

1 **Title:** *Campylobacter jejuni* transcriptome changes during loss of culturability in  
2 water

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## 33 Abstract

34

35 **Background:** The natural environment serves as a potential reservoir for

36 *Campylobacter*, the leading cause of bacterial gastroenteritis in humans.

37 However, little is understood about the mechanisms underlying variations in

38 survival characteristics between different strains of *C. jejuni* in natural

39 environments, including water.

40 **Results:** We identified three *Campylobacter jejuni* strains that exhibited

41 variability in their ability to retain culturability after suspension in water at two

42 different temperatures (4°C and 25°C). Of the three, strains *C. jejuni* M1

43 exhibited the most rapid loss of culturability whilst retaining viability. Using

44 RNAseq transcriptomics, we characterised *C. jejuni* M1 gene expression in

45 response to suspension in water by analyzing bacterial suspensions recovered

46 immediately after introduction into water (Time 0), and from two sampling

47 time/temperature combinations where considerable loss of culturability was

48 evident, namely (i) after 24 h at 25°C, and (ii) after 72 h at 4°C. Transcript data

49 were compared with a culture-grown control. Some gene expression

50 characteristics were shared amongst the three populations recovered from

51 water, with more genes being up-regulated than down. Many of the up-regulated

52 genes were identified in the Time 0 sample, whereas the majority of down-

53 regulated genes occurred in the 25°C (24 h) sample.

54 **Conclusions:** Variations in expression were found amongst genes associated

55 with oxygen tolerance, starvation and osmotic stress. However, we also found

56 upregulation of flagellar assembly genes, accompanied by down-regulation of

57 genes involved in chemotaxis. Our data also suggested a switch from secretion

58 via the *sec* system to via the *tat* system, and that the quorum sensing gene *luxS*

59 may be implicated in the survival of strain M1 in water. Variations in gene

60 expression also occurred in accessory genome regions. Our data suggest that

61 despite the loss of culturability, *C. jejuni* M1 remains viable and adapts via

62 specific changes in gene expression.

63

64 **Keywords:** transcriptome, *C. jejuni*, RNA Seq, environment, survival, *luxS*, viable

65 but non-culturable

## 66 Background

67 *Campylobacter jejuni* is the most common bacterial cause of gastroenteritis in  
68 Europe and the USA [1-3]. Although campylobacteriosis is primarily considered  
69 to be a zoonotic infection, mostly transmitted to humans through consumption of  
70 contaminated poultry [4], it is clear that the environment can play a role in  
71 transmission either directly, for example via unchlorinated drinking water [5], or  
72 indirectly, via farm animals that acquire the pathogen from the environment [4,  
73 6]. Indeed, contaminated groundwater is considered to be a potential source of  
74 transmission of *Campylobacter* to poultry flocks and to humans directly [7-15].  
75

76 The survival mechanisms employed by *Campylobacter* outside of the host  
77 are not well understood. Genomic studies suggest that the ability of *C. jejuni* to  
78 regulate gene expression in response to environmental stresses may be limited  
79 because of the lack of many of the stress response mechanisms possessed by  
80 other bacterial species [6, 16, 17]. However, despite its apparent limited ability  
81 to respond to stress, *Campylobacter* can survive in the environment and remain  
82 infectious [18]. It has been suggested that *Campylobacter* spp. can survive in  
83 natural water by entering a viable but non culturable (VBNC) state [19]. During  
84 the VBNC state, *Campylobacter* form coccoid-shaped cells with an intact cell  
85 membrane, which remain viable according to various measures of metabolic  
86 activity, but are unable to grow on routine culture media [20]. Bacteria in this  
87 state are capable of causing infections or colonising a host and can be returned to  
88 a state of culturability [21].

89 Previous studies have shown that survival of *Campylobacter* in natural  
90 water samples or ground water is highly dependent upon temperature and  
91 strain origin [22-24]. Low temperatures (around 4°C) enhance the survival of  
92 *Campylobacter*, whereas at increased temperatures (20-25°C) viability declines  
93 rapidly [22, 25]. Survival times for different *C. jejuni* isolates in water can vary  
94 from a few days at ambient temperatures to four months at 4°C [26].

95 Many factors could contribute to the variations observed between  
96 *Campylobacter* strains with respect to survival in water [18]. These include the  
97 ability to metabolize available nutrients and to deal with oxidative and osmotic  
98 stress [25, 27]. Previous epidemiological studies have suggested that some strain

types, defined using schemes such as Multi Locus Sequence Typing (MLST), are more commonly found in the environment [28-30]

There are significant gaps in our knowledge concerning variations in the survival of different *C. jejuni* strain types in the environment. These could be due to differences either in gene content or in gene expression. Variations in the gene content of *C. jejuni* strains, linked to virulence potential, have been described previously [31]. These include variations in surface structures, such as glycosylation patterns of flagellin or the structures of lipooligosaccharides, but also include metabolic traits [32]. Genes supporting oxygen-independent respiration and the catabolism of amino acids and peptides are particularly over-represented in strains that are robust colonisers of poultry, compared to strains that do not colonise well [30, 31, 33-36]. Little is known about how variations in gene expression contribute to the ability to survive in the environment. A better understanding of the occurrence and behaviour of *Campylobacter* in water, and the mechanisms underlying variations has implications for food safety and public health [37].

In this study we demonstrate how strains of *C. jejuni* adjust differently to an aquatic lifestyle by comparing the culturability of *C. jejuni* strains after prolonged exposure to water. We show that *C. jejuni* strain M1, though rapidly losing culturability on standard laboratory media, retains viability in water and, using strand-specific Illumina RNA Seq analysis, we identify gene expression changes occurring during the survival process.

## Results and Discussion

### Loss of culturability during survival in water

In this study, we focused on three *C. jejuni* strains exhibiting differences in their ability to retain culturability during incubation in water at specific time points and temperatures (24 h at 25°C and 72 h at 4°C). The ability to retain culturability in water was tested using three biological replicates for each of the three strains of *C. jejuni*: M1 (ST137, clonal complex ST45, associated with severe human infection) [38], 1336 (ST841, a representative of the water/wild-life clade)[30], and strain 414 (ST3704, associated with bank voles) [39]. Clear variations were observed between the three strains with respect to the ability to

132 retain culturability on Columbia Blood Agar (CBA) containing 5% (v/v)  
133 defibrinated horse blood after exposure to sterile distilled water at two  
134 temperatures: 25°C and 4°C (Figure 1). At 4°C (72 h), whereas only  
135 approximately 17% of strain M1 cells were still culturable, for strains 1336 and  
136 414 much higher levels (64% and 48% respectively) remained culturable. At  
137 25°C (24 h), whereas only approximately 1.2% of strain M1 cells remained  
138 culturable, for strains 1336 and 414 approximately 71% and 82% respectively  
139 remained culturable. At these two sampling points, the survival of strain M1,  
140 based on culturability on CBA media, was significantly lower ( $p < 0.01$ ; 2-tailed  
141 Student's t-test) than for either of the other two strains.

