Cloning, sequencing and molecular analysis of the *Campylobacter jejuni* *groESL* bicistronic operon

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The *groESL* bicistronic operon from the enteric pathogen *Campylobacter jejuni* was cloned and sequenced. It consists of two ORFs encoding proteins with molecular masses of 9.5 and 57.9 kDa, which showed a high degree of homology to other bacterial GroES and GroEL proteins. Northern blot analysis suggested that the *groESL* operon is transcribed as a bicistronic mRNA, and its steady-state level was markedly increased after temperature upshift. By primer extension assay, one potential transcription start point preceding the *groESL* genes could be demonstrated, and a putative promoter region compatible with both *Escherichia coli* and *C. jejuni* σ^{70} consensus sequences was identified. A conserved inverted repeat, which is believed to be involved in the regulation of the *groESL* genes, was found between the –10 promoter box and the *groES* translation start site. The complete coding region of *groEL* was fused with pET-22b(+) and expressed in *E. coli* as a His₆-tagged recombinant protein (rCjHsp60-His). After purification, the protein was recognized by an anti-HSP60 monoclonal antibody. ELISA and Western immunoblotting experiments showed that IgG and IgA antibody responses against rCjHsp60-His were not significantly increased in sera from 24 patients with sporadic *Campylobacter* infection when compared to sera from 16 healthy controls.

Keywords: *Campylobacter jejuni*, heat shock, *groESL* operon

INTRODUCTION

Campylobacter jejuni, a microaerophilic Gram-negative rod, is now recognized as a leading cause of human gastroenteritis throughout the world (Mishu Allos & Blaser, 1995). The main symptoms provoked by *C. jejuni* infection are watery or bloody diarrhoea, abdominal pain, and fever. Extraintestinal complications include rheumatic manifestations such as reactive ar-

thritis (Peterson, 1994), as well as Guillain-Barré syndrome, an acute inflammatory disorder of the peripheral nervous system and putative autoimmune disorder (Mishu Allos, 1997). It is highly desirable that the pathogenic mechanisms underlying *C. jejuni* infection, and efficient vaccination strategies, are investigated. However, the molecular characterization of genes involved in the pathogenicity and immunogenicity of *C. jejuni* is still in its infancy, possibly due to difficulties encountered in cloning of *Campylobacter* DNA (Taylor, 1992).

A variety of environmental stimuli are known to trigger the synthesis of a set of highly conserved proteins, named stress or heat-shock proteins (HSPs). The most intensively studied HSPs are those of the 60 kDa (GroEL) and 70 kDa (DnaK) families, which, according to their major function, are designated molecular chaperones. In cooperation with the 10 kDa GroES protein, GroEL prevents misfolding and aggregation of

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Abbreviations: HSP, heat-shock protein; IR, inverted repeat.

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partially denatured proteins through an ATP-dependent process. The GroES–GroEL complex is also involved in oligomer assembly, phage morphogenesis, translocation of proteins, and general protease activity (summarized by Zeilstra-Ryalls *et al.*, 1991; Craig *et al.*, 1993). In most bacteria studied so far, the genes encoding GroES and GroEL homologues are transcribed as a bicistronic operon, and transcription is rapidly elevated when cells are shifted to high temperatures. However, the regulatory mechanisms involved in the heat-shock response seem to differ between bacterial species. In *Escherichia coli*, heat induction is mediated by the alternative sigma factor σ^{32} , allowing for the coordinate expression of genes that belong to the so-called σ^{32} regulon (Bukau, 1993). In most Gram-positive bacteria, e.g. *Bacillus subtilis*, as well as in some Gram-negative species, regulation of *groESL* expression seems to be completely different from that in *E. coli*. The class I heat-shock genes (*groE* and *dnaK*) from *B. subtilis* are preceded by an inverted repeat (IR), which is believed to function as a *cis*-acting negative control element (Hecker *et al.*, 1996). This IR has been found in numerous phylogenetically distant bacteria (Segal & Ron, 1996), and growing evidence indicates that it interacts with a suppressor encoded by the *hrcA* gene (Yuan & Wong, 1995; Schulz & Schumann, 1996).

In vitro exposure of *C. jejuni* to heat or an alkaline pH shock results in induction of several proteins, including GroEL and GroES HSPs (Wu *et al.*, 1994). In another recent report, it was shown that synthesis of a *C. jejuni* GroEL homologue was increased during growth in an aerobic environment. Small amounts of GroEL were observed in culture supernatants, possibly indicating that *C. jejuni* GroEL is, at least partially, secreted (Takata *et al.*, 1995). Extracytoplasmic location of GroEL homologues has been reported for some bacterial species and may play a role in pathogenesis (Garduño *et al.*, 1998; Scorpio *et al.*, 1994; Engraber & Loos, 1992). HSPs of many pathogenic micro-organisms were initially investigated because of their immunodominant properties (reviewed by Kaufmann & Schoel, 1994). In *C. jejuni*, stress-inducible GroEL- and GroES-like proteins have been shown to be targets of serum IgG and mucosal IgA during *Campylobacter* infection in the rabbit model (Wu *et al.*, 1994). Secretory IgA confers protective immunity against *C. jejuni* infections in this animal (Konkel & Cieplak, 1996), thus suggesting a possible role for *Campylobacter* HSPs in host immunity.

We are interested in the heat-shock response of *C. jejuni* and in the role of its HSPs as putative immune targets in infectious and autoimmune diseases. As part of this study, we cloned and sequenced the genes encoding the GroES and GroEL homologues from *C. jejuni*. Furthermore, we analysed the transcription of the *groESL* bicistronic operon under heat-shock conditions. In order to elucidate the role of the GroEL HSP in human *C. jejuni* infection, the GroEL protein was expressed in *E. coli*, and the antibody response of patients with sporadic *C. jejuni* infections against the purified proteins was studied.

