The *hrcA* and *hspR* regulons of *Campylobacter jejuni*

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The human pathogen *Campylobacter jejuni* has a classic heat shock response, showing induction of chaperones and proteases plus several unidentified proteins in response to a small increase in growth temperature. The genome contains two homologues to known heat shock response regulators, HrcA and HspR. Previous work has shown that HspR controls several heat-shock genes, but the *hrcA* regulon has not been defined. We have constructed single and double deletions of *C. jejuni* *hrcA* and *hspR* and analysed gene expression using microarrays. Only a small number of genes are controlled by these two regulators, and the two regulons overlap. Strains mutated in *hspR*, but not those mutated in *hrcA*, showed enhanced thermotolerance. Some genes previously identified as being downregulated in a strain lacking *hspR* showed no change in expression in our experiments.

INTRODUCTION

Most bacteria show a heat shock response (HSR) to a sudden, small increase in temperature. This consists of rapid upregulation of a set of proteins, the heat-shock proteins (Hsps), most of which reduce the intracellular level of unfolded and aggregated proteins. The major Hsps fall into two categories: molecular chaperones which promote correct protein folding (Hartl *et al.*, 1992; Hartl, 1996; Lund, 2001; Georgopoulos, 2006) and ATP-dependent proteases that degrade unfolded polypeptides (Neuwald *et al.*, 1999; Suzuki *et al.*, 1997; Dougan *et al.*, 2002). Both groups also have roles in protein quality control within the cell under normal conditions, and their importance is shown by their high conservation.

Bacteria regulate the HSR in diverse ways. *Escherichia coli* and many other proteobacteria use the alternative sigma factor σ_{32} (Grossman *et al.*, 1984; Yura & Nakahigashi, 1999), which directs RNA polymerase to promoters of heat-shock genes, and is regulated by a number of mechanisms including a feedback loop that reduces the HSR once the stress has ended (Gamer *et al.*, 1996; Tomoyasu *et al.*, 1998; El-Samad *et al.*, 2005). Most

bacteria use one or more of a number of repressors to regulate the HSR (Narberhaus, 1999). One of these is the HrcA/CIRCE system, where transcription from heat shock promoters is limited by the binding of HrcA repressor to the CIRCE (controlling inverted repeat of chaperone expression) element, which has the consensus TTAGCACTC-N9-GAGTGCTAA; Zuber & Schumann, 1994). This system has been studied extensively in *Bacillus subtilis* and is the most widespread bacterial heat-shock regulation system, with examples in both Gram-positive and Gram-negative organisms (P. A. Lund and I. Barber, unpublished data). HrcA instability at elevated temperatures leads to dissociation of HrcA from the CIRCE element which causes increased expression of heat-shock genes (Watanabe *et al.*, 2001; Hitomi *et al.*, 2003). A feedback loop involving GroES and GroEL acts to fold HrcA to its active conformation under non-heat-shock conditions (Mogk *et al.*, 1997; Reischl *et al.*, 2002; Roncarati *et al.*, 2007).

Another negative regulator of the HSR is HspR. This repressor binds the HAIR (HspR associated inverted repeat) sequence which has the consensus CTTGAGT-N7-ACTCAAG (Bucca *et al.*, 1995, 1997; Grandvalet *et al.*, 1997). In this case, sensitivity to temperature requires the chaperone DnaK (Bucca *et al.*, 2000). HspR and DNA containing the HAIR sequence form a complex, with the presence of DnaK increasing the affinity of HspR for its target (Bucca *et al.*, 2000). After heat shock, unfolded proteins compete with HspR for DnaK, resulting in decreased HspR activity and derepression of the HSR.

Campylobacter jejuni, an important human pathogen, has a small genome of 1.6 Mb with a small number of regulator genes (Parkhill *et al.*, 2000; Gundogdu *et al.*, 2007). It contains homologues of *hrcA* and *hspR* but no σ_{32} . A number of its proteins are expressed at higher levels

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Abbreviations: c.f.u., Colony-forming units; CIRCE, controlling inverted repeat of chaperone expression; HAIR, HspR associated inverted repeat; Hsp, heat-shock protein; HSR, heat shock response; MH, Mueller–Hinton.

The array design is available in BμG@Sbase (accession no. A-BUGS-9) and ArrayExpress (accession no. A-BUGS-9). Fully annotated microarray data have been deposited in BμG@Sbase (accession no. E-BUGS-88) and ArrayExpress (accession no. E-BUGS-88).

