Diverse roles for HspR in Campylobacter jejuni revealed by the proteome, transcriptome and phenotypic characterization of an hspR mutant

Marianne Thorup Andersen,1 Lone Brøndsted,1 Bruce M. Pearson,2 Francis Mulholland,2 Mary Parker,2 Carmen Pin,2 Jerry M. Wells2 and Hanne Ingmer1

Correspondence
Hanne Ingmer
hi@kvl.dk

1Department of Veterinary Pathobiology, The Royal Veterinary and Agricultural University, Stigbøjlen 4, DK-1958 Frederiksberg C, Denmark
2Institute of Food Research, Colney, Norwich NR4 7UA, UK

**INTRODUCTION**

Campylobacter jejuni is one of the most frequently diagnosed food-borne human pathogens to cause gastroenteritis, and contaminated poultry is thought to be one of the main food vehicles. In rare cases, C. jejuni triggers Guillain-Barré syndrome, an autoimmune disorder of the peripheral nervous system (Nachamkin et al., 1998). The pathogenesis of C. jejuni is not fully understood, but may involve damage to the intestinal epithelium by invasion of host cells and toxin production (Wassenaar, 1997; Wooldridge & Ketley, 1997). Characterized virulence factors include those involved in adhesion and invasion, as well as motility, which enables the spiral-shaped bacterium to move into the mucus layer, presenting the bacterium to the intestinal epithelial cells (Szymanski et al., 1995; Guerry et al., 2000). In addition, the ability to cope with the unfavourable conditions encountered during transfer from the intestinal tract of poultry to the human body is crucial to the success of C. jejuni as a human food-borne pathogen. Despite the apparent sensitivity of Campylobacter to stresses in the laboratory, their infectivity does not seem to be compromised by exposure to environmental stresses during transmission from animals to humans (Ketley, 1997). Accordingly, a better understanding of the mechanisms by which C. jejuni overcomes the stresses encountered in the food chain is important for the identification, assessment and control of food hazards.

In general, exposure of bacteria to stressful conditions leads to induction of a set of heat-shock proteins that often fall...
into one of two categories, the chaperones and the ATP-dependent proteases. Inspection of the C. jejuni NCTC 11168 genome sequence has revealed the presence of several heat-shock protein homologues also found in other bacteria, including the major chaperones GroEL, GroES, GrpE, DnaK and DnaJ, and several ATP-dependent proteases (Parkhill et al., 2000). In many other Gram-negative bacteria, the response to heat stress is mediated by a specialized set of heat-shock sigma factors, such as σ32, but in C. jejuni, there are only two alternative sigma factors, RpoN (σ54) and Flia (σ28), which both control expression of flagella genes (Nuijten et al., 1990; Guerry et al., 1991; Kinsella et al., 1997; Hendrixson et al., 2001; Hendrixson & DiRita, 2003; Jagannathan et al., 2001; Carrillo et al., 2004). Instead, C. jejuni possesses homologues of the HrcA and HspR regulators that negatively control the heat-stress response in other bacteria (Narberhaus, 1999). HrcA has been identified in more than 40 eubacteria, including proteobacteria and cyanobacteria (Zuber & Schumann, 1994; Narberhaus, 1999). Studies of Bacillus subtilis have shown that the transcriptional repressor HrcA requires the GroE chaperonin system for binding to the well-conserved CIRCE DNA element (Mogk et al., 1998; Reischl et al., 2002). Following exposure to stress, GroE levels are decreased due to its association with misfolded proteins, resulting in decreased binding of HrcA to DNA and activation of heat-shock gene expression.

The HspR/HAIR (HspR-associated inverted repeat) repressor/operator system is less widely utilized in the bacterial kingdom, but has been described in actinomycetes and in Helicobacter pylori, an organism closely related to Campylobacter (Tomb et al., 1997). Like HrcA, HspR is a transcriptional repressor that binds to at least three HAIR sequences in the promoter region of regulated genes (Bucca et al., 1997; Grandvalet et al., 1997, 1999). Similarly, environmental stress releases HspR from its DNA-binding element, resulting in expression of the target genes. In Streptomyces coelicolor, DnaK seems to function as a co-repressor in a complex with HspR, and the sequestering of DnaK by denatured or partially unfolded proteins under stress conditions may result in release of HspR and induction of gene expression (Bucca et al., 2003). In Streptomyces, the HspR regulon includes the chaperones dnaK, clpB and dnaJ, as well as the lon protease gene, while in H. pylori the regulon comprises dnaK, groES, groEL and clpA (Grandvalet et al., 1997; Spohn & Scarlato, 1999; Servant & Mazodier, 2001; Bucca et al., 2003). Interestingly, disruption of hspR in Streptomyces seems to have a limited impact on cell physiology (Grandvalet et al., 1997), whereas an hspR mutant in H. pylori is non-motile and has reduced levels of urease enzyme, suggesting that HspR or other components of the HspR regulon might influence cellular processes, in addition to the heat-shock response (Spohn & Scarlato, 1999). The aim of the present study was to characterize the phenotypic effects of an hspR mutation and identify members of the regulon by comparison of the proteome and transcriptome of an hspR mutant and a wild-type strain of C. jejuni.