METHODS

Bacterial strains and growth conditions. *C. jejuni* (clinical isolate, Lior serotype 11) was grown on agar plates for 18 h at 37 °C in a microaerophilic atmosphere (5% O₂, 10% CO₂). For heat-shock experiments, *C. jejuni* cells were harvested and subcultured into brain heart broth supplemented with 1% yeast extract (BHIYE) (Wu *et al.*, 1994). Thereafter, bacteria were shifted from 37 °C to 48 °C for 5–60 min. *E. coli* strains were grown at 37 °C on LB agar or in LB broth supplemented with ampicillin at a final concentration of 50 µg ml⁻¹, if required.

DNA techniques. Genomic DNA of *C. jejuni* was isolated as described by Wilson (1989). Plasmid DNA was purified using the QIAprep plasmid kit (Qiagen). DNA fragments were purified from agarose gels using QIAquick gel extraction kit (Qiagen). ³²P-labelling of DNA fragments was performed by random priming (Feinberg & Vogelstein, 1983). PCR products were ligated with *Srf*I-predigested pCR-Script SK(+) cloning vector (Stratagene) and transformed into *E. coli* XL-1 Blue MRF' Kan^r supercompetent cells (Stratagene).

Nucleotide sequencing. The DNA sequences of inserts of recombinant plasmids were determined in both strands with internal primers. The ABI Prism dye terminator cycle sequencing kit was used to generate fluorescence-based dideoxy-sequence reactions. DNA sequencing was performed on an ABI model 373A automated sequencer. Nucleic acid and predicted amino acid sequence data were analysed by the DNASIS software package (Hitachi).

Amplification of a gene fragment by PCR. Genomic DNA of *C. jejuni* (100 ng) was incubated with degenerate oligonucleotide primers 5' GC(A/T)AA(A/G)GA(A/G)-ATITT-(C/T)TC(A/C/G/T)GA 3' (Dg1) and 5' TCTTT(A/C/G/T)ACIA(A/G/T)(C/T)TGIGC-(A/C/G/T)CCCAT 3' (DgRv). DgRv was synthesized according to a highly conserved region of bacterial GroEL homologues (corresponding to aa 68–75 of *E. coli* GroEL), and Dg1 was derived from the published N-terminal sequence of a putative GroEL homologue of *C. jejuni* (Wu *et al.*, 1994; Takata *et al.*, 1995). PCR was conducted using a Perkin-Elmer GeneAmp 9600 PCR system with the following cycle parameters: 94 °C for 30 s, 52 °C (42 °C during the first five cycles) for 30 s, 72 °C for 1 min, for 40 cycles.

Southern blot analysis. Genomic DNA of *C. jejuni* was digested with *Eco*RI, *Pst*I, *Xba*I, *Nco*I, *Bam*HI, *Sac*I, *Sal*I, *Xho*I or *Bgl*II (New England Biolabs). The DNA was separated by agarose gel electrophoresis and transferred to nylon membranes (Hybond-N, Amersham) using standard techniques (Sambrook *et al.*, 1989). Membranes were hybridized with ³²P-labelled DNA fragments in 6 × SSC, 0.5% SDS, 50% formamide at 42 °C for 16 h. After high-stringency washing (0.2 × SSC, 0.1% SDS at 65 °C for 2 × 15 min), filters were autoradiographed at –80 °C for 16 h.

To screen for a second *groES* gene, a probe (pb2) was obtained by PCR. A 196 bp DNA product internal to the *C. jejuni* *cpn10* gene was amplified by means of oligonucleotide primers 5' TCAACCTTTAGGAAAGCGTGT 3' and 5' AAGTTTAA-TTTCTGTTCACCG 3', and ³²P-labelled. Low-stringency hybridization was performed in 6 × SSC, 0.5% SDS, 30% formamide at 42 °C, followed by washing under relaxed salt and temperature conditions (0.6 × SSC, 0.1% SDS at 50 °C for 2 × 15 min).

Colony screening. A *C. jejuni* plasmid library was constructed by ligating *Bgl*II-digested genomic DNA of *C. jejuni* with

plasmid vector pET-22b(+) (Novagen) and transforming *E. coli* XL-2 Blue MRF' ultracompetent cells (Stratagene). About 10^4 colonies were plated and replicated onto Hybond-N membranes following standard procedures (Sambrook *et al.*, 1989). Hybridization with the 32 P-labelled insert of pCRcpn1 (pb1), high-stringency washing and autoradiography were performed as described above.

Randomly primed PCR. In order to obtain the entire *groEL* gene, a semi-nested PCR was performed using two sequence-specific oligonucleotides oriented towards the unknown 3' end of the *groEL* gene and a random primer (RP) as reverse primer, respectively (method modified from Struck & Collins, 1994). For outer PCR with primer pair 5' CTGATGCTATGGAA-AAAGTGG 3'/RP and *C. jejuni* genomic DNA as template, the cycling conditions were as follows: 94 °C for 45 s, 48 °C for 60 s (42 °C for the first five cycles), 72 °C for 120 s, for 20 cycles. One microlitre of the outer reaction was used as template for inner PCR with primer pair 5' CGCGGTGT-TCTTAATATTCA 3'/RP under the following conditions: 94 °C for 30 s, 52 °C for 30 s, 72 °C for 120 s, for 25 cycles. Specific primers correspond to nt 1141–1161 and nt 1455–1475, respectively, of the *C. jejuni groE* nucleotide sequence described in this work. A 1.3 kb PCR product was amplified using random primer 5' ATACTTCAGTGAGACACAGAGAAA 3'. The amplification product was gel-purified, cloned, and sequenced.

RNA isolation and analysis. Total RNA was isolated from *C. jejuni* cells using TRIzol reagent (Gibco-BRL) according to the manufacturer's instructions. DNA contaminations were removed by DNase I treatment. For each sample, 10 µg total RNA was electrophoresed on denaturing 1.2% agarose/formaldehyde gels and transferred onto a nylon membrane by capillary blotting. Northern blots were subjected to hybridization with 32 P-labelled fragments internal to *C. jejuni groES* and *groEL*, respectively. A 0.24–9.5 kb RNA ladder (Gibco-BRL) was used as a size marker.