Three supplementary figures and two supplementary tables are available with the online version of this paper.

following a temperature rise, including homologues of GroEL (Wu *et al.*, 1994) and DnaJ (Konkel *et al.*, 1998). Some of the heat-inducible genes have been cloned and characterized, including *lon* (Thies *et al.*, 1998), *clpB* (Thies *et al.*, 1999a), the *groESL* operon (Thies *et al.*, 1999c) and the *dnaK* operon (Thies *et al.*, 1999b). The *groESL* and *dnaK* operons are preceded by sigma factor σ^{70} promoters and inverted repeat sequences similar to CIRCE, suggesting that they may be regulated by HrcA (Thies *et al.*, 1999b, c). The global response of *C. jejuni* NCTC 11168 50 min after a temperature increase was studied using microarrays (Stintzi, 2003), with the upregulated genes being divided into four groups according to their expression profiles. One group of 33 genes, including many of the major heat-shock genes, had the classic HSR expression profile, with high upregulation immediately after the temperature increase, followed by a gradual return to non-heat-shock levels (Stintzi, 2003). A *C. jejuni* *hspR* mutant had reduced motility and growth, increased sensitivity to high temperatures, and impaired adherence to and invasion of epithelial cells, suggesting a role for HspR in virulence (Andersen *et al.*, 2005). This strain showed an increase in the expression of 13 genes including the operons *hrcA*–*grpE*–*dnaK*, *groESL* and *cbpA*–*hspR*, and reduced expression of 17 genes, nearly half of which are involved in the flagellar apparatus. How HspR regulates this latter group of genes is unknown; it has not been previously shown to positively regulate genes in any bacterium. A connection between negative regulation of the HSR and positive regulation of motility-associated genes has also been described for *Helicobacter pylori* (Roncarati *et al.*, 2007). Stintzi *et al.* (2005) have reported the analysis of an *hrcA* mutant and shown that this gene may also have a role in virulence. Despite this, the regulon of HrcA in *C. jejuni* has yet to be defined.

Here, we describe strains of *C. jejuni* NCTC 11168 where the *hrcA* and *hspR* genes were deleted both individually and together. Changes in the transcriptomes of these strains have been analysed, as have changes in protein expression and phenotype. To ensure all changes seen were due to the deletion of regulators, complementation experiments in single knockout strains were also done.

METHODS

Bacterial strains, plasmids and growth conditions. All bacterial strains and plasmids used are described in Supplementary Tables S1 and S2, available with the online version of this paper. The *C. jejuni* strain was the NCTC 11168 strain that was used to sequence the genome (Parkhill *et al.*, 2000). Strains were grown on Mueller–Hinton (MH) media (Oxoid) at 37 °C (unless otherwise stated) in a variable atmosphere incubator (Don Whitley Scientific) in 10% CO₂, 5% O₂ and 85% N₂. Strains were subcultured no more than three times before use. *E. coli* DH5 α was grown aerobically at 37 °C in Luria–Bertani medium (Sambrook *et al.*, 1989). Kanamycin (50 μ g ml^{−1}), chloramphenicol (10–25 μ g ml^{−1}) or ampicillin (100 μ g ml^{−1}) were added as appropriate. Cultures on plates were grown as above or in a sealed gas jar with a CampyGen gas pack (Oxoid) in a temperature-controlled incubator. To assess thermotolerance, *C. jejuni* strains were

grown to mid-exponential phase (OD₆₀₀ 0.2) at 37 °C. Aliquots (250 μ l) were transferred to 1.5 ml centrifuge tubes which were sealed, removed from the microaerobic incubator and placed in a water bath at 50 °C, except for one set of tubes that was serially diluted and plated as the zero time point. Tubes were removed from the water bath at regular times, serially diluted and plated; plates were subsequently counted to calculate colony forming units (c.f.u.) ml^{−1}. Percentage survival of the strain was calculated relative to the zero time point.

Construction of *C. jejuni* mutant strains. Primers are listed in Supplementary Table S3. To construct a *C. jejuni* *hspR* null mutant, the upstream and downstream regions flanking the *C. jejuni* *hspR* gene were amplified using primers CJHSPRUPF and CJHSPRUPR, and CJHSPRDOWNF and CJHSPRDOWNR, respectively. The products were cloned separately into pGEM-T easy (Promega) to create plasmids pGEMhspRUP and pGEMhspRDOW. These plasmids were digested with *Bam*HI and *Pst*II, and the excised *hspR* upstream fragment was cloned into pGEMhspRDOW to create pGEMhspRUD. The chloramphenicol resistance cassette (*cat*) from pAV35 was ligated as a *Bam*HI fragment into pGEMhspRUD between the hspRUP and hspRDOW regions to create suicide vectors pGEMhspRKOF and pGEMhspRKOR, where the resistance cassette is in either the same or the opposite orientation to the *hspR* gene.