METHODS

Bacterial strains and growth conditions. Campylobacter jejuni strain NCTC 11168 strain NCTC 11168 was obtained from NCTC. Unless otherwise stated, C. jejuni was grown at 37 °C under microaerobic conditions (10% CO2, 85% N2, 5% O2) inside a MACS-MG-1000 controlled-atmosphere workstation (DW Scientific) on brain heart infusion (BHI) agar (Oxoid) or in BHI broth (Oxoid). Alternatively, the microaerobic environment was generated by CampyGen (Oxoid) and Blood Agar Base II (Oxoid) supplemented with 5% calf blood (Base II) was used. Escherichia coli DH5α (80lacZAM15 ΔlacZYA-argF)U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 was grown in Luria–Bertani broth or on Luria–Bertani agar (Difco). When appropriate, media were supplemented with 100 μg ampicillin ml⁻¹ or 20 μg chloramphenicol ml⁻¹.

Transformation. C. jejuni NCTC 11168 was transformed by electroporation essentially as described by Wassenaar et al. (1993a). To produce competent cells, C. jejuni NCTC 11168 was harvested from overnight-incubated agar plates with 2 ml ice-cold wash buffer (272 mM sucrose, 15%, v/v, glycerol) and subjected to four wash steps with repeated centrifugations (4 °C, 10 000 r.p.m., 10 min). After the final centrifugation, cells were resuspended in 1/10 volume wash buffer, resulting in a concentration of approximately 10⁶ c.f.u. ml⁻¹. A cell volume of 50 μl was electroporated (1-80 kV, 200 Ω, 25 F) with 1–5 μl plasmid DNA. Immediately after electroporation, 1 ml of recovery broth (10% glycerol, 2/3 Mueller–Hinton broth, 1/3 Brucella broth) was added and cells were plated on non-selective plates. After incubation overnight at 37 °C, cells were harvested with recovery broth and plated on selective plates. E. coli DH5α was transformed by standard methods (Cohen et al., 1972).

DNA manipulations. Extraction of chromosomal DNA from C. jejuni was performed using Fast Prep DNA (Bio 101), with the modification that cells were incubated in phosphate buffer, pH 7.0, containing 10 mg lysozyme ml⁻¹ and 20% sucrose for 80 minutes at 37 °C prior to DNA purification. Recombinant plasmid DNA from E. coli was isolated by using QIAGEN columns, as recommended by the supplier (Qiagen). Biolabs supplied restriction endonuclease enzymes and Klenow DNA polymerase. All enzymes were used as recommended by the supplier. PCR reactions were performed using Bioline tag DNA polymerase and buffer (DNA Technology). The following oligonucleotides were used in this study: HspR-A-F, 5'-CAAATCTAGAGCTTCACATTCCT-3'; HspR-B-R, 5'-GACGTCGCACTTAAGGTGTTGTGA-3'; HspR-C-F, 5'-CAGCCTTTAAAGGCGAGCTACTACGTTAAGATA-3'; HspR-D-R, 5'-GCAAGGAAAAATACGCGTATACGTTAAGATA-3'; CAT-up, 5'-GTCCTGAACTCTTATCGTGCG-3'; CAT-down, 5'-CGTGGCAAGAAGTCTTAAAGG-3'.

Construction of C. jejuni hspR mutant. C. jejuni NCTC 11168 chromosomal DNA was used as template for amplification. For construction of the hspR mutant, a 593 bp DNA fragment containing the 5’ end of the hspR gene and upstream sequences (primers HspR-A-F and HspR-B-R) and a 593 bp fragment carrying the 3’ end of the hspR gene and downstream sequences (primers HspR-C-F and HspR-D-R) were amplified. In a second round of PCR, the hspR fragments were joined by the splicing by overlap extension PCR method (Horton et al., 1993), creating a PCR fragment containing an in-frame deletion of 187 bp in hspR and introducing an AatI site between the upstream and downstream fragments. The ΔhspR PCR fragment was cloned in the TOPO TA cloning vector pCR2.1 (Invitrogen), resulting in plasmid pHspR-AD. Finally, pHspR-AD was digested with AatII, followed by treatment with Klenow, and
ligated to a DNA fragment containing the chloramphenicol acetyl transferase (cat) gene obtained from pRY109 (Yao et al., 1993) to generate pHsPR-ADcat, in which the cat gene was transcribed in the same direction as hspr. C. jejuni NCTC 11168 was transformed with pHsPR-ADcat (Δhspr::cat), and several chloramphenicol-resistant colonies were isolated. Chromosomal DNA was isolated from six different chloramphenicol-resistant colonies and used as template in PCR reactions to verify that the mutations were transferred by a double crossover event to the chromosome of C. jejuni NCTC 11168. Primers that annealed to sequences upstream and downstream of the region cloned in pHsPR-ADcat (primers HsPR-E-F and HsPR-F-R) were combined with primers CAT-up and CAT-down annealing internally in the cat gene. In each case, PCR fragments were obtained that verified the double crossover event, and one of these mutants, C. jejuni Δhspr::cat (MTA55), was used in subsequent experiments.

