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

Christopher W. Holmes,† Charles W. Penn and Peter A. Lund

School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK

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 σ32 (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 CTGGAGT-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 σ32. A number of its proteins are expressed at higher levels
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 $\sigma^{32}$ 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% CO$_2$, 5% O$_2$ and 85% N$_2$. Strains were subcultured no more than three times before use. E. coli DH5$\alpha$ was grown aerobically at 37 °C in Luria–Bertani medium (Sambrook et al., 1989). Kanamycin (50 µg ml$^{-1}$), chloramphenicol (10–25 µg ml$^{-1}$) or ampicillin (100 µg 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 thermostolerance, C. jejuni strains were grown to mid-exponential phase (OD$_{600}$ 0.2) at 37 °C. Aliquots (250 µl) 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 C/JHSRUPF and C/JHSRUPR, and C/JHSRPRDOWN and C/JHSPRDOWN, respectively. The products were cloned separately into pGEM-T easy (Promega) to create plasmids pGEMhspRUP and pGEMhspRDOWN. These plasmids were digested with BamHI and PvuI, and the excised hspR upstream fragment was cloned into pGEMhspRDOWN to create pGEMhspRUD. The chloramphenicol resistance cassette (cat) from pAV35 was ligated as a BamHI fragment into pGEMhspRUD between the hspRUP and hspRDOWN regions to create suicide vectors pGEMhspRKO 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 C/JHRCUAUPF and C/JHRCUAUPR, and C/JHRCADOWN and C/JHRCADOWN, respectively). The products were cloned separately into pGEM-T easy (Promega) to create plasmids pGEMhrcAUP and pGEMhrcADOWN. Plasmid pGEMhrcADOWN was digested with KpnI and EcoRI, and the excised hrcA downstream fragment was ligated into pUC9.1 to create pUCHrcADOWN. pGEMhrcAUP was digested with KpnI and BamHI to excise the hrcA upstream fragment, and this was ligated between the KpnI and BamHI sites of pUCHrcADOWN to create pUCHrcAUD. The kanamycin cassette from pMK30 (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 Cj0752DOWNR allowed an overlap PCR to be done on the products of these reactions with primers Cj0752UPF and Cj0752DOWN. The product of this second-stage PCR was ligated into pGEM-T-easy to create pGEMCWH01, which has BamHI and KpnI sites between the upstream and downstream fragments of Cj0752, to allow insertion of DNA fragments.

hspR was amplified using primers C/JHSPRF and C/JHSPRR and cloned into pGEM-T-easy, to give plasmid pGEMhspR. The cbpA promoter (the native promoter for the hspR gene) was amplified using primers C/PCBPAF and C/PCPBPAR. This product was digested with KpnI and Ncol, pGEMhspR was digested with BamHI and Ncol, and both were ligated into KpnI- and BamHI- digested pUC19.1 to give the plasmid pUCPCbpAhsR. pUCPCbpAhsR and pGEMCWH01 were both digested with KpnI and BamHI, and the

http://mic.sgmjournals.org
resulting PamA–hspR fragment was ligated into pGEMCWH01 to give pGEMhspRCNC. The aphA-3 kanamycin resistance cassette from pJM30 was inserted into pGEMhspRCNC on a BamHI fragment to create pGEMhrhRCOMP.

hrcA and its promoters were amplified using the primers CJHRCACOMP F and CJHRCACOMP R, and cloned into pGEM-T easy to give pGEMhrhACNC. This was digested with BamHI and KpnI, and the PamA–hrcA fragment was ligated into BamHI/KpnI-digested pGEMCWH01 to give pGEMhrhACNC. This was digested with BamHI, and the cat chloramphenicol resistance cassette from pAV35 was inserted to create pGEMhrhACOMP.

**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 aphA-3 kanamycin resistance cassette from pJM30 was inserted into pGEMhspRCNC on a BamHI fragment to create pGEMhrhRCOMP.

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: ΔhspRF (with a cat gene replacing all but the first 18 bp and last 57 bp of hspR), ΔhrcA (with an aphA-3 gene replacing all but the first 16 bp and last 99 bp of hrcA) and ΔhspR–ΔhrcA (combining the two deletions).