Primer extension mapping. For primer extension mapping of the transcription start site of the *C. jejuni groESL* operon, oligonucleotide 5' AGGCTGTTGTTTAGTTTCTTC 3' was used, which is complementary to nucleotides +46 to +67 relative to the *groES* start codon. The oligonucleotide was end-labelled with [γ - 32 P]ATP according to standard techniques (Sambrook *et al.*, 1989) and hybridized to 10 µg total RNA, isolated from *C. jejuni* cells heat-shocked for 30 min. The annealed primer was extended at 42 °C for 30 min using 25 U avian murine virus reverse transcriptase (Promega). The newly synthesized DNA was ethanol-precipitated, resuspended in 4 µl formamide loading buffer, and loaded onto a 8% sequencing gel together with the corresponding sequencing reaction of the upstream flanking region of the *groES* gene (T7 sequencing kit, Pharmacia).

Protein expression and purification. The coding region of the *C. jejuni groEL* gene was amplified by means of oligonucleotide primers 5' AAAAAAGGATAAACCATGGCAA-AAGAAATT 3' (*Nco*I site underlined) and 5' ATCATT-CCTCCCATGCCACC 3' using *C. jejuni* genomic DNA as template. The amplification conditions were as follows: 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 100 s, for 32 cycles, with 1 U Vent polymerase (New England Biolabs) per 100 µl reaction volume. The resulting PCR product was digested with *Nco*I and ligated with the prokaryotic expression plasmid pET-22b(+), which had been *Xho*I digested, blunt-ended with mung bean nuclease, and *Nco*I digested. The correct nucleotide sequence was confirmed by sequencing the insert. In order to cleave the pelB leader present upstream of the start

codon, the recombinant plasmid was *Nde*I/*Msc*I digested, blunt-ended with mung bean nuclease, and religated. Both plasmids were transformed into *E. coli* BL21(DE3) host cells (Novagen). Large-scale expression and non-denaturing purification by nickel/nitrilotriacetic (Ni-NTA; Qiagen) metal-affinity chromatography was performed as proposed by the manufacturer. Further purification was achieved by gel filtration and anion-exchange chromatography as described for the purification of the *E. coli* GroEL protein (Buchner *et al.*, 1991). The N-terminal amino acid sequence of the purified protein was determined by Edman degradation using an ABI 473A amino acid analyser.

Western blotting and ELISA. To obtain a crude *C. jejuni* protein extract, bacterial cells were harvested from agar plates, washed twice with PBS pH 7.4, and resuspended in 50 mM sodium phosphate pH 7.8, 300 mM NaCl. After repeated sonication, protein concentration was determined. For Western blot experiments, 25 µg protein lysate or 1 µg of the recombinant protein was used. SDS-PAGE was carried out on 15% polyacrylamide gels, from which proteins were transferred to nitrocellulose membranes (Towbin *et al.*, 1979). Blots were incubated either with human sera (diluted 1:200) or with a monoclonal antibody recognizing an epitope common to most prokaryotic and eukaryotic HSP60 proteins (clone LK-2; Stress Gen Biotechnologies). Bound antibodies were detected by peroxidase-conjugated second antibody (anti-IgG). For ELISA, microtitre plates were coated with 1 µg recombinant protein ml⁻¹. After blocking, plates were incubated with human sera in serial dilutions. Bound immunoglobulins were detected by incubation with peroxidase-coupled anti-human IgG and IgA antibodies.

RESULTS

Molecular cloning of the *groESL* bicistronic operon

Degenerate oligonucleotide primers based on the published N-terminal amino acid sequence of a putative *C. jejuni* GroEL homologue and on a highly conserved region of bacterial GroEL-like proteins, respectively, were used in a PCR. A single, 221 bp DNA fragment was amplified, cloned (then designated pCRcpn1), and sequenced. As confirmed by database search, the nucleotide sequence of the PCR product was similar to those of bacterial *groEL* genes. Southern blot analysis revealed a *Bgl*II-digested 3 kb fragment hybridizing to the radio-labelled PCR product. Therefore, a plasmid library was constructed by *Bgl*II digestion of *C. jejuni* genomic DNA

Fig. 1. Linear restriction maps of plasmids pETcpn4 (3.0 kb) and pCRcpn2 (1.3 kb). Only the inserts are depicted. Boxes indicate the *groES* and *groEL* genes described in this study. Black bars correspond to probes used in hybridization experiments.

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1
aatctgccatatttgcgatatttgccttgaattcaagcgttgaacacgttaaattaaatgcgtgatattacatctaactccgcactttctttgatcttttttgcgctatatttcca
121
ctatattattaccaaaacttgtgaaagtgatggtataaatagcaatcaaaagcacaacacaaaagctataactccataaaaaatctttttcattttatccttttagttttattataaa

241
ataacttttagtctataaaactaaactttataaatatttttaatttaagttatgactaaattcattttttatagtagatattacacttaaataaaagagtgctaaaatcaatatttt
-35 -10 >>> >>>>> <<<<<< <<<
*****
361
groES →
taaggatggacaaaaatgaattttcaaccttttaggaaagcgtgttctagtttaaacgcgtagaagaaactaaaacaacagcctcaggcataattataccagataatgctaaagaaagcct
M N F Q P L G K R V L V K R V E E T K T T A S G I I I P D N A K E K P

481
ttaatgggtgaagtagtagcagtaagtaagaatcactgatattgcaaatgggtgataaaatcgtgtttgctaaatcaggtggaacagaaattaaacttgataataatgaatatttagtt
L M G E V V A V S K E I T D I A N G D K I V F A K Y G G T E I K L D N N E Y L V

601
ttaaatttagatgatattcttaggaatttttaaaataatttataaaaaaggtataaaaaatggcacaagaaattattttttcagatgaagcaagaataaaactttatgaggcggttaaaaaac
L N L D D I L G I L K * M A K E I I F S D E A R N K L Y E G V K K

721
ttaatgacgcgtgtaaaagtaactatggggccagaggacgcaatgttttaatacaaaaaagcctttgtgtcctcaagcattactaaagatggcgtaaggtgttgctaaagatagagctta
L N D A V K V T M G P R G R N V L I Q K S F G A P S I T K D G V S V A K E V E L