Construction of the C. jejuni DNA microarray. Internal DNA fragments corresponding to unique segments of the individual ORFs in the annotated genome sequence of C. jejuni strain NCTC 11168 were amplified using gene-specific primers, as described in Pearson et al. (2003). DNA probes were spotted on GAP Slides (Corning) using an in-house Stanford-designed arrayer and employing the recommended software and protocols (for further details, see http://cmgm.stanford.edu/psrawn/mguide/). Each glass slide contained two arrays each, with duplicate probes (or features) for each gene.

RNA isolation and purification. Cultures (50 ml in BHI) were grown in triplicate to an OD<sub>600</sub> of 0.3, and bacteria were harvested by centrifugation at 3000 g for 20 min, resuspended in 1 ml Tris- Reagent (Sigma-Aldrich) and equilibrated at room temperature for 10 min. After centrifugation at 12000 g for 15 min, the aqueous phase was removed and applied to Qiagen RNeasy Mini columns for RNA purification, according to the manufacturer’s protocol. DNA removal was ensured by treatment with DNA-free (Ambion), and the quality and quantity of RNA were checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, www.agilent.com/chem/labonachip).

Microarray transcriptome analysis. Two independent RNA preparations (biological replicates) of each sample were labelled and hybridized to glass-slide microarrays. Labelled cDNA was prepared from 15 μg RNA using Stratascript RT (Stratagene) for incorporation of Cy3 and Cy5 dyes (Amersham). Labelled cDNA was purified using a Quick purification kit (Qiagen) and dried before being resuspended in 19.5 μl water, 2.25 μl human Cot1 DNA (InviGen), 4.5 μl 20 × SSC, 0.72 μl 1 M HEPES, pH 7.0, 0.68 μl 10 % SDS and 3 μl Denhardt. Samples were heated for 3 min in a boiling water bath, cooled at room temperature for 5 min and centrifuged at maximum speed in a microfuge for 2 min to remove any solid particles from the hybridization mixture. This mixture was put on the microarray slide, sealed with a coverslip in a GeneMachine hybridization chamber (Anachem) and incubated for 18 h at 63 °C. Following hybridization, microarray slides were washed briefly in hybridization chamber (Anachem) and incubated for 18 h at 63 °C. Following hybridization, microarray slides were washed briefly in 1× SSC/0.1 % SDS, 1× SSC/0.3 % SDS and 1× SSC/0.5 % SDS, washed twice with 1× SSC, washed twice with 0.2× SSC, and finally 0.05× SSC. Microarray slides were dried by centrifugation at 300 g for 15 min before scanning.

Microarray data analysis. Microarrays were scanned using an Axon 4000A microarray scanner and images were acquired using GenePix Pro 3.0 software (Axon). All microarray data were filtered to remove poor-quality data using four sequential cut-off values (Mark Reuter, IFR, personal communication). All data values were from features above 50 μm in diameter, with sum of medians above 50, regression coefficient squared values above 0.2 and with a sum of the signal:noise ratio greater than 3.

A control experiment (comparison of two mRNA samples from replicated independent cultures of the wild-type strain) was used to estimate the boundaries between genes that were equally and differentially expressed in the two samples (Holmes et al., 2005). For the analyses described here, this boundary would detect changes equivalent to about 3-3-fold greater intensity in one of the fluorescence channels. Applying this boundary to the control dataset gave an error rate for misclassification of approximately 0-43 % of the gene features that give a fluorescence signal above background. Approximately 84 % of the 1654 annotated gene probes gave a fluorescence signal above background under these experimental conditions.

All test hybridization datasets (hspr mutant versus wild-type) were normalized (Holmes et al., 2005). The normalized data from each independent array were then unified in one single dataset and reanalysed. The genes that potentially had increased or decreased expression levels were first identified as those with mean intensities outside the boundaries specified above, and were further tested statistically by an F test. According to this test, the genes were classified as either differently or equally expressed in both populations. Genes identified in these analyses were annotated using on-line databases (http://www.sanger.ac.uk/Projects/C jejuni/).

Proteome analysis by two-dimensional (2D) gel electrophoresis. Campylobacter cells were harvested in late exponential phase by centrifugation (3000 g for 10 min) and washed with Tris-buffered saline, pH 7.5, prior to lysis by four times 1 min glass bead beating (106 μm or finer) in a lysis buffer containing 50 mM Tris, pH 7-5, 0-3 % SDS, 0.2 M DTT, 3-3 mM MgCl<sub>2</sub>, 16-7 μg RNase ml<sup>-1</sup> and 1-67 U Dnase ml<sup>-1</sup>. Following beating, the extract was kept on ice for 20 min before centrifuging at 18 500 g for 20 min; the supernatant was retained for further analysis. Protein concentrations were determined using the 2D Quant Kit (Amersham) according to the manufacturer’s instructions. Proteomic analysis of the cell-free extracts, including 2D electrophoresis, imaging, spot picking, digestion and MALDI-TOF analysis, was carried out as described in Holmes et al. (2005), using 100–125 μg protein per IGP strip.

