GroEL (Hsp60) of *Clostridium difficile* is involved in cell adherence

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Previous results have demonstrated that adherence of *Clostridium difficile* to tissue culture cells is augmented by various stresses; this study focussed on whether the GroEL heat shock protein is implicated in this process. The 1940 bp *groESL* operon of *C. difficile* was isolated by PCR. The 1623 bp *groEL* gene is highly conserved between various *C. difficile* isolates as determined by RFLP-PCR and DNA sequencing, and the operon is present in one copy on the bacterial chromosome. The 58 kDa GroEL protein was expressed in *Escherichia coli* in fusion with glutathione S-transferase and the fusion protein was purified from IPTG-induced bacterial lysates by affinity chromatography on glutathione–Sephadex. A polyclonal, monospecific antiserum was obtained for GroEL which established by immunoelectron microscopy, indirect immunofluorescence and immunoblot analysis that GroEL is released extracellularly after heat shock and can be surface associated. Cell fractionation experiments suggest that GroEL is predominantly cytoplasmic and membrane bound. GroEL-specific antibodies as well as the purified protein partially inhibited *C. difficile* cell attachment and expression of the protein was induced by cell contact, suggesting a role for GroEL in cell adherence.

Keywords: *Clostridium difficile*, adherence, pathogenesis, GroEL, heat shock

INTRODUCTION

Clostridium difficile is recognized as the major aetiological agent of nosocomial infections such as pseudomembranous colitis, and antibiotic-associated colitis and diarrhoea (Bartlett *et al.*, 1978; George, 1984). Several potential and putative virulence factors that could play a role in *C. difficile* pathogenesis have been identified. Virulence is mainly due to the production of two protein exotoxins: toxins A and B, although other toxic factors have been described (Lyerly *et al.*, 1988). There is considerable evidence that some strains are more virulent than others; a number of factors have been proposed to explain inter-strain diversity: (i) the capsule, an antiphagocytic factor (Davies & Borriello, 1990); (ii) proteolytic enzymes, which may play a role in

releasing suitable substrates from available protein sources for metabolism and which could be involved in mucus penetration (Poilane *et al.*, 1998; Seddon & Borriello, 1992); (iii) adhesins, which are involved in mucus and cell association (Borriello *et al.*, 1988a; Eveillard *et al.*, 1993; Karjalainen *et al.*, 1994); (iv) fimbriae, the role of which is obscure (Borriello *et al.*, 1988a); and (v) flagella, for which no role in *C. difficile* colonization has yet been assigned (Tasteyre *et al.*, 2000).

One aspect of *C. difficile* pathogenesis that has been studied by us is its interaction with target cells and identification of the bacterial factors involved in this process (Eveillard *et al.*, 1993; Karjalainen *et al.*, 1994). Adhesion and colonization of animal tissue by bacteria is an important step in establishing infection. It is probable that without attachment, *C. difficile* cannot colonize and will be quickly removed by non-specific host defence mechanisms. It is likely that after destruction of normal flora by antibiotic treatment, *C. difficile* finds itself in a stressful environment that could serve as a stimulus for cell attachment and subsequent

Abbreviations: GST, glutathione S-transferase; HSP, heat shock protein.

The nucleotide sequence of the *groESL* locus of strain 79-685 was assigned GenBank accession number AF093568; that of strain ATCC 53603 was assigned GenBank accession number AF159449. The nucleotide sequence of the 16S rDNA of strain 79-685 was assigned GenBank number AF072474.

colonization. We have previously observed that *C. difficile* cell adherence is enhanced by heat, osmotic and acid shock as well as iron insufficiency (Waligora *et al.*, 1999). Furthermore, attachment was partially inhibited by an antiserum raised to GroEL of *Mycobacterium leprae*. This evoked a potential role for heat shock proteins (HSPs) in adherence. Therefore, we were interested in finding out whether GroEL, a member of the Hsp60 family of chaperonins, plays a role in *C. difficile* colonization.

In this study we undertook the isolation of the *C. difficile* *groELS* operon from two strains, and characterization and expression of the *hsp60* gene. PCR amplification of the *groEL* gene coupled with RFLP analysis was used in an attempt to differentiate between clinical isolates. The role of Hsp60 in cell adhesion was examined by inhibition assays using antiserum and the purified Hsp60 protein, and by investigating the localization of the protein in various cell compartments.

METHODS

***C. difficile* strains and growth conditions.** *C. difficile* isolates investigated are presented in Table 1. The strains were grown under anaerobic conditions in Tryptone Glucose Yeast (TGY) infusion broth (Difco).