841
aagatagctcttgaataatgggtgtcttactcgtgaagagaagtagcgagtaaaacagcgtgatcaagcagcgatggaacaactactgcaacgggttttagctcatgcaattttcaagaag
K D S L E N M G A S L V R E V A S K T A D Q A G D G T T T A T V L A H A I F K E

961
gtttaagaatatcacagcaggtgcaaatcctatcgaggtataaaacgcgtgttgataaaagcttgcaagctatagtagcagaacttaaaaaactttctcgcgaagtaaaagataaaaaag
G L R N I T A G A N P I E V K R G M D K A C E A I V A E L K K L D R E V K D K K

1081
aaattgcacaagtgctacaatctcagccaactctgatgaaaaaatcggaatttaacgctgtatgctatggaagaaagtggtgttatcactgttgtaagagccaaaatcaa
E I A Q V A T I S A N S D E K I G N L I A D A M E K V G K D G V I T V E E P K S

1201
tcaatgatgaattaaatgtatgtgaaggtatgcaattttgacagaggttatttaagccctatttttatcactaatgcagaaaaatgacagtagagctttcaagcccttatatcctgcttt
I N D E L N V V E G M Q F D R G Y L S P Y F I T N A E K M T V E L S S P Y I L L

1321
ttgataaaaaaattacaaaatttaaaagatttattaccggttttagaacaattcaaaaaacaggcaaacacttttaattatcgctgaagatattgaaggtgaagcgcttgcaactttgg
F D K K I T N L K D L L P V L E Q I Q K T G K P L L I I A E D I E G E A L A T L

1441
ttgtaataaaacttcgcggtgttcttaattttcagcagtgaaagctccaggttttggcgatagaagaaaagctatgctgaagatatagcgatttttaacaggtggagaagtgattctg
V V N K L R G V L N I S A V K A P G F D R R K A M L E D I A I L T G G E V I S

1561
aagaacttggaagaactcttgaagtgcgactatacaagatcttggacaagctcttagtgtaactcgataaagacaatacaaccatagtaaatgtgtgcaggcgaaaaagcgaataattg
E E L G R T L E S A T I Q D L G Q A S S V I I D K D N T T I V N G A G E K A N I

1681
atgcgagagtcacatcaaatcaaaagcacaattgctgaacaaacttcagattatgacagagaaaaattacaagaagacttgcaaaattagtggtgtgtgttcagttattaaagtaggtg
D A R V N Q I K A Q I A E T T S D Y D R E K L Q E R L A K L S G G V A V I K V G

1801
caacaactgaaactgaaatgaaagaaaaagatcggttgacgatgctttaagcgctactaaagcagcagttgaagaaggtatagtaacgtgtgtgtgtgcagcgttatcaaaagcaa
A T T E T E M K E K K D R V D D A L S A T K A A V E E G I V I G G G A A L I K A

1921
aagctaaaaatcaaaacttgatctacaggtgatgaagcaattggcgacgatctgttgaaagagctttaagagcacctttaagacaattgctgaaaatgcaggattttgatgcaggtgtgg
K A K I K L D L Q G D E A I G A A I V E R A L R A P L R Q I A E N A G F D A G V

2041
ttgtaaatagcgtagaataatgctaaagatgaaacacaggtatttgatgctgcaaaaggtgaatatgttaatatgctgaaagtggaattatcgatcctgttaaagtagaaagtagcttt
V V N S V E N A K D E N T G F D A A K G E Y V N M L E S G I I D P V K V E R V A

2161
tactcaatgcagtttctgtagctagtatgcttttaaccacagaagcaacaattagtgaaattaaagaagataaacctactatgccagatatagcggtatgggaggaatgggtggcatgg
L L N A V S V A S M L L T T E A T I S E I K E D K P T M D P M S G M G G M G M G M

2281
>>>>>>> <<<<<<<<
gaggaatgatgtaatatctgcttaacctcttatcagaaggattttctctgctgattaccaatttaatttaaaaacactcccttgattttctacagattcgcaataatcttaatatca
G G M M *

2401
ttatcatcacaaactttttttaccaaagaagccctataccaaatcctccttgatcggtattaaatcttgatatctatcaaaaatgatttaaatgtgactttgaaattccacaacct
2521
gtatccgcgaataacttaaaaaattggctttcaaaattatagaattactccacctttttta

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Fig. 2. Nucleotide sequence of the *C. jejuni* *groESL* bicistronic operon. The deduced amino acid sequence is shown below the DNA sequence in one-letter code. The transcription start site is marked by a vertical arrow below the DNA sequence. Putative promoter sites similar to *E. coli* σ^{70} consensus sequence are underlined; those similar to *C. jejuni* σ^{70} consensus sequence are marked by asterisks. A potential 'UP' regulatory element is boxed. Ribosome-binding sites are double-underlined. Putative stem-loop structures are marked by converging arrows above the sequence.

and cloning the fragments into pET-22b(+). Colony blotting identified a positive transformant containing a 3 kb insert within its plasmid (further named pETcpn4; Fig. 1). A database homology search based on nucleotide sequence analysis of the cloned fragment showed that the entire *groES* gene and the 5' end of the *groEL* gene were present in pETcpn4. To obtain the missing 3' end, a 1.3 kb DNA product was amplified by randomly primed PCR. Cloning and sequencing revealed perfect

identity with the nucleotide sequence of pETcpn4 within the 148 bp overlapping region.