Physiological studies. C. jejuni strains were grown overnight on Base II agar plates at 37 °C under microaerobic conditions (Campygen, Oxoid), harvested in BHI medium and adjusted to an OD<sub>600</sub> of 0.1. Serial dilutions were made, and 10 μl of each dilution was spotted onto three Base II agar plates, which were incubated microaerobically (Campygen, Oxoid) for 3 days at 37, 42 or 44 °C. Alternatively, 10 μl of each dilution was spotted onto a Base II agar plate containing 10, 15 or 20 μg puromycin ml<sup>-1</sup> and incubated microaerobically (Campygen, Oxoid) for 5 days at 37 °C. Growth at 17–18 % O<sub>2</sub> was obtained by incubating in a candle light atmosphere (Wang et al., 1983).

Growth in liquid culture of C. jejuni strains was examined under microaerophilic conditions (5 % O<sub>2</sub> influx) by inoculating 50 ml pre-warmed BHI (37 or 42 °C) to a final OD<sub>600</sub> of 0-1. Two flasks were incubated at each growth temperature (i.e. 37 and 42 °C), one of which was shaken continuously and the other kept static, except for mixing at hourly intervals when aliquots were taken for OD<sub>600</sub> measurement.

Light and electron microscopy. C. jejuni was grown overnight in sterile filtered Mueller–Hinton broth (MH, Oxoid) under microaerophilic conditions (5 % O<sub>2</sub> influx) at 37 or 42 °C. Bacterial cells were examined either under the light microscope (100× magnification) or by electron microscopy. For the latter, bacteria were negatively stained with saturated aqueous uranyl acetate and photographed in a JEOL EX/B transmission electron microscope at 80 kV.

Motility/chemotaxis assay. C. jejuni strains were grown overnight, harvested in BHI medium and adjusted to an OD<sub>600</sub> ml<sup>-1</sup> of 0-1. One microlitre was spotted onto the centre of heart infusion...
broth (Difco) motility plates containing a low concentration (0-25%) of agar, and the motility of C. jejuni was examined after 48 h of microaerobic incubation at 37 °C.

**Autoagglutination assay.** Autoagglutination assays with the wild-type and hspR mutant strains were performed essentially as described by Misawa & Blaser (2000). Briefly, C. jejuni cells were grown overnight on Base II agar plates, harvested in MilliQ water and washed once by centrifugation before being resuspended in 10 mM PBS, pH 7-2, to a final concentration of 1 OD₆₀₀ ml⁻¹. Cell suspensions were then incubated under aerobic conditions at 25 °C, and 1 ml from the top of the suspension was collected to measure OD₆₀₀. C. jejuni cells normally agglutinate and precipitate, causing a decrease in the OD. The mean percentage decrease in OD₆₀₀ and standard deviation were calculated using data from three independent assays.

**Adherence and invasion assay.** INT 407 cells were cultured in Eagle’s minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified 5% CO₂ incubator. For the adherence and invasion assay, approximately 1 x 10⁸ bacterial cells were added to a monolayer of 5 x 10⁵ INT407 epithelial cells (m.o.i. 200) and incubated for 2 h. In order to determine the number of adherent bacteria, the epithelial cell monolayers were washed three times with 10 mM PBS, pH 7-2, and then lysed with a solution of 0-1% (v/v) Triton X-100. The suspensions were serially diluted and the number of viable adherent bacteria was determined by enumeration of bacterial colonies after plating on Base II agar. To measure the number of internalized bacteria, the epithelial cell monolayers were incubated with bacteria, as described above, and then treated with EMEM containing 250 µg gentamicin ml⁻¹ for 2 h at 37 °C in 5% CO₂ in order to kill extracellular bacteria. The epithelial cells were then washed three times with PBS, lysed with 0-1% (v/v) Triton X-100 and plated to enumerate the internalized viable bacteria, as described above. The values obtained represented the mean count and standard deviation calculated using samples from four replicate wells.

**RESULTS**

**Inactivation of HspR affects cell morphology in C. jejuni**

In the genome sequence of Campylobacter jejuni NCTC 11168, gene Cj1230 is annotated as hspR. Sequence analysis (BLASTP) confirmed that Cj1230 encodes a 124 amino acid product with extensive homology (63% identity over 120 amino acids) to the transcriptional regulator HspR from H. pylori. In C. jejuni, hspR is located 19 bp downstream of cbpA (Cj1229), which encodes a predicted protein with homology to the DnaJ chaperone (Ueguchi et al., 1994). While no obvious promoter-like sequences are present immediately upstream of hspR, the gene is preceded by a Shine–Dalgarno sequence (AAGGAAA) located 8 nucleotides upstream of the start codon. In H. pylori, cbpA and hspR form an operon, and the promoter elements identified upstream of H. pylori cbpA are also conserved in C. jejuni (data not shown).

With the aim of determining the role of HspR and members of the HspR regulon in C. jejuni, we substituted the central 186 bp corresponding to nucleotides 83 to 268 of hspR with a chloramphenicol resistance gene marker that is transcribed in the same direction as hspR (see Methods). The growth rate of the ΔhspR mutant was identical to wild-type cells at 37 °C, as determined both by OD₆₀₀ measurement and by colony forming units (data not shown); however, when examined by microscopy, we found that the cells of the HspR mutant strain were highly elongated, and this was more pronounced at 42 °C than at 37 °C (Fig. 1).

