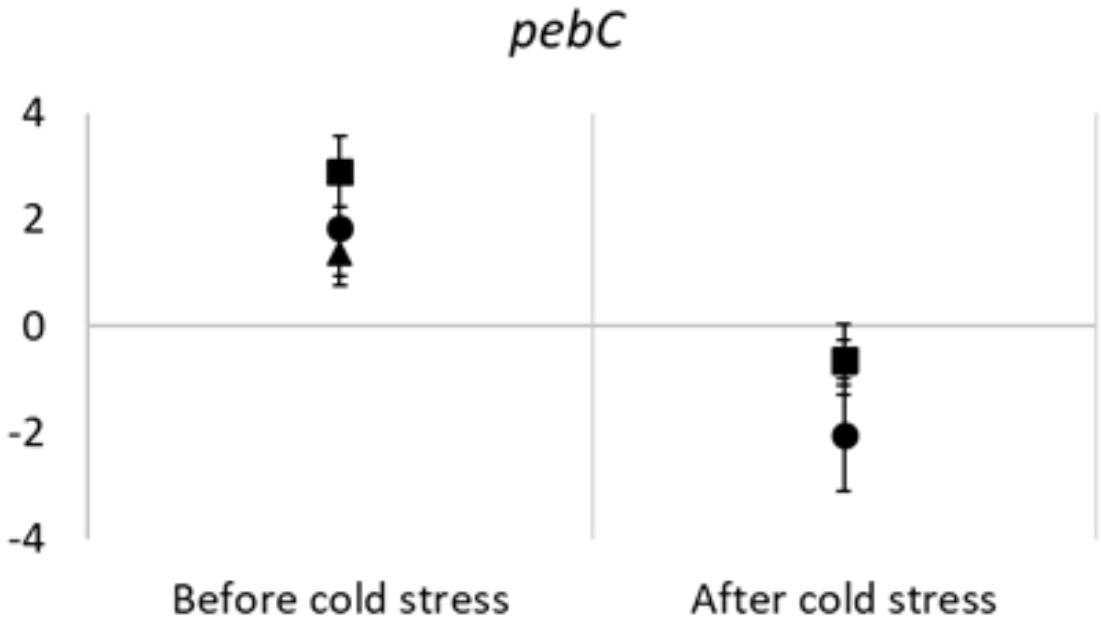
853 **Figure 4.** Representative genes from each cluster which were differentially expressed after
854 application of the consecutive heat and cold stress (open bars, grey bars and black bars correspond to
855 46, 51 and 54°C, respectively) for the three strains of *C. jejuni* C09MJLT205, RM121 and
856 C97anses640). For each strain and each gene, different letters (a-c) show significant differences
857 between the different temperatures ($p \leq 0.05$).