142 We further investigated viability of cells using LIVE/DEAD staining at  
143 Time 0, 4°C (72 h) and 25°C (24 h). Whereas the prevalence of culturable cells on  
144 CBA media declined rapidly, especially for strain M1, at both 4°C and 25°C, the  
145 percentage of viable cells according to LIVE/DEAD staining remained high  
146 throughout the experiment (Figure 1). Hence, although strain M1 rapidly loses  
147 culturability on CBA media during exposure to water, the majority of cells  
148 remain viable, indicative of the VBNC state when this medium is used.

149

## 150 **Overview of differential gene expression during survival of *C. jejuni* M1 in** 151 **water**

152 In order to better understand the process by which strain M1 remains viable but  
153 loses culturability, we analysed the transcriptome of cells recovered from water  
154 survival experiments. *C. jejuni* M1 gene expression in water was investigated,  
155 using Illumina RNA Seq under two key experimental conditions: i) 25°C for 24h,  
156 ii) 4°C for 72 h, selected because of the high viability but low culturability  
157 exhibited by strain M1 at these sampling points (Figure 1). In addition, two  
158 controls were included in the study. The first control involved suspending an  
159 inoculum of *C. jejuni* M1 in 100 ml of sterile distilled water and recovering the  
160 cells immediately; this control will be referred to as the "Time 0" sample. It  
161 should be noted that the bacterial cells in the Time 0 sample were pre-exposed  
162 to water at room temperature for approximately 20 min due to the sample  
163 processing time, prior to RNA extraction. The second control consisted of cells  
164 cultured in Mueller Hinton Broth (MHB) for 24 h at 37°C under microaerophilic

165 conditions; this transcriptome will be referred to as the “MHB Control”. All initial  
166 inocula were taken from the same starter culture.

167 The transcriptomics data were analysed using two separate approaches  
168 to determine differential expression of genes: (i) based on counts per million  
169 (cpm) and (ii) using BitSeq analysis.

170 A summary of all M1 RNA Seq data in counts per million (cpm) is shown  
171 in Additional file 1. It is apparent from these data that significant transcriptome  
172 changes occurred during the preparation of the Time 0 sample compared to the  
173 MHB Control, indicating that *C. jejuni* gene expression changes in response to  
174 suspension in water occur rapidly. Figure 2 shows comparisons of genes up  
175 (Figure 2A) or down (Figure 2B) regulated 2-fold or more relative to the MHB  
176 Control during the different experimental conditions. Additional file 2  
177 summarizes genes that are up- or downregulated 2-fold or more across all three  
178 test conditions compared to the MHB control.

179 Figure 2 clearly shows that more genes were upregulated (58 genes)  
180 across the three time-point/temperature combinations [Time 0, 25°C (24 h) and  
181 4°C (72 h)], than downregulated (5 genes). The biggest response in terms of  
182 upregulation in gene expression was observed during the early exposure to  
183 water at Time 0. The biggest response in terms of downregulation occurred at  
184 25°C (24 h) (Figure 2).

185 Among genes upregulated across all three conditions were several  
186 flagellar genes (*flgBCDEGHF*) and *flaG*), as well as the *flmA* (*pseB*) gene, which is  
187 also involved in flagellar assembly and glycosylation; *flmA* mutants are non-  
188 motile and accumulate intracellular flagellin [40]. Four of the genes upregulated  
189 across all three conditions were hypothetical ones with their function currently  
190 unknown (Additional file 2). The list of genes downregulated 2-fold included  
191 three members of the *nap*-operon.

192 The results of the Bayesian Inference of Transcripts from Sequencing data  
193 (BitSeq) [41] analysis are summarized in Additional file 3. 546 genes were  
194 statistically significantly upregulated at Time 0, compared to the MHB Control,  
195 whereas 204 genes were downregulated; 872 genes showed no significant  
196 change. In the survival experiments, at 25°C after 24 h in sterile distilled water,  
197 161 genes were upregulated, 557 genes were downregulated and 904 showed no

significant change, compared to the MHB Control. At 4°C after 72 h, 202 genes were upregulated, 301 were downregulated and 1119 showed no significant change in expression compared to the MHB Control.

## **Differential expression of known stress response genes**

Despite its lack of many of the conventional pathways possessed by other enteropathogenic bacteria, *Campylobacter* does adapt quickly to environmental stressors [42, 43]. During adaptation to water, *C. jejuni* M1 must potentially counter oxidative and short term aerobic stress, hypo-osmotic stress, starvation and temperature shock in the different experimental conditions tested. Additional file 4 summarises the expression profiles of a number of previously characterized stress response genes compared to the MHB Control, across the experimental conditions tested. Figure 3 highlights the expression of known stress response genes and also includes all of the genes that showed significant up- or down-regulation ( $\geq 2$ -fold) compared to MHB Control (Additional file 2).

## **Oxidative, short term aerobic and temperature stress responses**

Catalase (*katA*) and superoxide dismutase (*sodB*) genes were significantly upregulated in all three experimental conditions compared to the MHB control (Figure 3, Additional file 4). These genes are involved in both oxidative stress and freeze-thaw stress response [44]. Despite an apparent lack of cold shock proteins, *Campylobacter* maintains its metabolic rate at low temperatures (4°C) and appears to survive better at 4°C than at 25°C [45, 46]. In addition, an ankyrin-containing protein CJM1\_1347 (Cj1386) has recently been identified in *C. jejuni*, encoded by a gene based directly downstream of the *katA* gene, and this is thought to be involved in the same detoxification pathway as catalase [47]; our data indicated significant upregulation of this gene as an early response to exposure to water in the Time 0 sample (Figure 3).

Previous studies have suggested that expression of the catalase (*katA*) gene, increased after exposure to oxidative stress, but not the superoxide dismutase (*sodB*) gene, the main antioxidant defence of most organisms [48]. It is essential for *C. jejuni* to counter atmospheric oxygen tensions and the resulting damage to nucleic acid through the toxicity of reactive oxygen species (ROS).

231 Unlike the three classified types of superoxide dismutases (SODs) present in  
232 *Escherichia coli*, *C. jejuni* only possess one (SodB) [49, 50]. It has been suggested  
233 that both SodB and catalase may play an important role in intracellular survival  
234 of *C. jejuni* [51, 52].

235 The *htrA* gene was significantly downregulated during the early response  
236 to exposure to water (Time 0). However, downregulation was not evident at the  
237 other time points. HtrA is important for stress tolerance and survival of Gram-  
238 negative bacteria generally [53, 54] and is required for both heat and oxygen  
239 tolerance [55]. It has been reported previously that *htrA* is downregulated in *C.*  
240 *jejuni* in response to low nutrient and especially oxidative stress [56]. HtrA, a  
241 periplasmic serine protease, displays both chaperone and protease properties,  
242 both implicated in the ability to tolerate stress, though the chaperone activity  
243 was identified as more important for resistance to oxidative stress [57].

244 *Campylobacter* cells show signs of heat stress at temperatures of 46°C and  
245 above; these conditions accelerate the transition of spiral cells to coccoid shaped  
246 ones. Arguably our experimental conditions tested for cold shock conditions,  
247 encountered by *Campylobacter* in the natural environment outside the host, with  
248 test temperatures below the ideal growth temperatures of 37 to 42°C. We did,  
249 however, observe changes in the expression levels of several heat shock proteins  
250 at 25°C including the chaperones *groLS* and *dnaK* the encoding genes are  
251 summarized in Additional file 4.