**Genome-wide transcriptional profile of C. jejuni mutant ΔhspR**

Since HspR is a negative regulator of the heat-shock response in other bacteria, we predicted that a comparison of the transcriptome of the hspR mutant with the wild-type strain would reveal gene members of the HspR regulon that had been depressed in the mutant. The transcriptome of the hspR mutant and wild-type strain were compared in exponential-phase growth by DNA microarray analysis, as described in Methods. These experiments were repeated twice using independently isolated samples of RNA (i.e. two biological replicates), and hybridization was performed in duplicate (i.e. two technical replicates).

The results presented in Table 1 show that the transcript levels of 13 and 17 genes were increased and decreased, respectively, in the hspR mutant strain compared to the wild-type strain. Transcript levels of the putative heat-shock operon genes hrcA, grpE and dnaK, as well as two downstream genes encoding hypothetical proteins (Cj0760 and Cj0761), were substantially elevated (4-8- to 28-2-fold) in the hspR mutant, suggesting that this operon is negatively regulated by HspR (Table 1). While the functions of the hypothetical proteins are unknown, Cj0760 shows homology to hydrolases of the metallo-beta-lactamase superfamily. Interestingly, the gene encoding the ATP-dependent chaperone ClpB, which interacts with DnaK to reactivate proteins that have become aggregated after heat shock

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**Fig. 1.** Electron micrographs of wild-type and hspR mutant cells. Electron microscopy of (a) C. jejuni NCTC 11168 (wild-type), (b) C. jejuni MTA55 (ΔhspR), both grown at 37 °C, and (c) C. jejuni MTA55 (ΔhspR) grown at 42 °C. All cells were collected in the late-exponential growth phase. Bars, (a) and (b), 500 nm; (c), 1000 nm.
Table 1. Results of a DNA microarray-based comparison of relative transcript levels in the hspR mutant and the wild-type strain

The gene number is according to the Sanger Centre annotation for strain NCTC 11168. The fold change in the transcript level of hspR mutant cells relative to wild-type cells is shown; a negative fold change value indicates that expression is decreased in the mutant relative to the wild-type.

<table>
<thead>
<tr>
<th>Gene no.</th>
<th>Gene name</th>
<th>Fold change in hspR mutant</th>
<th>Proposed gene function</th>
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<td>flgE</td>
<td>-4.7</td>
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<td>-3.5</td>
<td>Hypothetical protein CJ0044c</td>
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<td>thiC</td>
<td>-3.9</td>
<td>Thiamin biosynthesis protein ThiC</td>
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<td>flgH</td>
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<td>Putative flagellar L-ring protein precursor</td>
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<td>flgG2</td>
<td>-4.9</td>
<td>Putative flagellar basal-body rod protein</td>
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<td>flaD</td>
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<td>Putative flagellin</td>
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<td>CJ0986c</td>
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<td>Putative integral membrane protein</td>
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<td>CJ1242</td>
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<td>-3.4</td>
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<td>Flagellin</td>
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<td>hspR</td>
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<td>Putative heat-shock transcriptional regulator</td>
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(Goloubinoff et al., 1999), was also transcribed at substantially higher levels (21.8-fold) in the hspR mutant. In the C. jejuni strain NCTC 11168, clpB appears to be transcribed as a single cistron from its own promoter. In addition, higher amounts of hspR transcripts were present in the hspR mutant (4.0-fold) compared to the wild-type strain. In the hspR mutant strain, hspR was inactivated by replacement of an internal fragment with the cat gene; thus, increased transcription from either the upstream clpA promoter or the cat gene promoter could increase the amount of labelled cDNA hybridizing to the hspR probe on the microarray. Polypeptide deformylase, the essential enzyme that cleaves the formyl group of the N terminus of nascent polypeptide chains, was also expressed at 4.0-fold higher levels in the hspR mutant.

Of the genes that were reduced in expression in the absence of HspR, eight were predicted to be involved in assembly of the flagellar apparatus, including genes encoding flagellin (FlaA and FlaB) and a putative flagellin (FlaD), as well as the ring protein (FlgH), hook protein (FlgE, FlgE2) and basal body protein (FlgG2) (Table 1). In addition, genes encoding acetyl-coenzyme A synthetase and a sodium/proline transporter were also transcribed at lower levels in the hspR mutant compared to the wild-type.

Proteome analysis of the hspR mutant

The effect of HspR on protein expression was investigated by comparing the proteomes of hspR mutant and wild-type cells. Proteins were visualized by Sypro-Ruby staining and quantified by the use of a multiverse fluorimeter (ProExpress) and the Proteomweaver 2D gel analysis software package. Digital images from 2D gels of protein samples of the hspR mutant and wild-type strain indicating the differentially expressed proteins are shown in Fig. 2.
Analysis of the gels using Proteomweaver 2D gel analysis software followed by manual filtering and editing identified six of the matched spots as being significantly higher in the hspR mutant (Table 2). Apart from the cat gene, which used a selectable marker for inactivation of hspR, all other proteins increased in amount in the hspR mutant were linked to the heat-shock response (i.e. DnaK, GrpE, GroEL, GroES and ClpB). The measured increase in these heat-shock proteins ranged from 3.7- to 35-fold (Table 2). Curiously, none of the proteins encoded by the flagella-associated genes transcribed at lower levels in the hspR mutant were identified by proteomic analysis. Possible reasons for this include poor solubility of the flagella proteins due to high stability of the flagella apparatus, or the hydrophobic nature of the membrane-associated flagella components.