To construct a *C. jejuni* *hrcA* null mutant, the upstream and downstream regions of DNA flanking the *C. jejuni* *hrcA* gene were amplified (using primers CJHRCaupF and CJHRCaupR, and CJHRCadownF and CJHRCadownR, respectively). The products were cloned separately into pGEM-T easy (Promega) to create plasmids pGEMhrcAUP and pGEMhrcADOWN. Plasmid pGEMhrcADOWN was digested with *Kpn*I and *Eco*RI, and the excised *hrcA* downstream fragment was ligated into pUC19.1 to create pUChrcADOWN. pGEMhrcAUP was digested with *Kpn*I and *Bam*HI to excise the *hrcA* upstream fragment, and this was ligated between the *Kpn*I and *Bam*HI sites of pUChrcADOWN to create pUChrcAUD. The kanamycin cassette from pJMK30 (*aphA*-3) was then ligated between the *hrcA* upstream and downstream regions of this plasmid to create suicide vectors pUChrcAKOF and pUChrcAKOR where the resistance cassette is in the same and opposite orientations to the *hrcA* gene, respectively.

Plasmids were transformed into *C. jejuni* as described by van Vliet *et al.* (1998). Double homologous recombination and deletion of the appropriate region of the *C. jejuni* NCTC 11168 genome were confirmed by PCR and were stable without antibiotic selection.

Construction of strains expressing *hspR* and *hrcA*. To construct a suicide vector for insertion of genes onto the *C. jejuni* chromosome, the upstream and downstream regions of the pseudogene Cj0752 were amplified using primer pairs Cj0752UPF and Cj0752UPR, and Cj0752DOWNF and Cj0752DOWNR, respectively. Homologous sequences in primers Cj0752UPR and Cj0752DOWNF allowed an overlap PCR to be done on the products of these reactions with primers Cj0752UPF and Cj0752DOWNR. The product of this second-stage PCR was ligated into pGEM-T-easy to create pGEMCWH01, which has *Bam*HI and *Kpn*I sites between the upstream and downstream fragments of Cj0752, to allow insertion of DNA fragments.

hspR was amplified using primers CJHSPRF and CJHSPRR and cloned into pGEM-T-easy, to give plasmid pGEMhspR. The *cbpA* promoter (the native promoter for the *hspR* gene) was amplified using primers CJPCBPAF and CJPCBPAP. This product was digested with *Kpn*I and *Nco*I, pGEMhspR was digested with *Bam*HI and *Nco*I, and both were ligated into *Kpn*I- and *Bam*HI- digested pUC19.1 to give the plasmid pUCPcbpAhspR. pUCPcbpAhspR and pGEMCWH01 were both digested with *Kpn*I and *Bam*HI, and the

resulting P_{cbpA} -*hspR* fragment was ligated into pGEMCWH01 to give pGEMhspRCNC. The *aphA-3* kanamycin resistance cassette from pJM30 was inserted into pGEMhspRCNC on a *Bam*HI fragment to create pGEMhspRCOMP.

hrcA and its promoter were amplified using the primers CJHRCACOMPF and CJHRCACOMPR, and cloned into pGEM-T easy to give pGEMhrcACNC. This was digested with *Bam*HI and *Kpn*I, and the P_{hrcA} -*hrcA* fragment was ligated into *Bam*HI/*Kpn*I-digested pGEMCWH01 to give pGEMhrcACNC. This was digested with *Bam*HI, and the *cat* chloramphenicol resistance cassette from pAV35 was inserted to create pGEMhrcACOMP.