Nucleotide sequence of the *groESL* bicistronic operon

Sequence analysis of the relevant region of pETcpn4 and of the cloned 1.3 kb PCR product (further named pCRcpn2; Fig. 1) revealed the presence of two contiguous ORFs (Fig. 2). The first ORF (ORF1) is 258 bp

Cj	MNFQPLGKRVLVKRVETKTASGIIIPDNAKEKPLMGVEVVAV-----SKEI--TD---IANGDKIVFAK-YGGTEIKLDNNEYLVNLDDIL	
Hp	.K....E...E.L.ENK.S.....V.K.....HK.--SEGCKCVKE.V.A.G.-.K.A..V..GV..M..E.E...	
Ca	.KIR...D..VI..L.AEE..K...VL.SS.....Q.A.V...GPGGVVDG-...QMQ---VKT...VF.S.-.S....V..E.L.I.RQ...	
Ba	MADIK.R..HD..V.R...SEAK..G.....T....QE....AGAGARDEAG.LV-PL----VKA..RVL.G.-WS...V.IGGEDL.IMKES...	
Ec	..IR..HD..I...K.VETKS.G..VLTGS.AA.STR...L..GNRILEN-G.VKPL.----VKV..IVI.NDG..VKSE.I..E.V.IMSES...	
	** ** * ** * ** *	
Cj	GILK	<i>C. jejuni</i>
Hp	..VGSGSCCHTGNHDKHKAKEHEACCHDHKKH	<i>H. pylori</i> (54.4 %)
Ca	..VEE	<i>C. acetobutylicum</i> (53.3 %)
Ba	..VG	<i>B. abortus</i> (46.2 %)
Ec	A.VEA	<i>E. coli</i> (37.2 %)
	*	
		} GroES
Cj	M-AKEIIFSDEARNKLYEGVKKLNDVAVKVTMGPRGRNVLIQKSFAGPSITKDGVSVAKEVELKDSLENMGASLVREVASKTDQAGDGTITATVLAHAIF	
Hp	-.K...S...L.F...RQ.H.....Y.....SCPVA...Q..K.D.....A.....YS..	
Ca	..Q.LYGE...RSMQK..D..A.T...L..K...VLD.K...L..N...I...I..E.PY...Q..K...T..N.V.....L..Q..I	
Ba	.A.DVK.GRT..E.MLR..DI.A...L..K...V.E...R.....E.KF...QML...N.T.....GQ..V	
Ec	.A..DVK.GND..V.MLR..NV.A...L..K...VLD...T.....R.I..E.KF...QM.K...AN.A.....Q..I	
	* * * *	
Cj	KEGLRNITAGANPIEVKRGMDKACEAIVAEKLSREVKDKKEIAQVATISANSDEKIGNLIADAMEKVGKDGVTITVEEPKSIINDELNVVEGMQFDRGYL	
Hp	P...IN...A.KK.GG.E..T.....HN..K.....A.G.E...D.....	
Ca	R...K.V...MLIRN.IRL.VDKT.EG...V.KN.NG.ED..R..S...-A.PE..K.....NE.....S..MGT..D.....	
Ba	Q..AKAVA..M..MDL..I..L.VNEV...L.KAKKINTSE.V...G...AEAE..KM..E..Q...NE.....A.TAET..E.....	
Ec	T...KAVA..M..MDL..I...VT.A.E...A..VPCS.S.A...G...TV.K...E..D...E.....DGTGLQ..D.....	
	** ** * * * * * * * * * * * * * * * * *	
Cj	SPYFITNAEKMTVELSSPYILLFDDKITNLKDLLPVLEQIQKTGKPLIIAEDIEGEALATLVVKNLGRVNLISAVKAPGFGDRRKAMLEDIAILTGEV	
Hp	...V...AQ.DNA...T...SSM..I..L..KTM.E.....T.....A.....E..K...V...Q..	
Ca	..MV.DQ...EAV.DD...IT...A.IQEI..L...VQQ..K...D.V.....TF.CV.....D..R.....	
Ba	...V..P...VAD.EDA...HE..LS..QA...AVVQ.S...V.....G.K.A.....Q..	
Ec	...NKP.TGA...E..F...A...S.IREM...LEAVA.A...V...A...TI..IVKVA...Q...T...T..	
	*** * *	
Cj	ISEELGRTLESATIQDLGQASSVIIDKNTTIVNGAGEKANIDARVNQIKAQIAETTSYDREKLQERLAKLSGGVAVIKVGATTETEMKEKKDRVDDAL	
Hp	LS..N.EVEF..K.-KIV.....D.K.HSHDVKD..A..T...S.....K.....AS.V.....	
Ca	KD.KDVKE...S.E..K.S.E.....R.D.SA.HD..A..RG..E...F.....A...V...AS...L..R.M.IE...	
Ba	...D..IK...V.LDM..R.KK.S.S.E...D...Q..E...G...Q..E.....A.....R..GA..V.V.....	
Ec	...I.ME..K..LE...KR.V.N..T...ID.V..E.A.QG..A..RQ..E.A.....V...A...A.V...A..E...	
	*** * * *	
Cj	SATKAAVEEGIVIGGGAALIKAKAKIK-LDIQG-DEAIGAAIVERALRAPLRQIAENAGFDAGVVVNSVENAKDENTGFDAAKGEYVNMLESIGIIDPVKV	
Hp	R.AQ.VH-.N.HD-.KV.YE.IM..IK..A...I...Y.G...E..K-HEGHE..N.SN.K..D.FKE...L..	
Ca	A.....IA...T.Y.NVLPEVRE.TSDEP.VQV.IN.IVK..EE.V...A...LEGS.IIEKII.S-EKGI...LHEK..D..SV..V..T..	
Ba	N..R.....A...T..LR.ST..TAKGVNA-.QEA.IN..R..IQ..A...TT...EE.S.I.GKILENTS.TF.YNT.N...GDILSL..V....	
Ec	H..R.....V.A...V...RVAS.LAD.RGQNE.QNV.IKVAL..ME...VL.C.EEPS..A.T.KGG-.G.Y.YN..TE..G..IDM..L..T..	
	** ***** * * * * * * * *	
Cj	ERVALLNAVSVASMLLTTEATISEI-KE-DK--PTMPDM-SGMGGMGMGMGM	<i>C. jejuni</i>
Hp	..I..Q...S.L...VH...-E.AA.A...-G.....	<i>H. pylori</i> (75.6 %)
Ca	T.S..Q..A...TF...CAVAD.-P.K...-E..GG-APGM...Y	<i>C. acetobutylicum</i> (61.5 %)
Ba	V.T..Q..A...GL.I...M.A.L-PK-KD--AAPAG.PG.....DF	<i>B. abortus</i> (64.1 %)
Ec	T.S..QY.A...GLMI...CMVTDLP.N-.A--ADLGAA-G.....	<i>E. coli</i> (62.9 %)
	* *	
		} GroEL