Table 2. Proteomic analysis of hspR mutant cells

<table>
<thead>
<tr>
<th>Protein identification</th>
<th>Cj no.</th>
<th>Mol. wt</th>
<th>pI</th>
<th>Mowse score for ID</th>
<th>Sequence coverage for ID</th>
<th>wt 37 °C, mean spot intensity</th>
<th>hspR mutant 37 °C, mean spot intensity</th>
<th>hspR mutant 37 °C, induction compared to wt (-fold)</th>
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<tr>
<td>ATP-dependent clpB protease ATP-binding subunit</td>
<td>Cj0509C</td>
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<td>12%</td>
<td>0.807</td>
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<td>30-7085</td>
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<td>GrpE protein (HSP-70 cofactor)</td>
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<td>20412</td>
<td>4.83</td>
<td>144</td>
<td>51%</td>
<td>0.604</td>
<td>9-893</td>
<td>16-3914</td>
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<tr>
<td>Heat-shock protein DnaK</td>
<td>Cj0759</td>
<td>67491</td>
<td>4.98</td>
<td>205</td>
<td>28%</td>
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<td>107-732</td>
<td>34-8996</td>
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<td>10 kDa chaperonin (cpn10)</td>
<td>Cj1220</td>
<td>9452</td>
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<td>54%</td>
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<td>24504</td>
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<td>98</td>
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Fig. 2. Two-dimensional protein gel analysis of C. jejuni strain 11168 (a) and the hspR mutant (b) derived from this strain. Proteins were extracted from bacteria cultivated in BHI at 37 °C collected when OD₆₀₀ reached 0.4.
Temperature and oxygen influence growth in the absence of HspR

Previous studies have shown that increased expression of chaperones in some cases improves the ability of cells to adapt to heat stress (Stewart et al., 2001). As several chaperones were expressed more highly in the hspR mutant compared to the wild-type strain, we investigated the heat tolerance of the two strains. After 3 days growth at the optimum growth temperature of 42 ºC and at the lower temperature of 37 ºC, both strains produced similar numbers of colony forming units (c.f.u.) on agar plates (Fig. 3a). However, after growth at 44 ºC, significantly fewer colonies were recovered from the hspR mutant compared to the wild-type, indicating that in C. jejuni HspR is important for growth at high temperature (Fig. 3a).

To determine if HspR is important for growth under other conditions that lead to the accumulation of misfolded proteins, we examined the ability of the hspR mutant and the wild-type strain to form colonies in the presence of the aminoacyl-tRNA analogue pyromycin, which causes premature termination of protein synthesis, leading to puromycyl-containing polypeptides (VanBogelen et al., 1987; Goldberg, 1972). When exposed to various concentrations of puromycin, we found no differences between the mutant and the wild-type in the ability to form colonies (data not shown), suggesting that the heat sensitivity is not a result of reduced tolerance to misfolded proteins.

During the course of these experiments, we also noted that the temperature sensitivity of the hspR mutant was related to oxygen concentration. The growth rate of wild-type and hspR mutant cells was identical at 42 and 37 ºC when cultivated in shaken liquid cultures with 5 % O₂. However, we found that the hspR mutation conferred oxygen sensitivity, as mutant cells formed colonies at a significantly lower frequency than the wild-type at 37 ºC in the presence of 17–18 % O₂ (Fig. 3b). These results show that HspR is required for oxygen tolerance in C. jejuni.

HspR is required for normal motility/chemotaxis

DNA microarray analysis revealed that the transcript levels of eight flagella-associated genes were reduced in the hspR mutant. This finding prompted us to investigate the presence of flagella in the mutant cells by electron microscopy. DNA micrographs revealed that both strains were spiral-shaped and possessed flagella of a similar length (Fig. 1, and data not shown). However, on agar motility plates there was an obvious reduction in the motility of the hspR mutant compared to wild-type cells both at 37 ºC (Fig. 4) and at 42 ºC (data not shown). This result was reproducibly obtained in independent experiments and also confirmed...
by examination of the motility of the strains by light microscopy (data not shown).

In *C. jejuni*, expression of flagella has been correlated with the ability to autoagglutinate (Misawa & Blaser, 2000; Golden & Acheson, 2002). When autoagglutination was examined in static cultures, wild-type cells formed a distinct pellet at the bottom of the tube that was not apparent with the *hspR* mutant. The OD$_{600}$ of the wild-type culture was reduced to $9.5\% \pm 1.2\%$ of the initial value, while that of the mutant was only reduced to $14.9\% \pm 0.9\%$. Thus, we concluded that the *hspR* mutation results in a minor reduction in the ability of *C. jejuni* to autoagglutinate.