RNA isolation and microarray transcriptome analysis. Each microarray experiment was performed independently in triplicate to provide biological replicates. Cultures (50 ml) of *C. jejuni* NCTC 11168 and the strain under investigation were inoculated from an overnight liquid culture to a starting OD_{600} of 0.05 and grown in parallel to mid-exponential phase (OD_{600} 0.2) at 37 °C as described above. Samples of 5 ml were then added to 10 ml RNeasyprotect solution (Qiagen), incubated at room temperature for 10 min, then centrifuged at 4500 *g* to pellet the cells. RNA preparation, conversion to labelled cDNA for cDNA/cDNA hybridization, the use of Lucidia spikes and slide hybridization and washing were done as described by Constantinidou *et al.* (2006). A dye swap experiment confirmed that there was no bias due to differential labelling. RNA purity and concentration were determined using the 2100 Bioanalyser and the RNA 6000 LabChip (Agilent) according to the manufacturers' protocols. *C. jejuni* microarray slides (BjG@S, St George's Hospital, London; Dorrell *et al.*, 2001) were used for all experiments. Slides were scanned using a GenePix 4000B confocal laser scanner (Axon) to obtain the highest intensity without saturation.

Microarray data analysis. Microarray images were analysed using GenePixPro v.3.0 software (Axon). Poor-quality/damaged spots were flagged before the programme was used to generate spot and background intensity data. Further analysis was performed using the GeneSpring V6.2.2 package (Silicon Genetics), using the Gene Spring error model cut-off value. Upon importing the data into GeneSpring, background-corrected values were automatically calculated for each spot. Data for poor spots were discarded at this stage. The data were then normalized with the Lowess locally weighted regression algorithm using 40 % of the data for smoothing to eliminate dye bias. Values below 0.01 were taken as 0.01. The effectiveness of the normalization was checked using two methods: MA scatter plots of log-transformed intensity ratio against average intensity of both channels and scatter plots of Cy3 versus Cy5 log-transformed intensity, inspecting both for linearity. These data were first filtered using flags (removing genes that were flagged as bad in two or more slides) and then on signal intensity [removing the unreliable low-intensity data in both the control channel (Cy3) intensity and the signal channel (Cy5)] to produce a list of genes considered to be reliable and suitable for use in further analysis. Genes were filtered on expression level to remove non-changing genes with expression in the mutant strain between 0.5 and 2 times that of the wild-type. Changing genes were then filtered on confidence using a *t*-test and applying the Benjamini & Hochberg (1995) false discovery rate algorithm with a maximum significance cut-off at 0.05 to reduce false-positives. The array design is available in BjG@Sbase (accession no. A-BUGS-9; <http://bugs.sgul.ac.uk/A-BUGS-9>) and ArrayExpress (accession no. A-BUGS-9). Fully annotated microarray data have been deposited in BjG@Sbase (accession no. E-BUGS-88; <http://bugs.sgul.ac.uk/E-BUGS-88>) and ArrayExpress (accession no. E-BUGS-88).

Protein analysis. To standardize protein extracts, cells from 1 ml of mid-exponential phase culture of a measured OD_{600} were pelleted by

centrifugation at 15 000 *g* and resuspended in Laemmli sample buffer calculated to give the equivalent of a culture OD_{600} of 3.0, before being boiled for 5 min. Total cell extracts were separated by SDS-PAGE in 12 % (w/v) acrylamide gels (Laemmli, 1970). Proteins were visualized by staining with Coomassie Brilliant Blue or transferred to PVDF membrane by electrophoresis in 10 mM CAPS, pH 11.0, 10 % methanol. Immunoblotting was done using the ECL plus chemiluminescent detection kit (Amersham) following the manufacturer's protocol. Polyclonal antibodies against ClpB and DnaK were generous gifts from Bernd Bukau (Zentrum für Molekulare Biologie, University of Heidelberg); anti-GroEL was a monoclonal antibody raised in our laboratory. Immunoreactive protein bands were detected on Biomax light film (Kodak). Bands were quantified relative to several dilutions of purified protein run on the same gels by scanning and analysis using Quantity One software v. 4.2.3 (Bio-Rad).

RESULTS AND DISCUSSION

Microarray analysis reveals genes regulated by HspR and HrcA

The operons encoding the major chaperone proteins of *C. jejuni* and their putative regulators are shown in Fig. 1. To define the HspR and HrcA regulons, three mutant strains were constructed: $\Delta hspR$ (with a *cat* gene replacing all but the first 18 bp and last 57 bp of *hspR*), $\Delta hrcA$ (with an *aphA-3* gene replacing all but the first 16 bp and last 99 bp of *hrcA*) and $\Delta hspR$ - $\Delta hrcA$ (combining the two deletions).

No genes in the $\Delta hrcA$ strain were expressed at a significantly lower level than in the parent, and only three were significantly increased (see Table 1). Two of these are co-transcribed and encode the chaperonin GroEL and co-chaperonin GroES, both important Hsps. The third upregulated gene, Cj0168c, encodes a putative integral membrane protein that is conserved among *Campylobacter* spp., and shows 34 % amino acid identity to a MotA/TolQ/ExbB proton channel protein from *Shewanella frigidimarina*, but otherwise has no obvious homologues.