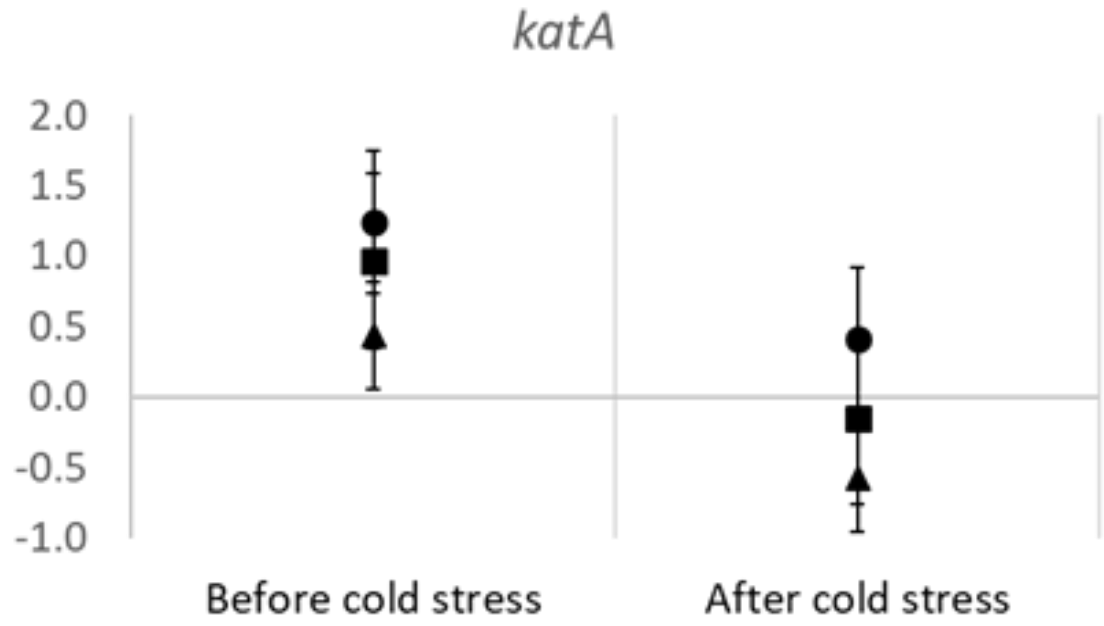
858

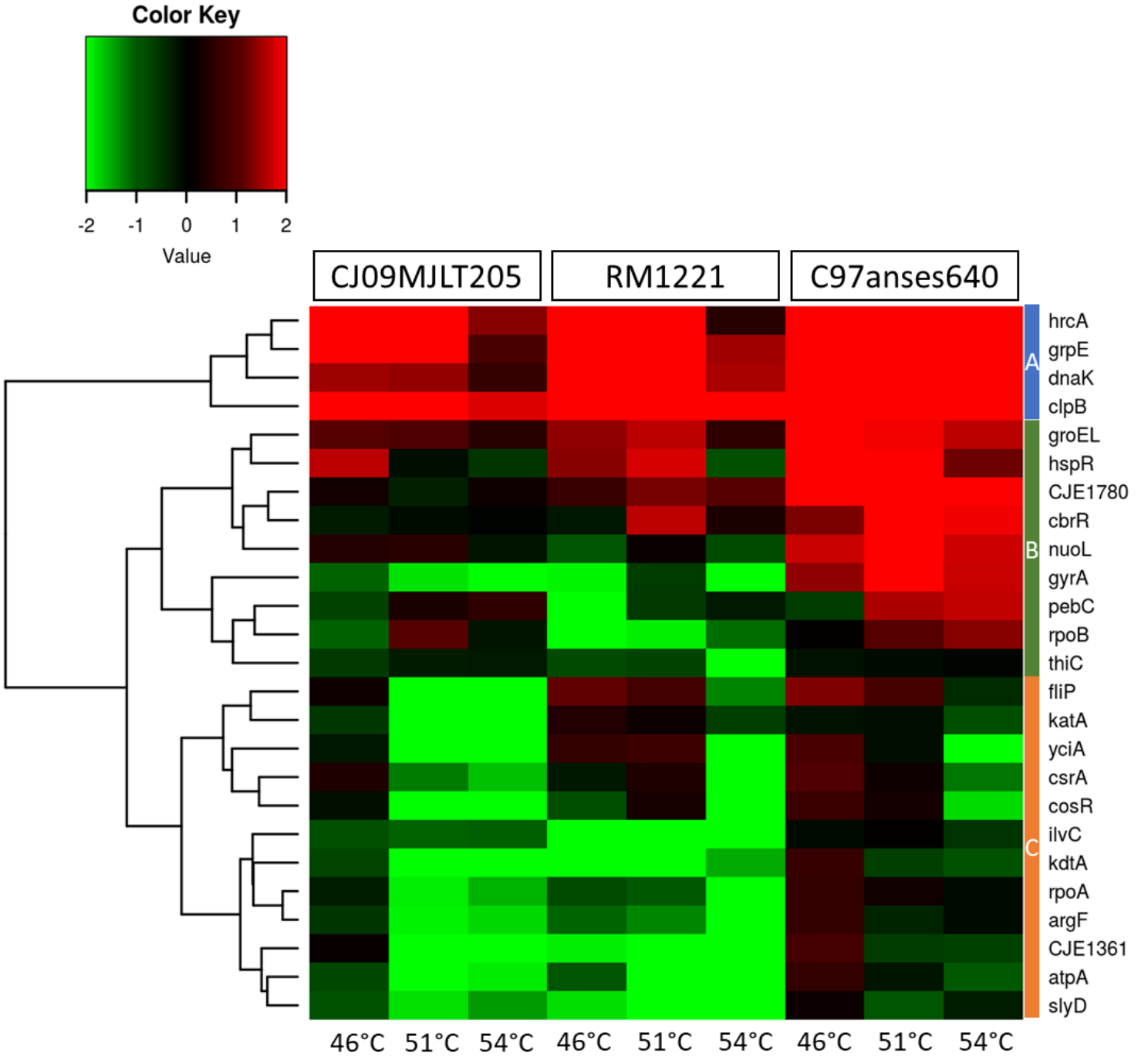

~1,600 genes
C. jejuni whole genome
Keywords
~1100 articles
Involved in stress response?
No
Rejection
~700 genes
REGULATORS
13 genes
120 articles
Yes
Highlighted in 2 ≠ lab?
No
Rejection
430 genes
Yes
Metabolic pathway classification
Involved in ≥ 2 stresses?
No
The only representative of a metabolic pathway
No
Rejection
Yes
Yes
RELEVANT GENES
27 genes
TARGET GENES
40 genes

