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

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*Campylobacter jejuni* is a leading cause of bacterial gastroenteritis in the developed world. The role of a homologue of the negative transcriptional regulatory protein HspR, which in other organisms participates in the control of the heat-shock response, was investigated. Following inactivation of *hspR* in *C. jejuni*, members of the HspR regulon were identified by DNA microarray transcript profiling. In agreement with the predicted role of HspR as a negative regulator of genes involved in the heat-shock response, it was observed that the transcript amounts of 13 genes were increased in the *hspR* mutant, including the chaperone genes *dnaK*, *grpE* and *clpB*, and a gene encoding the heat-shock regulator HrcA. Proteomic analysis also revealed increased synthesis of the heat-shock proteins DnaK, GrpE, GroEL and GroES in the absence of HspR. The altered expression of chaperones was accompanied by heat sensitivity, as the *hspR* mutant was unable to form colonies at 44°C. Surprisingly, transcriptome analysis also revealed a group of 17 genes with lower transcript levels in the *hspR* mutant. Of these, eight were predicted to be involved in the formation of the flagella apparatus, and the decreased expression is likely to be responsible for the reduced motility and ability to autoagglutinate that was observed for *hspR* mutant cells. Electron micrographs showed that mutant cells were spiral-shaped and carried intact flagella, but were elongated compared to wild-type cells. The inactivation of *hspR* also reduced the ability of *Campylobacter* to adhere to and invade human epithelial INT-407 cells *in vitro*, possibly as a consequence of the reduced motility or lower expression of the flagellar export apparatus in *hspR* mutant cells. It was concluded that, in *C. jejuni*, HspR influences the expression of several genes that are likely to have an impact on the ability of the bacterium to successfully survive in food products and subsequently infect the consumer.

**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, cfpB and dnaJ, as well as the lon protease gene, while in H. pylori the regulon comprises dnaK, groES, groEL and cfpA (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 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-MG1000 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 (λaacZΔM15 ΔlacZYA-argF)U169 recA1 endA1 hsdR17 supE44 thi-1 gyr96 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 107 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). Bioslabs 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'-GGCCGTGCCCTAATTGATTTTGGTGA-3'
- HspR-B-R, 5'-GACGTCGCCTTAAGGTTTGTGGATGTA-3'
- HspR-D-R, 5'-CACTCTAGAGCTTCACATTCCT-3'

PCR reactions were performed using Bioline taq DNA polymerase and buffer (DNA Technology). The following oligonucleotides were used in this study:

- HspR-A-F, 5'-GGCCGTGCCCTAATTGATTTTGGTGA-3'
- HspR-B-R, 5'-GACGTCGCCTTAAGGTTTGTGGATGTA-3'
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- HspR-D-R, 5'-CACTCTAGAGCTTCACATTCCT-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 Aattl site between the upstream and downstream fragments. The hspR PCR fragment was cloned in the TOPO TA cloning vector pCR2.1 (Invitrogen), resulting in plasmid pHspR-AD. Finally, pHspR-AD was digested with Aattl, 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 11618 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 11618. 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 11618 were amplified using gene-specific primers, as described in Pearson et al. (2003). DNA probes were spotted on GAPSII slides (Corning) using an in-house Stanford-designed arrayer and employing the recommended software and protocols (for further details, see http://cmgm.stanford.edu/pbrown/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_{600} 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 12,000 g for 15 min, the aqueous phase was removed and applied to Qiagen RNeasy Mini columns. The RNA was eluted in 500 μl of water, 2-25 μl human Cot1 DNA (Invitrogen), 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 pre-warmed (60 °C) 1 × SSC/0-3 % SDS to remove the coverslip and then washed twice for 5 min in each of the following buffers: (a) 1 × SSC/0-03 % SDS (pre-warmed to 60 °C), b) 0-2 × SSC and finally c) 0-05 × SSC. Microarray slides were dried by centrifugation at 300 g for 15 min before scanning.

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 Qiaquick purification kit (Qiagen) and dried before being resuspended in 10-20 μl of water. Samples were then heated for 3 min, the aqueous phase was removed and applied to Qiagen RNeasy Mini columns. The RNA was eluted in 500 μl of water, 2-25 μl human Cot1 DNA (Invitrogen), 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 pre-warmed (60 °C) 1 × SSC/0-3 % SDS to remove the coverslip and then washed twice for 5 min in each of the following buffers: (a) 1 × SSC/0-03 % SDS (pre-warmed to 60 °C), b) 0-2 × SSC and finally c) 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 GenePixPro 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 MgCl₂, 16-7 μg RNase ml⁻¹ and 1-67 U Dnase ml⁻¹. 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 IPG 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_{600} 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⁻¹ and incubated microaerobically (Campygen, Oxoid) for 5 days at 37 °C. Growth at 17–18 % O₂ 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 % O₂ influx) by inoculating 50 ml pre-warmed BHI (37 or 42 °C) to a final OD_{600} 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_{600} measurement.