**The adherence and invasion ability of the *hspR* mutant is impaired**

In *C. jejuni*, the flagella and motility are critical both for invasion of gastrointestinal epithelial cells and for colonization of the mucus lining of the gastrointestinal tract (Yao et al., 1994). As the *hspR* mutation reduced expression of a number of flagella genes, we determined the ability of mutant and wild-type cells to adhere to and invade INT-407 epithelial cells. Using an m.o.i. of 200 to maximize the number of internalized bacteria (Hu & Kopecko, 1999), we calculated that the number of *hspR* mutant bacteria adhering to the epithelial cells was only $6\%$ of that obtained with the wild-type strain (data not shown). Furthermore, the invasion capacity of the mutant was reduced to only $4\%$ of that measured for the wild-type strain. Thus, both adherence and invasion properties were reduced in the *hspR* mutant.

**DISCUSSION**

Heat-shock proteins are among the most highly conserved proteins in nature, and diverse regulatory mechanisms have evolved for controlling their synthesis. We have examined the role of a *C. jejuni* homologue of the putative negative heat-shock regulatory protein HspR in controlling gene expression in *C. jejuni*. Microarray comparison of the transcriptome of *hspR* mutant cells and the wild-type strain revealed that the transcript levels of $13$ and $17$ genes were increased and decreased, respectively, in the *hspR* mutant. In the absence of HspR, transcript levels of all genes in the *hrC-A-grpE-dnaK* operon were substantially increased, suggesting that this heat-shock operon is normally regulated negatively by HspR. The *hspR* gene was also more highly expressed in the mutant, suggesting that it may be auto-regulated, as previously observed in *H. pylori* (Spohn & Scarlato, 1999). The promoter for *hspR* probably lies upstream of *cbpA* (Cj1229) as there is only a $19$ bp intergenic region between *cbpA* and *hspR*. Although the *cbpA* gene was not classified as being differentially expressed in the *hspR* mutant, the relative transcript amounts were $2\times2$-fold higher in the *hspR* mutant than in the wild-type. This value is under the statistical cut-off used for differentially expressed genes (see Methods), but might possibly be biologically significant.

The proteome and transcriptome results also indicated that, in contrast to *H. pylori*, the *Campylobacter* HspR controls expression of *clpB*, the latter encodes a putative molecular chaperone that in *E. coli* cooperates with DnaK to remove aggregated proteins (Goloubinoff et al., 1999). Furthermore, the proteome analysis revealed that the GroES and GroEL proteins were also produced in higher amounts in the *hspR* mutant, although they were not classified as being differentially expressed by DNA microarray analysis of the transcriptomes. However, there was a $1.9$-fold increase in the amount of transcript of both the *groEL* and *groES* genes in the *hspR* mutant compared to the wild-type. Thus, as in the case of *cbpA*, it is possible that these changes in transcript levels were biologically relevant, even if below our statistical cut-off. This notion was supported by inspection of the *C. jejuni* NCTC 11168 genome for sequences that resemble the HspR binding site.
HAIR, reported for *Streptomyces albus* G (CTTGAGT-N7-ACTCAAG, Grandvalet et al., 1999) and for *H. pylori* (Delany et al., 2002), as we found putative HisR binding sites in the promoter region of *groEL-groES* (CTTGGC-TTTATCAACTCAGA) as well as in front of *hrcA-gpE-dnaK* (ATCAAGTGGAAATTCTCAAG) and *cbbA-hspR* (CTTGAATGAATAGCACTTTAAT). In contrast, we were not able to identify HAIR-like sequences in association with other of the regulated genes.

The biological importance of HisR at elevated temperature was revealed by the finding that *C. jejuni* is unable to form colonies at 44 °C in the absence of HisR. In contrast, *hspR* mutant cells of *Mycobacterium tuberculosis* were more temperature resistant than wild-type cells (Stewart et al., 2001), while disruption of *hspR* in *Streptomyces albus* G gave a marginal growth defect at 30 °C but not at 37 °C (Grandvalet et al., 1997). Interestingly, the major heat-shock proteins are overexpressed in all three cases, suggesting that they are not responsible for the observed differences. Additional evidence for the role of HisR in the *C. jejuni* heat-shock response comes from a recent DNA microarray analysis of cells shifted from 37 to 42 °C, which shows that the heat-shock gene clusters with altered transcript levels overlap with those that we showed to be de-repressed in the absence of HisR (Stintzi, 2003).

In *C. jejuni*, HisR controls expression of the other negative heat-shock regulator, HrcA, and putative binding sites for HrcA (Schulz & Schumann, 1996) precede *dnaK* and *groEL* (data not shown), indicating a complex interrelationship between the HrcA and HisR regulons. Likewise, it has been shown that both *dnaK* and *groEL* are co-regulated by HisR and HrcA in *H. pylori* (Spohn et al., 2004). Coregulation of heat-shock genes has also been observed in *Staphylococcus aureus*, and it was hypothesized that the cooperative activity of both HrcA and CtsR is required to maintain low heat-shock gene expression in the absence of stress (Chastanet et al., 2003). Similarly, certain heat-shock genes in *C. jejuni* may require both HisR and HrcA to ensure a low level of expression in the absence of stress. To address the respective roles of the two regulatory proteins in controlling gene expression in *C. jejuni*, we also set out to delete the *hrcA* gene, but numerous attempts proved unsuccessful (data not shown), suggesting that *hrcA* or a gene product encoded by the *hrcA* regulon may be essential for growth in the laboratory. We conclude that the *C. jejuni* HisR homologue encodes a negative regulator of the expression of several major chaperones in *C. jejuni*.