Isolation of *C. difficile* *groEL* by PCR, and nucleotide sequencing. Genomic DNA of *C. difficile* strains 79-685 and ATCC 53603 was isolated using the Puregene kit (Gentra) and resuspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) at 1 µg ml⁻¹. Initial amplification of a 600 bp fragment of the *groELS* operon of strains 79-685 and ATCC 53603 was carried out using primers based on the *Clostridium perfringens* *groEL* gene (GenBank accession no. X62914) (Rusanganwa

et al., 1992). PCR was carried out in a 100 µl reaction volume containing 5 µl each primer at 20 pmol µl⁻¹ (primers 5'-GGNGAYGGNACNACNACNGCNACNGT-3' and 5'-TCNCCRAANCCNGGNGCYTTNACNGC-3' where N = A/G/C/T; Y = C/T; R = A/G, W = A/T), 8 µl 25 mM MgCl₂, 8 µl of a mix of dATP, dTTP, dGTP and dCTP (2.5 mM each), 10 µl 10× reaction buffer (Promega) and 0.2 µl *Taq* polymerase (Promega) at 5 U µl⁻¹. The amplification was performed in a Perkin Elmer Cetus Thermal Cycler 480: 95 °C 5 min followed by 34 cycles of denaturation (95 °C 1 min), annealing of primers (55 °C 1 min) and polymerization of DNA (72 °C, 2 min). The last cycle was carried out at 72 °C for 10 min. The presence of an amplified product was confirmed by electrophoresis in a 1% agarose gel.

The entire operon from the translation-initiation codon of GroES until the stop codon of GroEL was subsequently obtained by PCR using primers designed on basis of alignment of the 5' ends of the *groES* gene of *Clostridium thermocellum* (P48212 and P48223), *C. perfringens* (P26821) and *Clostridium acetinobutylicum* (M74572), and the 3' ends of the *groEL* gene of the same bacteria (primers 5'-ATGARWATYARACCA-YTWGGWGACAFRG-3' and 5'-TTARTACATTCCGYC-CATKCCCCRTWCC-3'). Finally, sequences upstream of the operon were obtained by amplification with *groEL*-specific internal primer (5'-CAATAACAGCTAATACATCAC-3') and a promoter-specific primer (5'-TGCTTCTGCAGGTACAGCTAAT-3'), and sequences downstream were obtained with the primer pair 5'-GGAATAGTAGCTGGTGGAGG-3' and 5'-TACTGATTATCTAAATATGTG-3'. These primers were designed on the basis of the genome sequence of *C. difficile* strain 630 that recently became available on the Internet (<http://www.sanger.ac.uk>).

Amplified products were purified by the Wizard Gel Extraction Kit (Promega) and the nucleotide sequences of both strands of the amplified products were obtained by using the *Taq* DyeDeoxy and Big Dye Terminator Cycle sequencing kits purchased from Perkin Elmer. The labelled extension products were analysed with an ABI PRISM 310 Genetic Analyser (Perkin Elmer). Additional primers were designed to obtain additional internal sequence.

Southern blotting. DNA of *C. difficile* strains was prepared as described above. DNA (2 µg) was digested with *Hind*III and *Acc*I according to instructions provided by the supplier (Pharmacia), the fragments separated through a 0.8% agarose gel and electrically transferred to a nylon membrane (Roche). A 340 bp amplified PCR product of the *groEL* gene of *C. difficile* strain 79-685 was used as a probe, labelled in a standard PCR reaction (see above) with 25 µl of the following nucleotide mix: 25 µl 1 mM digoxigenin-11-dUTP (Roche), 4.6 µl 10 mM dTTP, 7.1 µl 10 mM dATP, 7.1 µl 10 mM dGTP, 7.1 µl 10 mM dCTP (Promega) and 2.5 µl sterile water. Hybridization was carried out overnight at 42 °C with 1 µg probe (ml DIG Easy Hyb)⁻¹ (Roche). The subsequent washing steps and detection with CSPD (disodium 3-{4-methoxy-spiro[1,2-dioxetane-3,2'-(5'-chloro) tricyclo(3.3.1.1^{3,7})decan]-4-yl} phenyl phosphate; Roche) were carried out as recommended by the manufacturer.

RFLP analysis. A 1448 bp amplicon encompassing *groEL* was obtained by PCR as described above on genomic DNA from 12 strains using primers 5'-ATGARWATYARACCA-YTWGGWGACAFRG-3' and 5'-TACAACAGCTACTCCTCCAGC-3'. The DNA was digested with eight restriction enzymes under the conditions recommended by the supplier (New England Biolabs). These digests were then subjected to electrophoresis in a 1% agarose gel.

Table 1. *Clostridium difficile* strains used in this study

ND, Not determined.

Strain	Toxigenicity	Serogroup	Origin
79-685	+	S3	Institut de Bactériologie*
VPI 10463	+	G	Dr T. D. Wilkins†
ATCC 43594	+	A	Dr M Delmée‡
ATCC 43593	—	B	Dr M Delmée‡
ATCC 53596	+	C	Dr M Delmée‡
ATCC 53598	—	D	Dr M Delmée‡
ATCC 53598	A—B+	F	Dr M Delmée‡
ATCC 53599	+	G	Dr M Delmée‡
ATCC 53600	+	H	Dr M Delmée‡
ATCC 53601	—	I	Dr M Delmée‡
ATCC 53602	—	K	Dr M Delmée‡
ATCC 53603	—	X	Dr M Delmée‡
630	+	ND	Dr P. Mullany§

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† Virginia Polytechnic Institute, Blacksburg, VA, USA.