252

## 253 **Osmotic stress**

254 While a number of studies have contributed to our understanding of hyper-  
255 osmotic stress responses [58-60], mainly related to food preservation and *in vivo*  
256 environments, hypo-osmotic stress responses are still poorly understood.  
257 Introducing the bacteria into water (Time 0) leads to a marked influx of water  
258 into the cells due to the osmotic gradient. Hence, *C. jejuni* needs to react quickly  
259 to prevent cell lysis. Genes implicated in responding to hyper-osmotic  
260 conditions include *htrB*, *ppk* and a sensor histidine kinase (CJM1\_1208). Both  
261 CJM1\_1208 and *ppk* were downregulated at all three time points (Figure 3,  
262 Additional file 4).



263 The obvious response to changes in osmolarity, exhibited by many  
264 bacteria, is to pump out both water and solutes. Three stretch-activated  
265 mechano-sensitive (Msc) channels have been described in *E. coli*: MscM (mini),  
266 MscS (small) and MscL (large). A homologue for MscL has been described in *H.*  
267 *pylori* [61-63]. Kakuda *et al.* recently identified two putative mechanosensitive  
268 channels in the strain 81-176 (*Cjj0263* and *Cjj1025*), corresponding to  
269 CJM1\_0221 (mechanosensitive ion channel family protein) and CJM1\_0980  
270 (putative membrane protein) respectively in M1 [64]. Both genes were  
271 upregulated during the early response at Time 0, compared to the MHB control,  
272 with CJM1\_0980 showing statistically significant upregulation (PPRL=0.965).  
273 Interestingly, both genes were downregulated at 4°C (72 h), suggesting that *C.*  
274 *jejuni* M1 had adjusted to the hypo-osmotic conditions after 24 h (Figure 3).

275

## 276 **Iron acquisition**

277 Iron acquisition is a vital process for bacterial survival and persistence. Due to  
278 the toxic potential of free iron, storage and uptake are tightly regulated.  
279 The putative hemin uptake gene cluster *chuABCD* and *Cj1613c* (*CJM1\_1550*) are  
280 regulated by the ferric uptake repressor (Fur), which in turn is governed by the  
281 availability of free iron [65]. In this study, the *chuABCD* genes were upregulated  
282 >2 fold across all three experimental conditions tested, compared to the MHB  
283 control; *chuA* and *chuC* were statistically significantly upregulated over all three  
284 conditions, whereas *chuB*, *chuD* and the heme oxygenase gene *CJM1\_1550*  
285 (*Cj1613c*) were only upregulated at Time 0. These findings strongly support the  
286 importance of iron regulation during water survival. The *fur* gene was  
287 statistically significantly downregulated at 25°C (24 h) compared to the MHB  
288 control, but did not vary significantly between the non-control samples.

289

## 290 **Starvation response**

291 The stringent response is rapidly induced during starvation and stationary  
292 phase. Classically, RpoS is the global regulator for the stationary phase in  
293 bacteria. In lacking the *rpoS* gene, *C. jejuni* presents an RpoS-independent  
294 response to starvation and stationary phase. Inorganic polyphosphate (poly-P) is  
295 a linear polymer of phosphate residues linked by phosphoanhydride bonds

which provide a high energy to the cell [66]. Poly-P, a source of energy and an essential molecule for survival during starvation, is synthesized by mediating the key enzyme polyphosphate kinase 1 (*ppk*) [67]; we observed that *ppk* was down-regulated in response to water (Figure 3; Additional File 4). Interestingly, it has been reported that a mutant *C. jejuni* ( $\Delta ppk$ ) showed decreased ability to enter a VBNC state due to lack in poly-P synthesis [16, 68]. Our observations of down-regulation appear to contradict this notion and may be indicative of variations between strains.

Even if glucose was available, *Campylobacter* spp. are incapable of using glucose as an energy source and have a very restricted carbohydrate catabolism (non-saccharolytic), a characteristic that distinguishes them greatly from other gastrointestinal pathogens. Phosphoenolpyruvate carboxykinase (PCK) (CJM1\_0407), an essential enzyme in gluconeogenesis, was significantly upregulated in the early response (Time 0) compared to the MHB Control (Additional file 2).

311

### 312 **Quorum sensing**

The transfer from an exponentially growing culture into water suddenly presents *C. jejuni* M1 cells with very low cell density conditions. Bacterial communities can communicate, in a density-dependent manner, through sensing autoinducers, extracellular signal molecules, produced by members of the community. The LuxS product autoinducer 2 (AI-2) is found in over 55 species, and is common to both Gram positive and Gram negative bacteria [69, 70]. Compared to the MHB control, we observed a significant upregulation of *luxS* expression at Time 0, and at both 25°C (24 h) and 4°C (72 h) (Additional file 2). The gene encoding CosR (CJM1\_0334), a known positive regulator of *luxS* [71], was significantly upregulated at 4°C (72 h) compared to 25°C (24 h), but not found to be upregulated otherwise.

324

### 325 **Protein translocation and secretion**

One of the overarching factors important in stress survival is the ability to transport proteins across membranes. The twin-arginine translocation (TAT) system in particular is vital for stress survival [72]. Our data show that

329 components of the TAT system were upregulated at both Time 0 and at 4°C (72  
330 h), compared to the MHB control (Figure 4), suggesting an increased need for  
331 translocation of proteins across the cell membrane at these time points and/or  
332 temperatures. In contrast, components of the Sec pathway were downregulated  
333 in all three conditions (Figure 4), confirming the crucial role of TAT during  
334 stressful conditions.

335       The TAT system is an inner membrane translocase that transports  
336 proteins folded in the cytoplasm across the inner membrane. In contrast to Sec,  
337 which cannot accept tightly folded pre-proteins for translocation, the TAT  
338 system can translocate folded enzymes. Several substrates for the *C. jejuni* TAT  
339 system have been identified, including PhoX [73-76], the only alkaline  
340 phosphatase identified in *Campylobacter* species. Upon transport into the  
341 periplasm PhoX becomes active, providing *Campylobacter* with that vital energy  
342 source, inorganic phosphate (Pi). The gene encoding PhoX (CJM1\_0145) was  
343 significantly upregulated at Time 0 compared to the MHB control.

344

### 345 **Motility and Chemotaxis**

346 We observed the upregulation of many flagellar assembly genes across all  
347 experimental conditions (Figure 5); however, expression of both the flagellar  
348 motor genes and components of the chemotaxis pathway (Figure 6) was either  
349 downregulated or unchanged, indicating an alternative role to motility, as has  
350 been suggested previously [77]. The structural components of flagella are  
351 important for the secretion of virulence factors such as the *Campylobacter*  
352 invasion antigen (CiaB) [78]. However, under the conditions tested here, *ciaB*  
353 expression was not significantly upregulated (Additional file 3). It has been  
354 suggested that *C. jejuni* could also play an important role in adhesion in the early  
355 stages of biofilm formation [79]. Further work is needed to determine whether  
356 the gene expression changes that we have seen in water are indicative of the  
357 bacteria aggregating as a precursor to forming biofilms.