Fig. 1. Organization of chaperone genes in *C. jejuni* NCTC11168, showing putative HAIR and CIRCE sites.

Table 1. Genes significantly changed in expression in $\Delta hrcA$, $\Delta hspRF$ and $\Delta hspR$ - $\Delta hrcAi$ strains

NC, No significant change seen; ABS, gene not present in strain.

Gene	Fold change (<i>t</i> test <i>P</i> value)			Function
	$\Delta hrcA$	$\Delta hspR$	$\Delta hspR$ - $\Delta hrcA$	
<i>groES</i>	4.442 (0.016)	3.504 (0.029)	7.446 (0.011)	Hsp. 60 kDa chaperonin
<i>groEL</i>	3.747 (0.015)	3.276 (0.021)	4.791 (<0.001)	Hsp. 10 kDa chaperonin
Cj0168c	2.122 (0.01)	NC	NC	Unknown. Putative integral membrane protein
<i>clpB</i> *	NC	53.68 (<0.001)	76.9 (<0.001)	Clp protease ATP-binding subunit
<i>grpE</i> *	NC	35.71 (0.003)	6.879 (<0.001)	Hsp. DnaK cofactor
<i>dnaK</i> *	NC	33.80 (0.002)	9.296 (<0.001)	Hsp. Molecular chaperone
<i>hrcA</i> *	ABS	28.51 (0.002)	ABS	HSR
<i>cbpA</i>	NC	6.019 (<0.001)	7.256 (<0.001)	Curved DNA-binding protein, similar to DnaJ
Cj0760*	NC	5.075 (<0.001)	NC	Unknown hypothetical ORF
Cj0761*	NC	2.723 (0.015)	NC	Unknown
Cj1295	NC	2.574 (<0.001)	3.222 (<0.001)	Unknown
Cj1500	NC	NC	4.77 (<0.001)	Probable integral membrane protein. YedE family
<i>argC</i>	NC	NC	2.235 (<0.001)	Probable <i>N</i> -acetyl-gamma-glutamyl-phosphate reductase
<i>sdhC</i>	NC	NC	0.488 (0.023)	Putative succinate dehydrogenase iron-sulphur protein
Cj1626c	NC	NC	0.477 (<0.001)	Unknown. Putative periplasmic protein
<i>flaC</i>	NC	NC	0.435 (<0.001)	Flagellin-like exported protein
Cj0200c	NC	NC	0.432 (0.015)	Unknown. Putative periplasmic protein
<i>sdhB</i>	NC	NC	0.43 (0.027)	Putative succinate dehydrogenase subunit C
Cj1219c	NC	NC	0.291 (0.006)	Unknown. Putative periplasmic protein

*Changes in expression were also detected in $\Delta hspRF$ by Andersen *et al.* (2005).

Interestingly, this protein is also upregulated by iron limitation (Holmes *et al.*, 2005). As HrcA binds to the CIRCE consensus sequence, we searched for CIRCE sequences in the *C. jejuni* NCTC 11168 genome using fuzznuc, part of the EMBOSS software suite. Only one CIRCE sequence, upstream of the *groESL* operon, was identified. Its sequence (TTATCACTC-N9-GAGTGCTAA) is identical to the consensus sequence apart from one base (T instead of G at position 4). Another putative CIRCE sequence previously identified upstream of the *dnaK* operon (Thies *et al.*, 1999b) was not identified by this search because the spacer between the inverted repeats is only 8 bp long, and the fact that the *dnaK* operon showed normal expression in the $\Delta hrcA$ mutant suggests that this sequence is not functional. No CIRCE sequence was identified upstream of the gene Cj0168c, suggesting that its upregulation must be indirect.