Fig. 3. Comparison of GroES and GroEL proteins from *C. jejuni* with those from other bacterial species. The derived amino acid sequences from *C. jejuni* are aligned with those from *H. pylori* (Hp), *C. acetobutylicum* (Ca), *B. abortus* (Ba), and *E. coli* (Ec). Amino acid residues identical to those of *C. jejuni* proteins are marked by dots. Positions identical in all five bacterial species are marked by asterisks. Overall identity is given after the species name. Gaps are introduced to maximize similarity.

long and encodes a polypeptide of 86 aa with a predicted molecular mass of 9.5 kDa and a calculated pI of 5.2. This polypeptide (further called *C. jejuni* GroES) exhibits considerable homology to GroES-like proteins of other bacterial species. In particular, overall identity to GroES homologues of *Helicobacter pylori* (Suerbaum et

al., 1994), *Clostridium acetobutylicum* (Narberhaus & Bahl, 1992), *Brucella abortus* (Gor & Mayfield, 1992), and *E. coli* (Hemmingsen et al., 1988) was calculated as 54.4%, 53.3%, 46.2%, and 37.2%, respectively (Fig. 3). By comparing the N-terminal sequences of GroES and of a putative *C. jejuni* GroES homologue described by Wu

Fig. 4. Mapping of the *groESL* transcription start point. Primer extension products were generated by reverse transcription of total RNA from *Campylobacter* cells heat-shocked for 30 min. Lanes G, A, T and C show the dideoxy-sequencing ladder obtained with the same primer as that used for primer extension. Lane 1, primer extension reaction. Lane 2, control reaction (no RNA).

et al. (1994) we found an exchange of 5 of 18 amino acids. Therefore we proceeded to look for the presence of a second *groES* gene. Hybridization experiments under stringent or relaxed conditions, in which a 196 bp PCR product internal to the *groES* gene (pb2) was used as a probe, did not give evidence for an additional *groES* gene (data not shown).

The start codon AUG of the second ORF (ORF2) is located 20 bp downstream of the stop codon (TAA) of ORF1. ORF2 is 1635 bp long and encodes a polypeptide of 545 aa with a predicted molecular mass of 57.9 kDa and a pI of 4.9. The deduced amino acid residues were aligned with the complete sequences of GroEL homologues of *H. pylori* (75.6% identity), *B. abortus* (64.1% identity), *E. coli* (62.9% identity), and *C. acetobutylicum* (61.5% identity) (Fig. 3). As seen in many bacterial species, the C-terminus consists of three tandem repeats of the Gly-Gly-Met motif (McLennan *et al.*, 1993). The first 37 predicted amino acid residues of GroEL agree perfectly with the N-terminal sequence of a putative GroEL homologue of *C. jejuni* (Takata *et al.*, 1995). *groEL* appears to be a single-copy gene, because only one band was observed when pb1 was hybridized to genomic DNA digested with the restriction endonucleases mentioned in Methods (data not shown).

Features of the noncoding regions

Both ORF1 and ORF2 are preceded by putative ribosome-binding sites (AAGGAT), 8 and 5 nt upstream of the AUG start codon, respectively. Primer extension analysis identified a presumptive transcription start site (S) corresponding to an A residue located 49 nt upstream of the translation start codon of the *groES* gene (Fig. 4).

Fig. 5. Homology of the putative *groESL* promoter to both the *E. coli* (Hawley & McClure, 1983) and the *C. jejuni* (Wösten *et al.*, 1998) σ^{70} consensus sequence. Identical nucleotide residues are marked by vertical lines. The transcription start site is marked by an arrow.

A putative promoter upstream of the transcription start point is identical at 10 of 12 positions with the *E. coli* σ^{70} promoter consensus sequence (Fig. 5), including the preferred spacing of 17 bp (Hawley & McClure, 1983). In addition, regions very similar to the *C. jejuni* σ^{70} promoter (Wösten *et al.*, 1998) or resembling the *E. coli* σ^{32} consensus sequence (Cowing *et al.*, 1985) were found in the *groES* upstream flanking region (see Discussion). A very A+T-rich sequence between positions -40 and -60 might act as an 'UP' element additionally regulating promoter activity (DeHaseeth *et al.*, 1998; Estrem *et al.*, 1998). Furthermore, a sequence of dyad symmetry surrounding S was identified. Similar putative stem-loop structures (designated CIRCE elements, for controlling inverted repeat of chaperone expression) have been described in the 5' noncoding regions of many bacterial *groE* and *dnaK* genes (Zuber & Schumann, 1994; Hecker *et al.*, 1996). The *C. jejuni* sequence differs from the CIRCE consensus sequence (5' TTAGCACTC-N₉-GAGTGCTAA 3') in that it exhibits a G $\rightarrow$ T mismatch at the fourth position on the 5' branch of the hairpin. A second, 23 bp stem-loop structure 19 bp downstream of the *groEL* stop codon might function as a rho-independent transcription termination signal (Rosenberg & Court, 1979).

Transcription of the *groESL* bicistronic operon

The *in vivo* transcripts of the *groESL* bicistronic operon were detected by Northern blot analysis. Total RNA was isolated from *C. jejuni* cells grown for 18 h at 37 °C, subcultured to BHIYE broth, and subsequently heat-shocked for 5–60 min at 48 °C. A 32 P-labelled 0.5 kb *Bgl*II-*Nru*I fragment (pb3) internal to the *groEL* gene hybridized to a single, 2.0 kb transcript that did not change in size under different temperature conditions (Fig. 6). *groESL* mRNA was expressed at a low level in *C. jejuni* cells grown at 37 °C under microaerophilic conditions. Confirming the Northern blot results, immunoblotting revealed that lysates of these non-induced bacterial cells contained a protein with an apparent molecular mass of approximately 62 kDa that was recognized by an anti-HSP60 monoclonal antibody (LK-2) (data not shown). The 32 P-labelled PCR product (pb2) internal to *groES* (see above) also hybridized to the 2.0 kb mRNA species, thereby suggesting that *groES* and *groEL* form one operon. After shifting bacterial cells to 48 °C, there was a rapid increase in mRNA levels, which reached its maximum after 20–30 min (Fig. 6). Bacterial cells which were incubated for 60 min at

Fig. 6. Northern blot analysis. RNA was isolated from *C. jejuni* cells incubated for 5 min (3), 10 min (4), 15 min (5), 20 min (6), 30 min (7) and 60 min (8) at 48 °C. 1, RNA from *C. jejuni* grown under microaerophilic conditions at 37 °C; 2, RNA from *C. jejuni* cells incubated for 60 min at 37 °C under aerobic conditions in BHIYE. Filters were hybridized with a ³²P-labelled probe internal to *groEL* (pb3).