358       Studies in *H. pylori* have shown that the AI-2 encoded by *luxS*, which is  
359 highly upregulated across conditions in this study, targets expression of flagellar  
360 genes, and it has been suggested that *H. pylori* can regulate the composition of its

361 flagella in response to environmental clues [80, 81]. Hence, there may be a link  
362 between the LuxS and flagellar component expression changes that we observed.

363

### 364 **Electron transport pathways and metabolism**

365 The respiratory chain in *C. jejuni* is highly branched, with a number of potential  
366 electron acceptors, including fumarate, nitrate, nitrite, trimethylamine-N-oxide  
367 (TMAO) and dimethylsulphoxide (DMSO), involved in growth under severely  
368 oxygen-limited conditions [82]. In our study, three genes (*napG*, *napH*, *napB*)  
369 encoding enzymes that play an important role in major electron transport pathways  
370 were down-regulated in response to the water environment (Figure 3; Additional file  
371 2). *nrfA* was also down-regulated in all three conditions, whilst *nrfH* was  
372 significantly down-regulated in two of the three conditions compared to the MHB  
373 control. These genes encode nitrate (Nap) and nitrite (Nrf) reductases involved in the  
374 use of nitrate and nitrite as electron acceptors. The Nap nitrate reductase is a two  
375 subunit enzyme comprising NapA and NapB, requiring NapD for proofreading. It is  
376 thought that the iron-sulphur proteins NapH and NapG assume the role of  
377 electron door to the NapAB complex [83]. The nitrite reductase NrfA is the  
378 terminal enzyme in the reduction of nitrite to ammonia, and is thought to play a  
379 role in defence against nitrosative stress [83]. It is thought that NrfH is the sole  
380 electron donor to NrfA [83]. Hence, our data suggest that nitrate or nitrite  
381 electron acceptors are not being utilised during survival in water.

382 The expression of many of the genes involved in central carbon  
383 metabolism was down-regulated in water. These included the genes encoding  
384 succinate dehydrogenase (SdhABC), malate dehydrogenase (Mdh) and formate  
385 dehydrogenase (FdhABC). Interestingly, the genes encoding PutP (proline  
386 transport) and PutA (proline dehydrogenase) were up-regulated, suggesting that  
387 proline metabolism was active. PutA catalyses the oxidation of proline to  
388 glutamate. Glutamine synthase (GlnA), which can catalyse conversion of  
389 glutamate to glutamine, and gamma-glutamyl transpeptidase (GGT), which can  
390 catalyse the hydrolysis of glutamine to form glutamate and ammonia, were also  
391 up-regulated. In *H. pylori*, it has been shown that the likely role of GGT is to  
392 supply the bacteria with glutamate for catabolism by the hydrolysis of  
393 extracellular glutathione or glutamine, with the substrates being hydrolysed in

the periplasm before glutamate is transported into the cell [84]. Hence, there is some evidence for metabolism involving proline, glutamine and glutamate, but the full pathway is not clear.

### **Pathogenicity and virulence factors**

Unlike other enteropathogenic bacteria, *C. jejuni* does not possess many conventional pathogenicity factors. The cells in VBNC state may play an important role in the pathogenicity of *C. jejuni*; for example, the expression of the virulence gene *cadF*, encoding an outer-membrane protein of *C. jejuni* involved in adhesion to intestinal fibronectin has still been detected at high levels up to the third week of entering a VBNC state [85]. We observed consistently high levels of expression of *cadF* across all conditions tested (Additional file 1). However, we did not detect any statistically significant changes in *cadF* expression (Additional file 3). In contrast, the genes encoding cytolethal distending toxin (*cdtABC*) were down-regulated in response to water (Additional file 3).

### **Do genomic Regions of Difference (RODs) in the *C. jejuni* M1 genome play a role in its enhanced survival in the experimental conditions tested?**

To further investigate the water survival response of *C. jejuni* M1, we identified RODs within the *C. jejuni* M1 genome compared to the other *C. jejuni* strains tested in this experiment, *C. jejuni* 414 and 1336 (Figure 1 A and B), as well as the well characterized reference strain NCTC11168 [30, 38, 86]. We identified a total of 45 RODs in the *C. jejuni* M1 genome; 13 of these RODs are variable regions in all four genomes tested. The results have been summarized in Additional file 5 where shared RODs have been highlighted.

The currently published *C. jejuni* M1 genome (CP001900) contains 234 CDS annotated as “hypothetical protein” or “putative uncharacterized protein”. Albeit many of the functions and products of these genes have been described since, for ease of discussion we re-annotated the genome using a combination of PROKKA [87], and searches in stringDB and BLAST (BLASTP and BLASTX). Putative functions and expression profiles are summarised in Additional file 5.

426 An overall summary of the M1 genome, including RODs, hypothetical genes, and  
427 BitSeq expression data is shown in Additional file 6.

428         There were a number of examples of genes, or clusters of genes, within  
429 RODs that were either up- or down-regulated (Additional file 5; Additional file  
430 7). It has been reported previously that the genomes of strains 1336 and 414  
431 lack some genes that are widely distributed in *C. jejuni* [30]. Hence, some RODs  
432 include genes that have known putative functions, such as the *cdtABC* genes,  
433 which are down-regulated in water. Since M1 behaves differently to strains  
434 1336 and 414 in water, it is possible that genes present in only strain M1 might  
435 contribute to these differences. For example, ROD-6 (comprising four putative  
436 genes) is absent from strains 414 and 1336, and is upregulated in water.  
437 However, the gene functions are not known. Further work would be needed to  
438 determine which, if any, RODs play a significant role in the strain M1 VBNC  
439 phenotype.

440

## 441 **Conclusions**

442 Our data suggest that *C. jejuni* M1 adapts rapidly to introduction into a water  
443 environment, instigating gene expression changes that allow it to adapt to the  
444 stressful conditions, whilst maintaining viability. In addition to the up-  
445 regulation of stress responses, a preference for secretion via the Tat pathway  
446 and the down-regulation of many (but not all) metabolic genes, the bacteria  
447 adapt in some more surprising ways. We observed down-regulation of  
448 chemotaxis genes coupled to up-regulation of flagellar genes, suggesting a role  
449 for flagella that is not linked to motility. In addition to the secretion of virulence  
450 factors, flagella have been implicated in autoagglutination and microcolony  
451 formation as a precursor to biofilm formation [88]. Hence, it is possible that the  
452 observed gene expression changes are indicative of the bacteria starting to  
453 instigate a lifestyle change from sessile to biofilm as a survival strategy. We also  
454 found evidence that the putative quorum sensing system protein LuxS plays a  
455 role during the adaptation to water. Genes in the accessory genomes, including  
456 genes encoding hypothetical proteins of no known function, may play a role in  
457 the variable survival phenotypes that are observed between strains. Hence,  
458 despite the loss of culturability, strain M1 remains viable and adapts to

suspension in water via multiple specific changes in gene expression. Further work is needed to ascertain which responses are shared by all *C. jejuni* strains, and which are specific to a sub-set of strains sharing the characteristics of M1.