The previous study of an *hspR* derivative of *C. jejuni* 11168 (Andersen *et al.*, 2005) was done under similar growth conditions to ours, but at a slightly higher OD₆₀₀ (0.3) and on cells grown in BHI. Our analysis of the $\Delta hspRF$ strain (see Table 1) revealed similarities and differences with this study. Increases were seen in the expression of several molecular chaperones; four of these (*clpB*, *hrcA*, *grpE* and *dnaK*) were upregulated in both studies. The most highly upregulated gene in both studies is *clpB*, a protein disaggregase that works together with DnaK (Mogk *et al.*,

1999). The cooperation between DnaK and ClpB makes it unsurprising that their genes are co-regulated in *C. jejuni* and many other bacterial species (e.g. Richmond *et al.*, 1999; Stewart *et al.*, 2002). The three next most upregulated genes in this study were *grpE*, *hrcA* and *dnaK*, which are likely to be co-transcribed. *grpE* encodes a nucleotide exchange factor which functions in the DnaK chaperone cycle. The other co-factor in the DnaK chaperone cycle is DnaJ, but upregulation of this gene was not seen. However, the *cbpA* gene, which is likely to be co-transcribed with *hspR* and encodes a DnaJ homologue (44 % similarity), does show higher expression in the $\Delta hspRF$ strain. We therefore suggest that CbpA is the main DnaJ protein in this strain. Andersen *et al.* (2005) saw a 2.2-fold increase in expression of *cbpA* which did not pass filtering, and they noted an increase of *hspR* expression in their *hspR* mutant. Andersen *et al.* (2005) speculate that *hspR* is probably co-transcribed with *cbpA* (as it is in *H. pylori*), and it is likely that this whole operon is normally repressed by HspR. The analysis of the HSR of *C. jejuni* NCTC 11168 by Stintzi (2003) found *dnaJ* to be upregulated only 1.4-fold, compared with the 5.1-fold induction of *cbpA*, consistent with our results. Also identified in both this study and that of Andersen *et al.* (2005) were two genes, Cj0760 and Cj0761, which are immediately downstream of *dnaK*. The function of these genes is unknown. We also saw elevated expression in $\Delta hspRF$ of *groEL* and *groES*. The increase was approximately 10-fold less than that of the genes in the

Table 2. HAIR sites in *C. jejuni* NCTC 11168

NA, Not applicable. All sequences had three mismatches compared with the consensus sequence (CTTGAGT-N₇-ACTCAAG).

Sequence*	Upstream of:	Position relative to ATG
cttt ag t— act aaac	<i>groES</i>	−131 to −111
cttg a ca— act aaag	<i>clpB</i>	−62 to −42
cttg a ca— act caat	<i>hrcA</i>	−75 to −55
cttt ag t— act aaat	<i>hrcA</i>	−125 to −105
cttg a tt— gct aaag	<i>cbpA</i>	−79 to −59
cttg ag t— act caag	Consensus	NA

*Matches to the consensus sequence are in bold.

dnaK operon, but was still statistically significant. These genes were not shown to increase in expression in the *hspR* mutant constructed by Andersen *et al.* (2005), but these authors did observe an increase in GroES and GroEL protein levels in this strain; our results are consistent with this.

Putative sequences with a match to the consensus HAIR sequence (Grandvalet *et al.*, 1997) have previously been identified upstream of the *groE*, *dnaK* and *cbpA* operons (Andersen *et al.*, 2005). In addition, we found a good match at position −62 to −42 of the *clpB* gene (Table 2). Thus, all of the genes or operons with higher expression in

$\Delta hspRF$ are preceded by HAIR sequences, except one: the gene Cj1295.

We saw no change in expression of the other genes which were upregulated in the study by Andersen *et al.* (2005). A further contrast between our results and those of Andersen and colleagues is that we found no genes to be significantly downregulated in $\Delta hspRF$, whereas they observed lower expression of several flagellar genes. These differences may be due to the use of different variants of *C. jejuni* NCTC 11168 in the two studies. The variant used by Andersen *et al.* was obtained directly from the NCTC, whereas the one used here was that used in the genome sequencing

Fig. 2. Comparison of expression levels of genes in the mutated and complemented strains. The expression level is relative to that of *C. jejuni* NCTC 11168. The dashed line represents a twofold change. (a) Genes in $\Delta hspR$ (filled) and *hspR* Comp (shaded). (b) Genes in $\Delta hrcA$ (filled) and *hrcA* Comp (shaded).

project (Parkhill *et al.*, 2000). This strain has an unknown culture history since acquisition from the NCTC. It lacks the typical spiral shape of *Campylobacter* and is non-motile, two features that were both present in the strain used by Andersen *et al.* (2005). Gaynor *et al.* (2004) identified a number of differences between the original clinical strain of *C. jejuni* 5636/77 (later renamed *C. jejuni* NCTC 11168) and the variant of this strain that was used in the genome sequencing project. The study by Gaynor *et al.* (2004) showed that there were no large genetic differences between the two variants. This consequently highlights the fact that laboratory passage can have profound effects on *C. jejuni* that are not always obvious at the genetic level. Interestingly, the upregulation of motility-associated genes in negative HSR mutants of *H. pylori* has recently been described (Roncarati *et al.*, 2007). In this case, however, HrcA is the regulator implicated in this positive regulation.