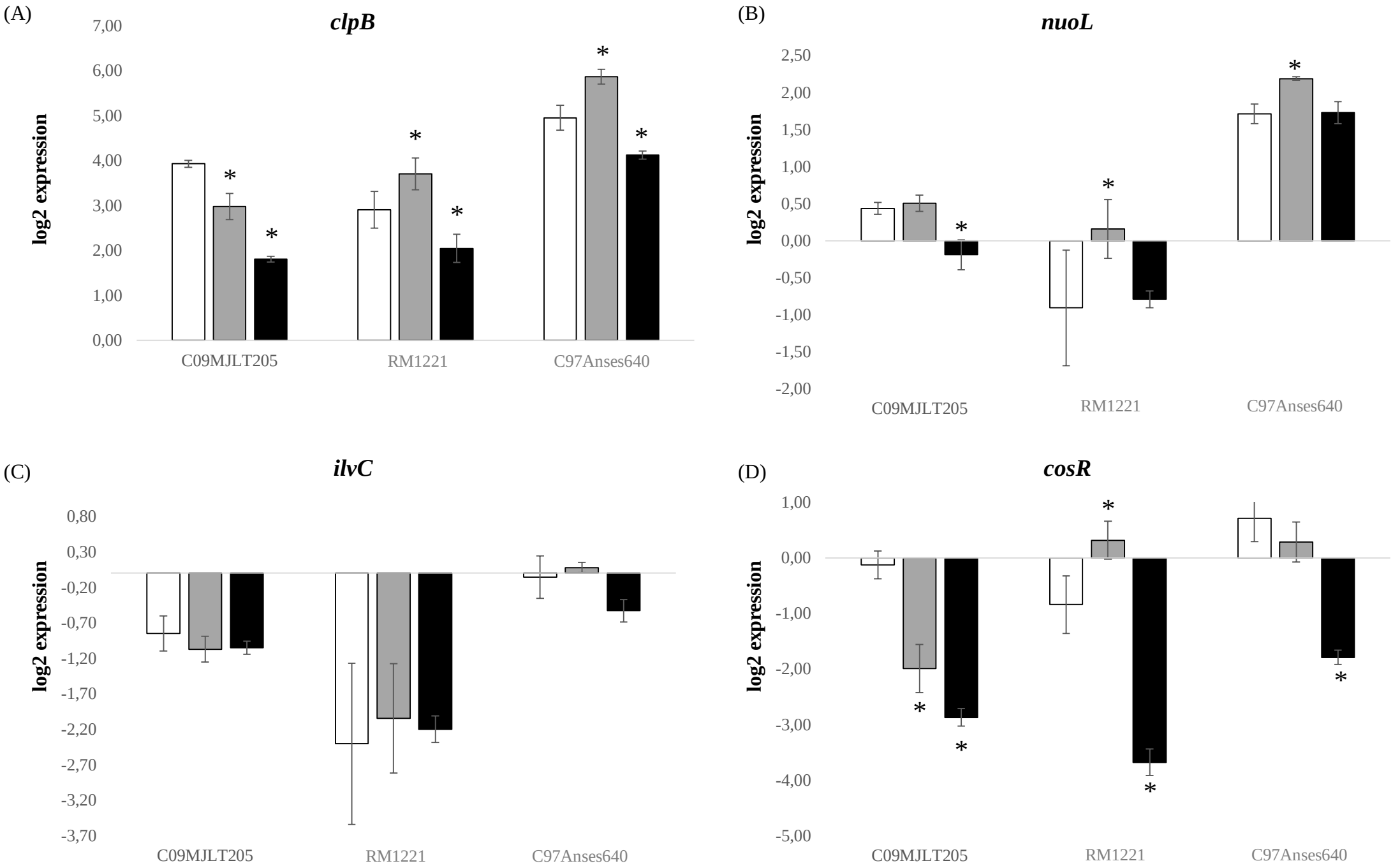

racR
2.0
1.5
1.0
0.5
0.0
-0.5
Before cold stress
After cold stress


lysR
3.0
2.0
1.0
0.0
-1.0
-2.0
Before cold stress
After cold stress


pebC
4
2
0
-2
-4
Before cold stress
After cold stress


katA
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
Before cold stress
After cold stress


Color Key
-2
-1
0
1
2
Value
CJ09MJLT205
RM1221
C97anses640
hrcA
grpE
dnaK
clpB
groEL
hspR
CJE1780
cbrR
nuoL
gyrA
pebC
rpoB
thiC
fliP
katA
yciA
csrA
cosR
ilvC
kdtA
rpoA
argF
CJE1361
atpA
slyD
A
B
C
46°C
51°C
54°C
46°C
51°C
54°C
46°C
51°C
54°C


(A)
clpB
log2 expression
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
C09MJLT205
RM1221
C97Anses640
(B)
nuoL
log2 expression
2,50
2,00
1,50
1,00
0,50
0,00
-0,50
-1,00
-1,50
-2,00
C09MJLT205
RM1221
C97Anses640
(C)
ilvC
log2 expression
0,80
0,30
-0,20
-0,70
-1,20
-1,70
-2,20
-2,70
-3,20
-3,70
C09MJLT205
RM1221
C97Anses640
(D)
cosR
log2 expression
1,00
0,00
-1,00
-2,00
-3,00
-4,00
-5,00
C09MJLT205
RM1221
C97Anses640