Fig. 7. Expression, purification and antigenicity of *Campylobacter* GroEL (rCjHsp60-His). Materials were electrophoresed in a 15% polyacrylamide gel and stained with Coomassie blue. Lanes: A, preinduction *E. coli* lysate; B, *E. coli* lysate 5 h after induction with 1 mM IPTG; C, eluates from Ni²⁺-chelating column; D, rCjHsp60-His after gel filtration and anion-exchange chromatography; E, purified rCjHsp60-His transferred to nitrocellulose and processed for Western blot analysis using an anti-HSP60 monoclonal antibody as described in Methods. Sizes of protein standards (LMW marker, Pharmacia) are shown to the left.

Expression and purification of recombinant *C. jejuni* GroEL

In order to express GroEL in a prokaryotic system, the entire coding region of the *groEL* gene was amplified and fused with the expression vector pET-22b(+). Recombinant His₆-Hsp60 proteins with and without pelB leader were expressed in *E. coli*, but as both were located in the cytoplasm, only the protein without pelB leader (designated rCjHsp60-His) was further used in this study. Purification of rCjHsp60-His to near homogeneity was achieved by metal chelate chromatography, followed by gel filtration and anion-exchange chromatography. SDS-PAGE analysis and Coomassie blue staining revealed a single band with an apparent molecular mass of about 62 kDa (Fig. 7). The N-terminal amino acid residues of the purified rCjHsp60-His were determined as AKELIFSDEA by Edman degradation and agreed perfectly with the predicted amino acid sequence (for comparison, the corresponding region of the GroEL protein of the expression host *E. coli* is AKDVKFGNDA). As was shown by Western blot analysis, rCjCpn60-His exhibited marked immunoreactivity to an anti-HSP60 monoclonal antibody (LK-2), thereby further verifying that the expressed protein is a GroEL homologue (Fig. 7, lane E).

It has been shown that a GroEL-like protein of *C. jejuni* is a B-cell antigen in the rabbit model of *Campylobacter* infection (Wu *et al.*, 1994). In this study, our aim was to investigate whether this HSP exerts similar immunological activity in humans with sporadic *C. jejuni* infections. The purified rCjCpn60-His was tested against sera from *C. jejuni*-infected patients using ELISA and Western immunoblotting. However, in ELISA experiments, the serum IgG response of 24 *C. jejuni*-infected patients (ELISA absorbance value 0.323 ± 0.267 ; mean $\pm$ SD) was not significantly increased compared to the sera of 16 healthy blood donors (absorbance 0.180 ± 0.137) (Mann-Whitney U-test). Similarly, in the serum IgA response, there was no significant difference between *C. jejuni*-infected individuals (absorbance 0.105 ± 0.106) and healthy controls (absorbance 0.058 ± 0.039). Immunoblot analysis of human IgG against rCjCpn60-His revealed that the recombinant protein was immunogenic only for a minority of *C. jejuni*-infected patients (5 of 24) as well as of healthy controls (3 of 16) (data not shown).

DISCUSSION

In this study, we describe the cloning and molecular characterization of the *groESL* bicistronic operon from *C. jejuni* and the recombinant expression of the GroEL protein. The organization of the *groESL* genomic region of *C. jejuni* was found to be similar to that of most bacterial species studied. The deduced amino acid sequences of the GroES and GroEL proteins exhibited a high degree of overall identity with homologous bacterial HSPs. Comparison with the *H. pylori* HspA and HspB proteins revealed the most striking homology (54.4% and 75.6% identity, respectively), reflecting the

37 °C in BHIYE exhibited about a two- to threefold increase in *groE* mRNA levels compared to cells grown under microaerophilic conditions.

close phylogenetic relationship of the two species (Vandamme & de Ley, 1991; Vandamme *et al.*, 1991). However, whereas the *H. pylori* HspA protein possesses additional C-terminal amino acid residues involved in nickel binding (Suerbaum *et al.*, 1994), no such motif could be found in the *C. jejuni* GroES protein.

The deduced N-terminal amino acid residues of GroEL agreed perfectly with published N-terminal sequences of a putative *C. jejuni* GroEL homologue, while GroES differed in 5 amino acid residues from the N-terminal sequence of a putative GroES homologue published by Wu *et al.* (1994). As the same authors observed that the GroEL- and GroES-like proteins can function separately in *C. jejuni*, we further investigated whether there is a second *groES* gene independent of the *groE* operon. Southern and Northern analyses, using a PCR product internal to *groES* as a probe, did not give evidence for a second *groES* gene. However, formally, the existence of a second *groES* gene differing considerably from the *groES* sequence described in this paper cannot be ruled out. Another explanation for the incongruity in the GroES sequences would be point mutations in the cloned DNA fragment, but resequencing of *groES* from PCR-amplified genomic DNA confirmed the nucleotide sequence obtained from plasmid clones. Alternatively, as Wu *et al.* (1994) used a *C. jejuni* strain serotyped as Lior 5 for their experiments (whereas in this study we used *C. jejuni* Lior serotype 11), the observed differences in the N-terminal amino acid composition of GroES may represent strain-to-strain variations.