## **Methods:**

### **Bacterial Growth conditions**

*Campylobacter* strains were stored on storage beads in glycerol broth (M Lab) at -80°C. *C. jejuni* strains were grown on sterile Columbia Blood Agar Base (CBA, Oxoid) with 5% (v/v) defibrinated horse blood (Oxoid) at 37°C for 48 h under microaerobic conditions (85% [v/v] N<sub>2</sub>, 5% [v/v] O<sub>2</sub>, and 10% [v/v] CO<sub>2</sub>), in a Whitley VA500 Workstation incubator (Don Whitley Scientific Ltd).

### **Preparation of cell suspensions for testing survival in sterile water**

For survival experiments, bacteria were sub-cultured on blood agar for 24 h at 37°C under microaerobic conditions. A 5 µL loop of cultured cells was taken, and suspended in 5 mL of Muller-Hinton Broth (MHB, Oxoid) supplemented with *Campylobacter* growth supplement (LAB M). Suspended bacterial samples were adjusted to a final optical density at 600 nm (OD<sub>600</sub>) of 0.05 [ $3.8 \times 10^7$  -  $3.5 \times 10^8$  Colony Forming Unit (CFU)/mL] (Spectronic Biomate 5).

### **Preparation and inoculation of sterile water sample**

Filtered-tap water (PUR1TE SELECT) was collected, and autoclaved at 121°C for 15 min. Aliquots of 99 mL (pH 6.5) of autoclaved water samples were transferred into 250 mL sterile borosilicate glass bottles with screw caps (Schott, Duran, Germany) in triplicate. These were inoculated with 1 mL from bacterial suspensions to a final concentration of  $8 \times 10^5$  -  $3.7 \times 10^6$  cells/mL. The inoculated samples were kept in the dark at 25°C (for 24h) or 4°C (for 72 h). An uninoculated sterile distilled water sample for each temperature was used as a control for the presence of contamination. A further control was used whereby the water was inoculated with 1 mL of bacterial suspension and the bacteria were collected immediately (time 0). All experiments were conducted using

three independent technical replicates and three biological replicates (strains M1, 1336 and 414).

#### **Enumeration of colony forming units (CFU)**

At time 0, 25°C (24 h), and 4°C (72 h), a 100 µL sample was taken and 10-fold dilutions were made in MHB supplemented with *Campylobacter* growth supplement. A 10 µL spot assay of appropriate dilutions was carried out on CBA plates in triplicate. The plates were incubated for 48h at 37°C under microaerophilic conditions, and the survival was then determined by enumerating the CFU/ mL.

#### **Cell survival by LIVE/DEAD staining**

Inoculated water samples were prepared as described above. At time 0, 25°C (24 h), and 4°C (72 h), inoculated water samples *C. jejuni* strains M1, 414 and 1336 were concentrated by centrifugation at 3893 × g for 20 min (3-16PK - SIGMA) in Falcon tubes (Corning, Appleton Woods). Supernatant was removed and approximately 1 mL remained at the bottom of each Falcon tube and was subsequently transferred into a 1.5 mL Eppendorf tube. Cells were pelleted by centrifugation at 5000 × g for 20 min. The pellet was re-suspended in 1 mL of sterile distilled water. Using the LIVE/DEAD BacLight, Invitrogen kit, 3 µL of the mixture (SYTO 9 green-fluorescent and propidium iodide red-fluorescent) were added for each 1 mL of bacterial suspension and this mixture was incubated for 15 min at room temperature. 5 µL of the cell suspension was then placed on a microscope slide, covered with a 22 × 22 mm cover slip and sealed.

The LIVE/DEAD BacLight kit (Invitrogen) contains two nucleic acid stains, SYTO 9 dye, which penetrates live cells (intact membranes) causing the cells to stain fluorescent green, and propidium iodide dye that cannot cross the cell membrane and therefore only stains cells red if the membranes are damaged and the cell is therefore presumed dead.

Enumeration of VBNC cells was carried out under a fluorescence microscope (Nikon ECLIPSE 80i). For each sample, three fields were enumerated at an average of 90-180 cells in each field. The percentage of viable cells was calculated as follows: % viable cells = [viable cell count (green cells)/total cell



count (green cells + red cells)] × 100. The experiment was conducted in three independent replicates.

### **RNA extraction from water survival experiments**

100 mL of inoculated water samples were prepared for *C. jejuni* M1 in triplicate at time 0, 25°C (24 h) and 4°C (72 h). Cells were concentrated by centrifugation at 3893 × g (3-16 pk -SIGMA) for 20 min at the corresponding temperature in 50ml Falcon tubes (Corning, Appleton Woods). Supernatants were removed but, for each sample, approximately 1 ml was retained at the bottom of each Falcon tube and subsequently combined per sample and transferred into a 1.5 mL Eppendorf tube. Cells were pelleted by centrifugation at 5000 × g for 10 min. A negative control was included using only sterile distilled water. Additionally a control of *C. jejuni* M1 grown in Mueller Hinton Broth was prepared. Cells were grown to a density of 1x10<sup>7</sup> in a 25 cm<sup>2</sup> cell culture flask with gas exchange lid (Corning) at 37°C microaerophilic conditions. Cells were collected by centrifugation at 3000xg in a microcentrifuge (Eppendorf). Once collected, cells were immediately resuspended in TRIzol solution (3 times TRIzol volume to 1 volume of cells)(Ambion) and stored at -80oC until further processing. TRIzol samples were allowed to reach room temperature and cells were disrupted using vigorous vortexing. The samples were then incubated at room temperature for 5 min. RNA was extracted using the Direct-zol RNA MiniPrep Kit (Zymo Research), following the manufacturer's instructions.

### **Illumina Library construction and sequencing**

116 ng of total RNA was depleted using the Illumina Ribo-zero rRNA Removal Kit (Bacteria) and purified with Ampure XP beads. Successful depletion was confirmed using Qubit and Agilent 2100 Bioanalyzer. All of the depleted RNA was used as input material for the ScriptSeq v2 RNA-Seq Library Preparation protocol. Following 15 cycles of amplification the libraries were purified using Ampure XP beads. Each library was quantified using Qubit and the size distribution assessed using the Agilent 2100 Bioanalyzer.

The final libraries were pooled in equimolar amounts using the Qubit and Bioanalyzer data. The quantity and quality of each pool was assessed by

Bioanalyzer and subsequently by qPCR using the Illumina Library Quantification Kit from Kapa on a Roche Light Cycler LC480II according to manufacturer's instructions. Sequencing was performed at the Centre for Genomic Research, University of Liverpool, on one lane of the Illumina HiSeq 2500 2x125 bp using v4 chemistry (Illumina).

562

### 563 **RNA Seq data analysis**

564 The raw FASTQ data files were trimmed for the presence of Illumina adapter sequences using Cutadapt (v1.2.1.) [89], using the -O 3 option. The reads were 565 further trimmed using Sickle (v1.200) (<https://github.com/najoshi/sickle>) with 566 a minimum window quality score of 20. Reads shorter than 10bp after trimming 567 were removed. 568

569 Sense and antisense overlaps between the annotation and mapped reads 570 were counted using the HTSEQ package [90], using the stranded and union 571 options. Read counts were then normalised and Differential Expression 572 calculated using EdgeR implemented in R (version 3.1.2 (2014-10-31), using 573 Loess-style weighting to estimate the trended dispersion values, heatmaps were 574 constructed using heatmap2 in R.