Light and electron microscopy. C. jejuni was grown overnight in sterile filtered Mueller–Hinton broth (MH, Oxoid) under microaerophilic conditions (5 % O₂ 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_{600} ml⁻¹ 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 OD<sub>600</sub> ml<sup>-1</sup>. Cell suspensions were then incubated under aerobic conditions at 25 °C, and 1 ml from the top of the suspension was collected to measure OD<sub>600</sub>. *C. jejuni* cells normally agglutinate and precipitate, causing a decrease in the OD. The mean percentage decrease in OD<sub>600</sub> 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% CO<sub>2</sub> incubator. For the adherence and invasion assay, approximately 1 x 10<sup>8</sup> bacterial cells were added to a monolayer of 5 x 10<sup>5</sup> 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<sup>-1</sup> for 2 h at 37 °C in 5% CO<sub>2</sub> 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 (AAGGAA) 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 Δ*hspR* mutant was identical to wild-type cells at 37 °C, as determined both by OD<sub>600</sub> 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 Δ*hspR***

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...
HspR in Campylobacter jejuni

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>
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<th>Proposed gene function</th>
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</tr>
<tr>
<td>CJ1230</td>
<td>hspR</td>
<td>4.0</td>
<td>Putative heat-shock transcriptional regulator</td>
</tr>
</tbody>
</table>

(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 clpB 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 multivariate fluoromager (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>
</tr>
</thead>
<tbody>
<tr>
<td>ATP-dependent clpB protease ATP-binding subunit</td>
<td>Cj0509C</td>
<td>95485</td>
<td>5.47</td>
<td>82</td>
<td>12 %</td>
<td>0.807</td>
<td>24.773</td>
<td>30.7085</td>
</tr>
<tr>
<td>GrpE protein (HSP-70 cofactor)</td>
<td>Cj0758</td>
<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>
</tr>
<tr>
<td>Heat-shock protein DnaK</td>
<td>Cj0759</td>
<td>67491</td>
<td>4.98</td>
<td>205</td>
<td>28 %</td>
<td>3.087</td>
<td>10.732</td>
<td>34.8996</td>
</tr>
<tr>
<td>10 kDa chaperonin (cpn10) GroES</td>
<td>Cj1220</td>
<td>9452</td>
<td>5.38</td>
<td>70</td>
<td>58 %</td>
<td>4.356</td>
<td>16.026</td>
<td>3.6794</td>
</tr>
<tr>
<td>60 kDa chaperonin (cpn60) GroEL</td>
<td>Cj1221</td>
<td>57991</td>
<td>5.02</td>
<td>320</td>
<td>54 %</td>
<td>31.434</td>
<td>141.34</td>
<td>4.4964</td>
</tr>
<tr>
<td>Chloramphenicol acetyltransferase (Cat)</td>
<td>24504</td>
<td>5.52</td>
<td>98</td>
<td>25 %</td>
<td>2.071</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**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°C and at the lower temperature of 37°C, both strains produced similar numbers of colony forming units (c.f.u.) on agar plates (Fig. 3a). However, after growth at 44°C, 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°C 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°C 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°C (Fig. 4) and at 42°C (data not shown). This result was reproducibly obtained in independent experiments and also confirmed.

Fig. 3. Temperature- and oxygen-dependent growth of wild-type and hspR mutant cells. (a) Growth was evaluated by plating dilutions of C. jejuni wild-type and hspR mutant cells on Base II agar plates and incubating at 37, 42 and 44°C under microaerophilic conditions (CampyGen). (b) Growth in a candle-jar atmosphere (17–18% O2) was evaluated after 3 days at 42°C.
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 ± 0.2 of the initial value, while that of the mutant was only reduced to 14.9 ± 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 hrcA-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 2.2-fold higher in the hspR mutant than in 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.
HspR homologue encodes a negative regulator of the C. jejuni for growth in the laboratory. We conclude that the C. jejuni shock genes in The motility of gene product encoded by the hspR showed that independently isolated to wild-type cells. By microscopy and motility assays, we revealed that transcription of eight flagella-associated genes was reduced in revealed that transcription of eight flagella-associated genes was reduced in the absence of HspR (Stintzi, 2003). In C. jejuni, flaA encodes the major flagellin, although the minor flagellin component Flab is also required for normal motility (Guerry et al., 1997). 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 HspR. In addition, genes involved in formation of the flagella hook (flgE, Cj0043; flgE2, Cj1729), flagella L-ring (flgH, Cj0687), P-ring (flgL, 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 HspR, 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 HspR, expression of flaA is dependent on $\sigma^{28}$ (FliA), while flaB and flgDE are expressed from a $\sigma^{54}$-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 $\sigma^{54}$ promoter sequences upstream of flgH, flgG2, flgI and flgE2 (Hendrixson & DiRita, 2003; Wösten et al., 2004). The finding that both $\sigma^{28}$- and $\sigma^{54}$-controlled genes are affected by the hspR mutation suggests that the effect of HspR on flagella gene expression is not mediated via a $\sigma^{54}$, or $\sigma^{28}$ specific factor, but rather involves a regulator affecting both of these regulons. Since HspR has not previously been shown to directly stimulate gene expression, we believe that expression of a putative negative regulator is controlled by HspR. 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 HspR 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 HspR (Stewart et al., 2002; Bucca et al., 2003).

The biological importance of HspR at elevated temperature was revealed by the finding that C. jejuni is unable to form colonies at 44 °C in the absence of HspR. 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 HspR 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 HspR (Stintzi, 2003).

In C. jejuni, HspR 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 HspR regulons. Likewise, it has been shown that both dnaK and groEL are co-regulated by HspR and HrcA in H. pylori (Spohn et al., 2004). Co-regulation 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 HspR 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 HspR 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 (Aldridge & Hughes, 2002).
In *C. jejuni*, motility is associated with pathogenesis, and colonization of the gastro-intestinal tract depends on FlaA expression (Wassenaar *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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**REFERENCES**


Delany, I., Spohn, G., Rappuoli, R. & Scarlato, V. (2002). In vitro selection of high affinity HspR-binding sites within the genome of *Helicobacter pylori*. Gene 283, 63–69.


Lüneberg, E., Glenn-Calvo, E., Hartmann, M., Bar, W. & Frosch, M. (1998). The central, surface-exposed region of the flagellar hook