Since *C. jejuni* grows optimally at 42 °C *in vitro*, heat shock was induced by shifting the temperature from 37 to 48 °C. However, Konkel *et al.* (1998) observed no differences in protein synthetic profiles of *Campylobacter* cells shifted from 37 to either 43 or 46 °C. Northern blot analysis indicated that the induction of the *groE* operon is regulated at the level of transcription. Following heat shock, there was a significant increase in the amount of a 2.0 kb mRNA species. We propose that this transcript is initiated at the transcription start point extending from *groES* to the inverted repeat downstream of *groEL*, which would correspond in length to the observed 2.0 kb mRNA. Thus, the *groE* gene seems to be transcribed bicistronically. Northern blot and immunoblot experiments demonstrated that *groE* is constitutively expressed in *C. jejuni* grown at 37 °C under microaerophilic conditions. In addition to heat stress, *groE* mRNA expression is induced in *C. jejuni* cells exposed to an aerobic atmosphere, confirming observations reported by Takata *et al.* (1995). As microaerophilic *Campylobacter* species have been found to be more susceptible to the toxic effects of oxygen and its derivatives than aerobic bacteria (Hoffman *et al.*, 1979), the induction of HSPs may help the bacterial pathogen to survive in an aerobic atmosphere environment outside suitable animal hosts.

Growing evidence suggests that there are two different, but not mutually exclusive, ways of regulating the heat-shock response in bacteria. The *groE* gene of *E. coli* is

under control of σ^{70} and σ^{32} promoters, allowing for constitutive and heat-induced protein expression, respectively (Bukau, 1993). In *C. jejuni*, the transcription start site of the *groE* gene is preceded both by a putative promoter similar to the *E. coli* σ^{70} consensus sequence and by a nucleotide sequence resembling the recently described vegetative promoter of *C. jejuni* (Wösten *et al.*, 1998). Based on 21 different promoter regions analysed, Wösten *et al.* (1998) established a consensus sequence for the *C. jejuni* σ^{70} promoter (TTTAA-GTnTT-N₁₅₋₁₉-TATAATT), which in its -35 region completely differed from that of *E. coli*. Interestingly, the -10 and the -35 boxes of the putative *groE* promoter are compatible with the *C. jejuni* as well as with the *E. coli* consensus sequences, possibly reflecting vegetative and stress-inducible expression of *groE*, respectively. The questions whether the two putative promoters correspond to different sigma factors, and how they are involved in *groE* expression under normal growth and stress conditions, require further analyses. Another sequence (GCACTTTCTTTGAT-N₂₁-CAC-TATATT), located approximately 200 bp upstream of the putative σ^{70} -like promoter, resembles the *E. coli* σ^{32} consensus sequence (TCTCnCCCTTGAA-N₁₃₋₁₇-CC-CATnTA). However, a corresponding transcription start site could not be detected. Furthermore, the idea that *E. coli* σ^{70} -like and σ^{32} -like promoters alternately govern *groE* expression is not supported by Northern hybridization results, since no difference in transcript size was found when comparing RNA from normally grown and heat-shocked bacteria. An element involved in *groE* regulation in Gram-positive and Gram-negative bacteria, a well-conserved inverted repeat (CIRCE element), was found between the putative -10 promoter box and the *groE* translation start site. The transcription start point was mapped within the 5' branch of the CIRCE hairpin. Therefore, the mRNA does not contain the entire stem-loop structure, suggesting that the IR operates at the DNA level. To our knowledge, a similar location of CIRCE in an organism with one *groE* gene has only been identified in *Synechococcus* sp. PCC 7942 (Webb *et al.*, 1990). To date, this is the first CIRCE element found in a member of the *Campylobacter* group (rRNA superfamily VI), which includes *Campylobacter*, *Helicobacter*, *Arcobacter*, *Wolinella* and '*Flexispira*' spp. (Vandamme *et al.*, 1991), and in a species positioned in the delta/epsilon subdivisions of the *Proteobacteria*.

In many pathogenic bacteria studied so far, including *Legionella pneumophila*, *Borrelia burgdorferi*, *Coxiella burnetii*, *Chlamydia trachomatis*, *Bordetella pertussis*, *H. pylori* and mycobacteria, the GroEL homologues are major antigens of the human immune response (Kaufmann & Schoel, 1994). It is noteworthy that most of these bacteria are intracellular pathogens or cause chronic infections in their human hosts. In *Campylobacter* infection, intraphagocytic survival is uncommon (Wassenaar *et al.*, 1997). In sera from patients with sporadic *Campylobacter* infection, we did not find a humoral response to *C. jejuni* GroEL which significantly differed from the antibody response of healthy controls.

By Western immunoblotting, we could demonstrate that a minority of patient sera (5/24) recognized the recombinant GroEL. However, since (i) there was also a detectable IgG response in some sera from healthy controls (3/16), and (ii) almost every human subject has been confronted with microbial organisms in the past, we cannot exclude that the reactions of the positive sera are directed against conserved regions common to microbial GroEL proteins. As *C. jejuni* GroEL is recognized by anti-*Campylobacter* rabbit serum IgG (Wu *et al.*, 1994), our results indicate a difference in the immune response between humans with sporadic *Campylobacter* infection and experimentally infected rabbits. A similar difference was observed in *Vibrio cholerae* infections. A major 61 kDa HSP (identified as a GroEL homologue) was immunogenic in rabbits, but did not react with sera from cholera patients (Sahu *et al.*, 1994). However, in order to assess the role of GroEL as an antigen in human *Campylobacter* infection more definitively, two further questions have to be answered. First, as GroEL evokes a secretory IgA response in rabbits, the local or mucosal immune response against GroEL in humans needs to be investigated. Second, it is generally accepted that the immune response of human subjects repeatedly infected with *Campylobacter* (e.g. young children in developing countries, chronic raw milk drinkers) is different from that of sporadically infected individuals. In developing countries, there is an early acquisition of 'semi-immunity' during the first 2 years of life, characterized by asymptomatic infections and rising titres of *Campylobacter*-specific antibodies (Glass *et al.*, 1983; Blaser *et al.*, 1986). Further studies are needed to determine whether immune responses against antigens common to different *Campylobacter* strains (as can be expected for the highly conserved GroEL protein) contribute to this protective immunity.

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