575 For pairwise Differential Expression analysis between samples, the data 576 were re-mapped to the *C. jejuni* M1 genome [38] using Bowtie2 [91], and parsed 577 using the BitSeq (Bayesian Inference of Transcripts from Sequencing data) 578 pipeline [41]. BitSeq takes into account biological replicates and technical noise, 579 and thereby calculates a posterior distribution of differential expression 580 between samples.

581 Statistical Significance of BitSeq results was visualized in Artemis [92]. 582 Regions of Difference between the genomes of M1 (CP001900) [38], 1336 583 (ADGL00000000), 414 (ADGM00000000) [30] and NCTC11168 (AL111168) [86, 584 93] were derived through pairwise genome comparisons in ACT [94]. Putative / 585 Hypothetical genes were selected in Artemis and a putative function was derived 586 from searches using BLASTX 587 ([http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE\\_TYPE=BlastSearch&LINK\\_LOC=blasthome](http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome)). 588 589

590 **Ethics Approval and Consent to Participate:** Not applicable

591 **Consent for Publication:** Not applicable

592 **Availability of data and materials:** Sequence data are available at the European  
593 Nucleotide Archive under Primary Accession PRJEB17925. Other data  
594 supporting the results are available as Additional Files. Bacterial strains will be  
595 made available on request.

596

597 **Competing Interests**

598 The authors declare that they have no competing interests.

599

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606

607 **Author' Contributions**

608 CB carried out the transcriptomics experiments. KM, CEJ carried out the initial  
609 water survival experiments. CN and AL carried out the laboratory work required  
610 for library preparation and Illumina sequencing. CB and IBG analysed the data.  
611 TH, NW and CW conceived the study. CB and CW wrote the paper. All authors  
612 read and approved the final manuscript.

613

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## Figure Legends

**Figure 1. Culturability of *C. jejuni* strains during survival periods in sterile distilled water.** The percentage of the original inoculum retaining culturability is shown at 0 h, 72 h and 240 h at 4°C and at 0 h, 24 h and 48 h at 25°C. The percentage of viable cells at 72 h at 4°C and 24 h at 25°C (indicated by the line graphs) was determined by LIVE/DEAD staining (BacLight, Invitrogen) for *C. jejuni*. The error bars represent a 5% error, three independent biological replicates were performed.

**Figure 2. Summary of gene expression changes.** (A) Venn diagram of Genes upregulated  $\geq 2$ -fold (log counts per million) compared to the MHB control. (B) Venn diagram of Genes downregulated  $\geq 2$ -fold (log counts per million) compared to the MHB control.

**Figure 3. Expression changes in stress-related genes.** The heatmap shows log fold change (logFC) calculated in edgeR compared to the MHB Control (blue = negative, red = positive, the white traceline in the columns indicates the size of the logFC measurement), and displays normalized counts per million (CPM) in numbers. It includes known stress response genes and genes up- or down-regulated  $\geq 2$ -fold compared to the MHB control based on normalized CPM (numbers displayed in the heatmap matrix) for MHB Control, Time 0, 24 h (25°C) and 72 h (4°C). Square brackets display the type of stress response the gene is involved in (if known). The Histogram in the Colour key indicates the distribution of the data included in the heatmap.

655 **Figure 4 Significant expression changes determined through BitSeq**  
 656 **analysis of Sec-dependent and twin-arginine (TAT) protein export pathway**  
 657 **components mapped in KEGG.** Results are shown for Time 0, 25°C (24 h) and  
 658 4°C (72 h) in sterile distilled water

659  
 660 **Figure 5. Significant expression changes determined through BitSeq**  
 661 **analysis of *C. jejuni* M1 flagellar assembly components mapped in KEGG.**  
 662 Results are shown for Time 0, 25°C (24 h) and 4°C (72 h) in sterile distilled  
 663 water.

664  
 665 **Figure 6. Significant expression changes determined through BitSeq**  
 666 **analysis of *C. jejuni* M1 chemotaxis components mapped in KEGG.** Results  
 667 are shown for Time 0, 25°C (24 h) and 4°C (72 h) in sterile distilled water.

## 670 **Additional files**

671  
 672 **Additional file 1.** Summary of normalised expression values for each gene in  
 673 average counts per million (cpm) determined in edgR.

674  
 675 **Additional file 2.** Table summarizing genes that are up or downregulated  $\geq 2$ -  
 676 fold compared to the MHB control in read counts per million (cpm) across all  
 677 three experimental conditions. Cpm displayed are normalised by the Trimmed  
 678 Mean of M-values (TMM) method, implemented in the edgR Bioconductor  
 679 package. In brackets the log-fold-change cpm relative to the MHB is shown. Cells  
 680 are shaded according to log-fold-change (yellow=negative, green=positive).

681  
 682 **Additional file 3.** Summary of Bayesian Inference of Transcripts from  
 683 Sequencing data (BitSeq) analysis [41] results. Results are shown in Probability  
 684 of Positive Log Ratio (PPLR), PPLR  $\geq 0.95$  shows a high probability that the  
 685 transcript is upregulated (green shading) compared to the first condition; PPLR  
 686  $\leq 0.05$  shows a very low probability that the transcript is upregulated compared

687 to the first condition and therefore the probability of the transcript being  
688 downregulated (red shading) is very high.

689

690 **Additional file 4.** Table showing gene expression of previously described stress  
691 response genes shown as average read counts per million (cpm). Fold change  
692 compared to the MHB Control, as determined by edgeR, the Paired student's T-  
693 test and PPLR determined through BitSeq analysis are shown in brackets.  
694 Stressor abbreviations: S, starvation; O, osmotic; OX, oxidative; A, aerobic; HS,  
695 heat-shock; F/T; freeze-thaw; I, iron stress; CD, cell density

696

697 **Additional file 5.** Regions of difference (RODs) identified between *C. jejuni* M1,  
698 414, 1336 and NCTC11168. RODs shaded in teal are divergent from M1 in the  
699 other three strains (414, 1336 and NCTC11168)

700

701 **Additional file 6. The *C. jejuni* M1 genome and maps a global overview of**  
702 **gene expression changes under the different test conditions in *C. jejuni* M1**

703 From the outside in: Track 1 *C. jejuni* M1 genome; Track 2 CDS forward strand;  
704 Track 3 CDS reverse strand; Track 4 hypothetical/ putative uncharacterized  
705 genes (CDS); Track 5 and 6 Regions of Difference (RODs) in the *C. jejuni* M1  
706 genome compared to *C. jejuni* 414 (turquoise), M1 compared to NCTC11168  
707 (magenta) and M1 compared to 1336 (blue); Track 7 operons in M1 as predicted  
708 by Rockhopper; Track 8 genes that are statistically significantly upregulated  
709 (green) or downregulated (red) at Time 0 only, compared to the Control;  
710 Track 9 genes that are statistically significantly upregulated (green) or  
711 downregulated (red) in 25°C (24 h) only compared to the Control; Track 10  
712 genes that are statistically significantly upregulated (green) or downregulated  
713 (red) in 4°C (72 h) only compared to the Control; Track 11 genes that are  
714 statistically significantly upregulated (green) or downregulated (red) in 25°C (24  
715 h) only compared to Time 0 ; Track 12 genes that are statistically significantly  
716 upregulated (green) or downregulated (red) in 4°C (72 h) only compared to  
717 Time 0; Track 13 genes that are statistically significantly upregulated (green) or  
718 downregulated (red) in 4°C (72 h) only compared to 25°C (24 h). Colouring of  
719 Tracks 2 and 3: replication related = bright red; efflux pumps = bright green;