The motility of *Campylobacter* is conferred by polar flagella, and approximately 40 genes are predicted to be involved in flagella biosynthesis or function (Yao et al., 1994; Parkhill et al., 2000). Interestingly, our DNA microarray analysis revealed that transcription of eight flagella-associated genes was reduced in *hspR* mutant cells when compared to wild-type cells. By microscopy and motility assays, we showed that independently isolated *hspR* mutants had reduced motility. Motility is correlated with the ability to self-associate or auto-agglutinate, and mutations affecting motility often influence auto-agglutination (Misawa & Blaser, 2000; Golden & Acheson, 2002). Likewise, we observed that the reduction in motility conferred by the *hspR* mutation was accompanied by a moderate decrease in the ability to auto-agglutinate. In general, flagellum biosynthesis is dependent on expression of the major structural component, flagellin, in addition to gene products constituting the flagella basal body, and switch- and hook-associated proteins (Alldridge & Hughes, 2002). In *C. jejuni*, *flaA* encodes the major flagellin, although the minor flagellin component FlaB is also required for normal motility (Guerry et al., 1991). Interestingly, the expression of both *flaA* (Cj1339c) and *flaB* (Cj1338c), as well as a third putative flagellin gene, *flaD* (Cj0887c), was reduced in the absence of HisR. In addition, genes involved in formation of the flagella hook (*flgE*, Cj0043; *flgE2*, Cj1729), flagella L-ring (*flgH*, Cj0687), P-ring (*flgI*, Cj1462) and basal body (*flgG2*, Cj0697) were all expressed at substantially lower levels in the mutant (3.5- to 6-7-fold). Several of these gene products are required for normal motility, as studies have shown that motility is reduced by mutations in *flaA*, *flgD*, *flgH*, *motA* (Hendrixson et al., 2001), *flaD*, *flaA*, *flgI* (Golden & Acheson, 2002), *flgE* (Kinsella et al., 1997), *flaD*, *flgE* (Colegio et al., 2001) *flaA*, *flaB* (Guerry et al., 1991) and *flgE2* (Konkel et al., 2004). Thus, the expression of a number of gene products involved in flagella formation is reduced in the absence of HisR, and this reduction is likely to be the reason for the impaired motility. In *H. pylori*, it was recently shown that the absence of *hspR* impaired motility, suggesting that *HspR* in this organism also is a central regulator of flagella biosynthesis (Spohn & Scarlato, 1999).

In *C. jejuni*, several factors are involved in expression of flagella genes (Jagannathan et al., 2001). Of the genes determined by our DNA microarray as being controlled by HisR, expression of *flaA* is dependent on σ^28* (FliA), while *flaB* and *flgDE* are expressed from a σ^34*-associated RNA polymerase, (Guerry et al., 1991; Kinsella et al., 1997; Lüneberg et al., 1998; Hendrixson et al., 2001). Additionally, sequence analysis of the *C. jejuni* genome revealed putative σ^34* promoter sequences upstream of *flgH*, *flgG2*, *flgI* and *flgE2* (Hendrixson & DiRita, 2003; Wösten et al., 2004). The finding that both σ^28* and σ^34*-controlled genes are affected by the *hspR* mutation suggests that the effect of HisR on flagella gene expression is not mediated via a σ^34*, or σ^28*, specific factor, but rather involves a regulator affecting both of these regulons. Since HisR has not previously been shown to directly stimulate gene expression, we believe that expression of a putative negative regulator is controlled by HisR. Such a regulator might also be involved in the heat-induced expression of several flagella genes that has observed in *C. jejuni* cells shifted from 37 to 42 °C (Stintzi, 2003). Interestingly, the positive effect of HisR on gene expression may be unique to *C. jejuni*, and perhaps *H. pylori*, as DNA microarray analysis of *M. tuberculosis* and *S. coelicolor* failed to reveal genes with expression stimulated by HisR (Stewart et al., 2002; Bucca et al., 2003).
In C. jejuni, motility is associated with pathogenesis, and colonization of the gastro-intestinal tract depends on FlaA expression (Wassenberg et al., 1993b; Morooka et al., 1985; Nachamkin et al., 1993). Accordingly, we found that both adherence and invasion of hspR mutant cells in INT407 epithelial cells were substantially reduced when compared to wild-type cells. Since our electron micrographs revealed intact flagella, the defective interaction with host cells is likely due to reduced motility rather than a lack of FlaA expression. In addition to the altered expression of flagella gene products that are known to function as adhesins (Yao et al., 1993) and to secrete virulence proteins (Konkel et al., 2004; Song et al., 2004), we also found that expression of a putative fibronectin/fibrinogen binding protein (Cj1349) was reduced in the absence of HspR. Previously, fibronectin binding by C. jejuni has been shown to be mediated by CadF, which also is required for maximal adherence to and invasion of INT407 cells (Monteville et al., 2003). However, the role of Cj1349 in these processes will be the focus of future investigations. In conclusion, C. jejuni HspR controls the expression of genes involved in diverse processes, including motility, stress tolerance, morphology and virulence, suggesting that either directly or indirectly it is a key regulator of adaptive responses both in the environment and inside host organisms.

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