No genes in the ΔhrcA 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/ToIQ/ExxB proton channel protein from Shewanella frigidimarina, but otherwise has no obvious homologues.

**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**

Fig. 1. Organization of chaperone genes in C. jejuni NCTC11168, showing putative HAIR and CIRCE sites.
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 (TATACCTC-N9-GAGTGCTAA) is identical to the consensus sequence apart from one base (T instead of G at position 4). Another putative CIRCE sequence, 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 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 ΔhspRF 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 disaggregate 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 ΔhspRF 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 dnaI 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 ΔhspRF of groEL and groES. The increase was approximately 10-fold less than that of the genes in the

### Table 1. Genes significantly changed in expression in ΔhrcA, ΔhspRF and ΔhspR–ΔhrcAi strains

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

*Changes in expression were also detected in ΔhspRF by Andersen et al. (2005).
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 ΔhspRF 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 ΔhspRF, 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

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

<table>
<thead>
<tr>
<th>Sequence*</th>
<th>Upstream of:</th>
<th>Position relative to ATG</th>
</tr>
</thead>
<tbody>
<tr>
<td>ctt tgt</td>
<td>groES</td>
<td>−131 to −111</td>
</tr>
<tr>
<td>ctt gca</td>
<td>clpB</td>
<td>−62 to −42</td>
</tr>
<tr>
<td>ctt gca</td>
<td>hrcA</td>
<td>−75 to −55</td>
</tr>
<tr>
<td>ctt gat</td>
<td>hrcA</td>
<td>−125 to −105</td>
</tr>
<tr>
<td>ctt gat</td>
<td>cbpA</td>
<td>−79 to −59</td>
</tr>
<tr>
<td>ctt gat</td>
<td>Consensus</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Matches to the consensus sequence are in bold.

---

**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 ΔhspR (filled) and hspR Comp (shaded). (b) Genes in ΔhrcA (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 ΔhspR-ΔhrcA is similar to that of ΔhspRF (Table 1). groES and groEL showed an increase in expression greater in ΔhspR-ΔhrcA 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 ΔhspR-ΔhrcA 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 ΔhspR-ΔhrcA 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 ΔhspRF deletion plus the hspR gene in the Cj0752 locus, driven by the cbpA promoter. Strain hrcA Comp contains ΔhrcA 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

---

http://mic.sgmjournals.org

---

**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 ΔhspRF; 3, C. jejuni hspR Comp; 4, C. jejuni ΔhrcA; 5, C. jejuni hrcA Comp; 6, C. jejuni ΔhspR–ΔhrcA; 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).
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 ΔhspRF and ΔhspR–ΔhrcA strains, and one band of 60 kDa in the ΔhrcA 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 identifies (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 ΔhspRF and ΔhspR–ΔhrcA strains, while GroEL showed 2.25-, 1.5- and 5-fold increases in ΔhspRF, ΔhrcA and ΔhspR–ΔhrcA, 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 ΔhspRF and ΔhspR–ΔhrcA strains. These results show broad agreement with the microarray experiments, including the much larger increase in expression of ClpB in the ΔhspR and ΔhspR–ΔhrcA 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 ΔhspRF, ΔhrcA and ΔhspRF–ΔhrcA strains

One of the two previously reported C. jejuni NCTC 11168 ΔhspR 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 ΔhspR 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 ΔhspR–ΔhrcA, 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 ΔhspR and ΔhspR–ΔhrcA 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 ΔhspRF and ΔhspR–ΔhrcA 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 ΔhrcA 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 clpA 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 ΔhrcA, △; C. jejuni ΔhspRF, ○; C. jejuni ΔhspR–ΔhrcA, ◊.
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.

ACKNOWLEDGEMENTS

C.W.H. was funded by a studentship from the Biotechnology and Biological Sciences Research Council. We are grateful to Nick Dorrell (London School of Hygiene and Tropical Medicine) and the Bacterial Microarray Group, St George’s, University of London, for arrays, and to Denise Waldron of the Bacterial Microarray Group for help with data submission.

REFERENCES


Edited by: H. Ingmer