720 chemotaxis = bright blue; hydrogenases = pale pink; iron or heme-related = light  
721 brown; hydrogenases = rose; chaperone = orange; lipoproteins = yellow;  
722 membrane or periplasmic proteins = turquoise; ATP- / ABC transporters = light  
723 green; ribosomal/RNA/ribonuclease = light blue; flagellar-related = magenta;  
724 hypotheticals = salmon

725

726 **Additional file 7.** Summary of hypothetical genes in the *C. jejuni* M1 genome and  
727 the corresponding BitSeq data. Green shading indicates PPLR  $\geq 0.95$ , red shading  
728 indicates  $\leq 0.05$ , grey shading indicates no statistically significant difference.

729

730 **Additional file 8.** Gel electrophoresis to show the quality of RNA samples.

731 Example samples are shown for a sample from 25 °C (24 h) (D1 3), Time 0 (D0  
732 2), 4 °C (72 h) (D3 1) and the Mueller Hinton Broth Control (C1 107). There was  
733 no evidence for RNA degradation in the 24 h or 72 h water samples compared to  
734 the time zero or control samples.

735

736

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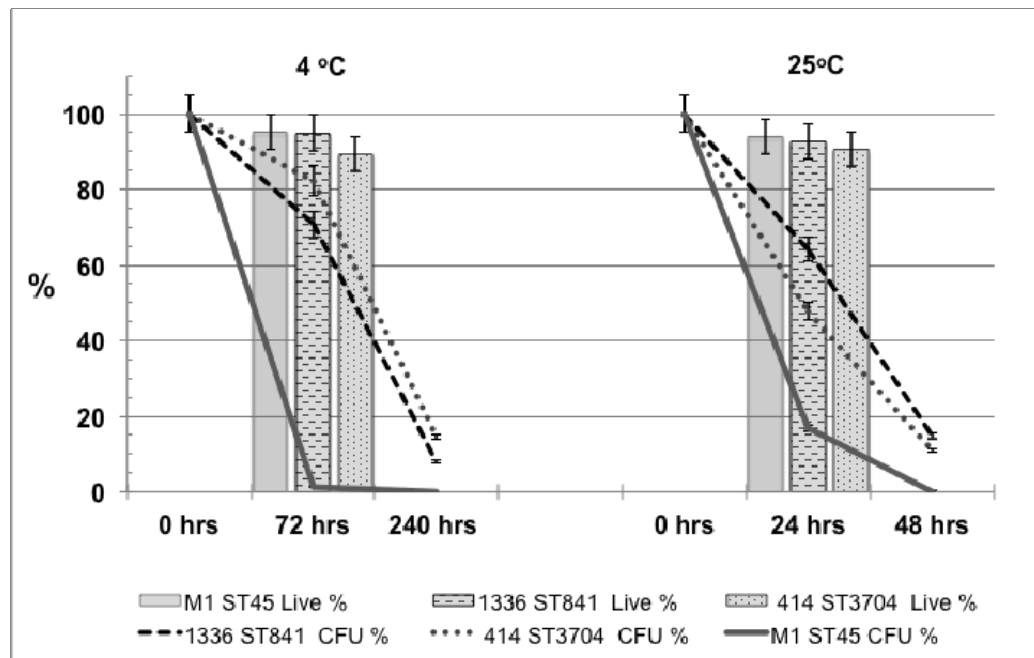
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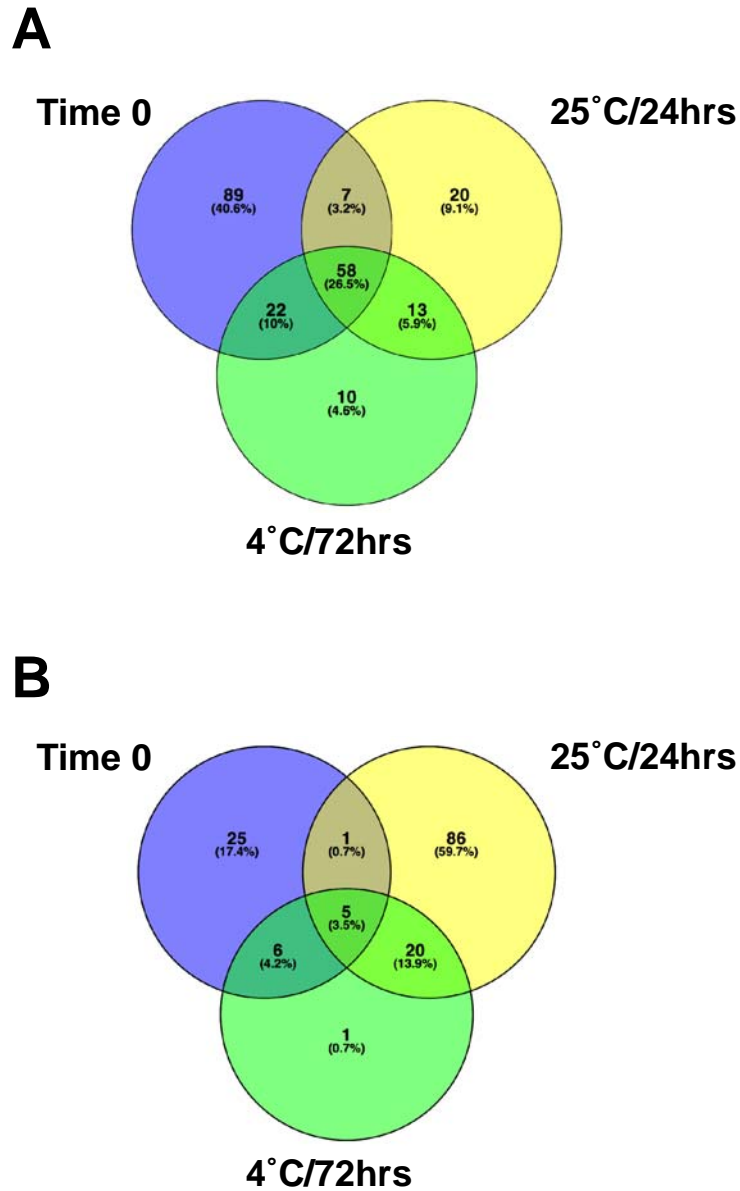
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**Figure 1** Culturability of *C. jejuni* strains during survival periods in sterile distilled water. The percentage of the original inoculum retaining culturability is shown at 0 h, 72 h and 240 h at 4°C and at 0 h, 24 h and 48 h at 25°C. The percentage of viable cells at 72 h at 4°C and 24 h at 25°C (indicated by the line graphs) was determined by LIVE/DEAD staining (BacLight, Invitrogen) for *C. jejuni*. The error bars represent a 5% error, three independent biological replicates were performed.