The list of overexpressed genes in the $\Delta hspR$ - $\Delta hrcA$ is similar to that of $\Delta hspRF$ (Table 1). *groES* and *groEL* showed an increase in expression greater in $\Delta hspR$ - $\Delta hrcA$ than in either of the two single knockout strains, suggesting that HrcA and HspR are both required for full repression of *groESL*. There are also a number of genes that are expressed at a lower level in $\Delta hspR$ - $\Delta hrcA$ compared with the wild-type. It is likely that these changes in expression are secondary effects, possibly due to the loss of regulation of the genes under the control of HspR and HrcA, rather than a direct effect of the loss of the two regulators, as none of them changed in expression in the single knockout strains. The gene showing the largest decrease in expression is Cj1219c, an uncharacterized gene encoding a putative periplasmic protein. Interestingly, it is divergently transcribed from the *groESL* operon. A flagellar gene (*flaC*) was downregulated in this strain, but this was not one of the flagellar genes downregulated in the study by Andersen *et al.* (2005). We noted that the *dnaK* and *grpE* genes appeared to be less strongly upregulated in the $\Delta hspR$ - $\Delta hrcA$ background than in the absence of *hspR* alone. As both of these genes are downstream of the *hrcA* ORF, we suspect this may be due to decreased stability of the mRNA for these genes, seen only when they are highly expressed due to the loss of HspR; this remains to be tested.

Complementation of gene deletions

To confirm that these changes were not due to polar effects or caused by secondary mutations, two strains were constructed where *hspR* or *hrcA* were reintroduced onto the *C. jejuni* genome using a novel suicide vector, pGEMCWH01. This plasmid contains ~400 bp fragments from the start and end of the pseudogene Cj0752, flanking a cloning site. Cj0752 resembles an insertion sequence element transposase, but contains stop codons in all three reading frames, making it one of few sites where heterologous DNA can be inserted onto the *C. jejuni* NCTC 11168 genome without disrupting a functional gene

(Elvers *et al.*, 2005). Strain *hspR* Comp contains the $\Delta hspRF$ deletion plus the *hspR* gene in the Cj0752 locus, driven by the *cbpA* promoter. Strain *hrcA* Comp contains $\Delta hrcA$ plus the *hrcA* gene and promoter at the Cj0752 locus. Gene expression in these strains was analysed using microarrays (Fig. 2). The expression of nearly all the genes which were changed in the knockout strains returned towards wild-type levels in *hspR* Comp and *hrcA* Comp, although significant overexpression was still seen in some cases, showing that complementation was not 100%. The elevated expression of Cj1295, the only gene upregulated in

Fig. 3. (a) Coomassie-stained SDS-PAGE gel of protein changes in *C. jejuni* strains. (b), (c) and (d) Immunoblots with anti-ClpB, anti-DnaK and anti-GroEL, respectively. Lanes: 1, *C. jejuni* NCTC 11168; 2, *C. jejuni* $\Delta hspRF$; 3, *C. jejuni* *hspR* Comp; 4, *C. jejuni* $\Delta hrcA$; 5, *C. jejuni* *hrcA* Comp; 6, *C. jejuni* $\Delta hspR$ - $\Delta hrcA$; 7, molecular mass markers (kDa). The arrows show bands that alter significantly in one or more tracks; these correspond well with the predicted molecular masses of ClpB (95.544 kDa), DnaK (67.418 kDa) and GroEL (57.971 kDa).

$\Delta hspRF$ not to have an upstream HAIR sequence, is unchanged in *hspR* Comp, making it very unlikely that this gene is directly regulated by HspR.

Validation of transcription analysis at the protein level

We compared protein levels in the different strains (Fig. 3a) to validate the transcriptional data and to investigate the extent to which transcriptional changes are reflected in changes in protein levels. The major increases seen were of three proteins with molecular masses of 60, 70 and 90 kDa in the $\Delta hspRF$ and $\Delta hspR\text{--}\Delta hrcA$ strains, and one band of 60 kDa in the $\Delta hrcA$ strain. These sizes correspond to the molecular masses of three major Hsps that were over-expressed in microarray experiments (GroEL, DnaK and ClpB homologues). Immunoblotting was used to confirm their identities (Fig. 3b–d), and duplicate gels were analysed to determine levels of induction, as described in Methods. DnaK was found to be expressed approximately 4-fold and 2.75-fold higher in the $\Delta hspRF$ and $\Delta hspR\text{--}\Delta hrcA$ strains, while GroEL showed 2.25-, 1.5- and 5-fold increases in $\Delta hspRF$, $\Delta hrcA$ and $\Delta hspR\text{--}\Delta hrcA$, respectively. Increases in ClpB could not be accurately quantified owing to low levels of expression in the wild-type strain, but it is clear from the Western blot that increases in expression of this protein were very high in the $\Delta hspRF$ and $\Delta hspR\text{--}\Delta hrcA$ strains. These results show broad agreement with the microarray experiments, including the much larger increase in expression of ClpB in the $\Delta hspR$ and $\Delta hspR\text{--}\Delta hrcA$ strains than either DnaK or GroEL, although the relative levels of change seen are different between transcriptome and proteome (as was also seen by Andersen *et al.*, 2005).

Phenotypic analysis of $\Delta hspRF$, $\Delta hrcA$ and $\Delta hspR\text{--}\Delta hrcA$ strains

One of the two previously reported *C. jejuni* NCTC 11168 $\Delta hspR$ strains was found to have a reduced growth rate under standard conditions when compared with the wild-

type (Stintzi *et al.*, 2005), whereas the other did not (Andersen *et al.*, 2005). We found no significant differences in the growth of our $\Delta hspR$ strain compared with 11168 when grown at 37 °C or 42 °C in MH broth (data not shown). However, when grown at 45 °C, all of these strains grew to a lower cell density than 11168, particularly $\Delta hspR\text{--}\Delta hrcA$, which also showed a slightly reduced growth rate at this temperature both in liquid and on solid media (see Supplementary Fig. S1).

The thermotolerances of the various strains were assessed. Fig. 4 shows an example of the enhanced survival of strains $\Delta hspR$ and $\Delta hspR\text{--}\Delta hrcA$ at 50 °C. A primary factor causing death in bacteria exposed to lethal temperatures is the loss of activity of essential proteins as they become aggregated (Weibezahn *et al.*, 2004). Pre-incubation at an elevated (non-lethal) temperature induces thermotolerance in many bacteria; in *E. coli*, deletion of *clpB* results in increased sensitivity to lethal temperatures (Squires *et al.*, 1991). Higher levels of ClpB in $\Delta hspRF$ and $\Delta hspR\text{--}\Delta hrcA$ may thus be the cause of their thermotolerance. However, involvement of the DnaK chaperone system cannot be ruled out. It is unlikely that the increase in cellular levels of GroES and GroEL caused the increase in thermotolerance as they were also overexpressed in $\Delta hrcA$ which showed a similar thermotolerance to the wild-type.

Overall, our results are consistent with a simple model of gene regulation by HspR and HrcA (see Supplementary Fig. S2). HrcA represses *groES* and *groEL*, by binding at the CIRCE sequence upstream of the *groE* operon. HspR represses (among others) the *dnaK* operon (which includes *hrcA*), the *cbpA* operon (which includes the gene for itself) and *clpB*. All of these operons become derepressed on heat shock. This will lead to higher levels of HrcA, which may lead to rapid downregulation the *groE* operon when heat shock is over. The fact that both repressors may require the function of the chaperones that they regulate (DnaK for HspR, GroEL for HrcA), as shown by studies in other organisms, means that the system is probably precisely tuned to respond optimally to heat shock.

Fig. 4. Thermotolerance of *C. jejuni* strains at 50 °C. The results of a single experiment are shown; absolute values varied between different experiments but the trends were always the same. *C. jejuni* NCTC 11168, ■; *C. jejuni* $\Delta hrcA$, △; *C. jejuni* $\Delta hspRF$, ○; *C. jejuni* $\Delta hspR\text{--}\Delta hrcA$, ◇.

This study extends the earlier study by Andersen *et al.* (2005) and shows differences to it in terms of the mutants that could be constructed, the transcriptional changes in the *hspR* mutant and the physiological behaviour of the mutant strains, despite the fact that the studies were done on ostensibly identical strains. While the differences may in part be accounted for by slight differences in culture conditions between the two studies, and assay and data analysis methods used, it is likely that changes that occur during the culture of identical strains in different laboratories may result in significant differences between strains, which in turn means that caution is necessary when comparing results from different studies.

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