**Figure 2 Summary of gene expression changes. (A)** Venn diagram of Genes upregulated  $\geq 2$ -fold (log counts per million) compared to the MHB control. **(B)** Venn diagram of Genes downregulated  $\geq 2$ -fold (log counts per million) compared to the MHB control.





## Protein Export in *C. jejuni* M1

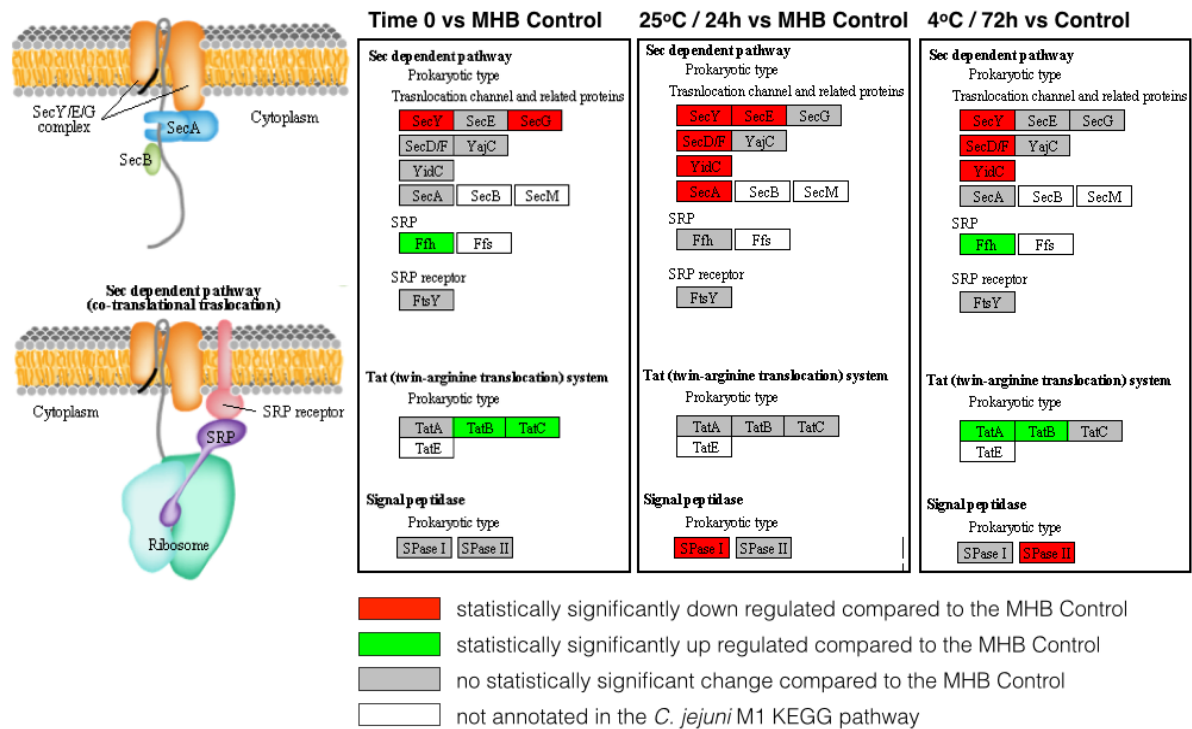


Figure 4. Significant expression changes determined through BitSeq analysis of Sec-dependent and twin-arginine (TAT) protein export pathway components mapped in KEGG. Results are shown for Time 0, 25°C (24 h) and 4°C (72 h) in sterile distilled water



## Chemotaxis in *C. jejuni* M1

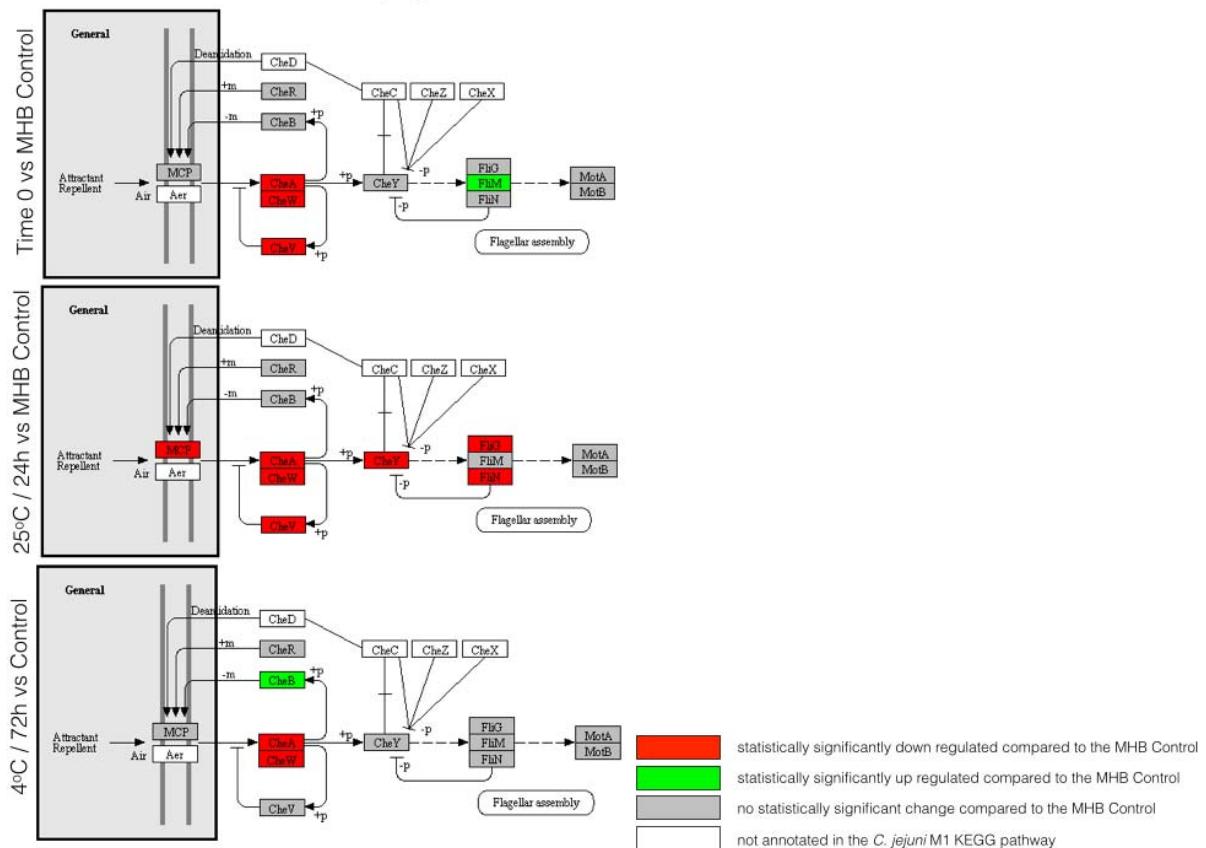


Figure 6. Significant expression changes determined through BitSeq analysis of *C. jejuni* M1 chemotaxis components mapped in KEGG. Results are shown for Time 0, 25°C (24 h) and 4°C (72 h) in sterile distilled water.