‡ Université Catholique de Louvain (UCL), Brussels, Belgium.

§ Eastman Dental Institute, London, UK.

Cloning of *C. difficile* 79-685 *groEL* into an expression vector. Two oligonucleotide primers, PGEX1 (5'-ATTGAATTC-GAGGGGTTTAAATGGCTA-3') and PGEX2 (5'-TTAG-TACATTCCGTCCATGCCCGTTCCT-3'), incorporating an *EcoRI* site in PGEX1 (shown in bold; the translation-initiation codon of GroEL is underlined), were synthesized and used to amplify by PCR the full-length coding region of *groEL* of strain 79-685 in a standard amplification reaction as described above. The resulting 1.6 kb DNA product was blunt-ended by Klenow [1 U in 30 µl One-Phor-All buffer (Pharmacia) for 2 h at 37 °C] and subsequently digested with *EcoRI*. The fragment was ligated (1 U T4 ligase; Life Technologies) into the *EcoRI* and *SmaI* sites of pGEX-6P-1 (Pharmacia) and transformed into *E. coli* XL0LR (Stratagene). A transformant was identified carrying a plasmid which, after nucleotide sequencing of the junction between vector and insert, was found to be capable of producing an in-frame fusion protein between glutathione S-transferase (GST) and GroEL. This plasmid was transformed into *E. coli* BL21.

Expression and purification of the fusion protein. An overnight culture of *E. coli* BL21 containing pGEX-6P-1-*groEL* grown in LB broth (Difco) containing carbenicillin (50 µg ml⁻¹) was diluted 1:100 into 4 l LB medium containing carbenicillin and the culture grown to OD₆₀₀ 0.6 at 37 °C. The expression of the fusion protein was induced by addition of 1 mM IPTG and the culture was continued for 12 h at room temperature. Bacteria were collected by centrifugation and resuspended in 200 ml 20 mM Tris/HCl, pH 7.5. The bacteria were lysed by incubation with freshly prepared lysozyme at 100 µg ml⁻¹ (Sigma) for 15 min at 30 °C, followed by sonication at intervals of 5 s for 1 h at 80% power (Biorblock Scientific 72442 Vibra Cell) and three successive freeze-thaw cycles. Insoluble material was removed by centrifugation at 14000 g for 10 min, and the fusion protein was purified from the supernatant by a single-step affinity chromatography using glutathione-Sepharose-4B and protocols from Pharmacia. A 2 ml bed volume was used for each 200 ml sonicate; the column was washed three times with 20 ml PBS, followed by cleavage of the GroEL moiety bound to glutathione-Sepharose with 80 U Prescission protease (Amersham Pharmacia Biotech) per 1 ml bed volume.

Antibodies. A rabbit polyclonal, monospecific GroEL antiserum was prepared. The band corresponding to the purified *C. difficile* GroEL protein was cut out of the PAGE gel and was lyophilized. Purified protein (200 µg) was injected with Freund's complete adjuvant into New Zealand White rabbits (AGROBIO), followed by four boosters with 100 µg protein in Freund's incomplete adjuvant at day 14, 28 and 42. The rabbits were killed and bled 21 d after the last injection. Antibodies were purified on protein A-Sepharose (Pharmacia-Biotech) according to the supplier's recommendations and used at a 1:2000 dilution in immunoblots.

Antiserum against *C. difficile* was produced as described previously (Karjalainen *et al.*, 1994). Monoclonal antibodies raised against the 65 kDa HSP of *M. leprae* were obtained from UNPD/World Bank/WHO Special Program for Research and Training in Tropical Diseases, Atlanta, GA.

N-terminal protein sequence. The purified *C. difficile* GroEL protein was electroblotted onto a PVDF membrane (Roche) with 50 mM Tris/HCl, 50 mM boric acid buffer and stained with 0.1% amido black (Sigma). The band was excised and N-terminal sequencing was performed by the Laboratory of Microsequencing of Proteins, Pasteur Institute, Paris.

Immunoelectron microscopy. After adsorption of a bacterial preparation to Formvar-coated grids (Sigma) for 5 min, the

grids were floated on PBS with 1% BSA for 30 min, then incubated with GroEL antibodies diluted 1:10 for 1 h and washed three times in PBS. They were incubated with a 1:20 dilution of protein A conjugated with 10 nm diameter colloidal gold particles (Sigma) for 1 h, washed three times with PBS and fixed with 3% glutaraldehyde. After three washings the grids were stained with phosphotungstic acid before observation by transmission electron microscopy.

Immunofluorescence. Bacteria were cultured for 24 h at 37 °C in TGY and shocked at 43 °C for 20 min. A drop of the PBS-washed culture was deposited on a microscope slide and dried. The slide was immersed in PBS (pH 7.2)/5% skimmed milk (1 h, room temp.), followed by a 1 h incubation with protein A purified anti-GroEL antibodies (dilution 1:2000 in the same solution). The slide was washed with PBS (pH 7.2)/0.1% skimmed milk and incubated for 15 min with tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG antibody (Immunotech; 1:20 dilution in PBS/5% skimmed milk). After washing, the specimens were examined with a 100× oil-immersion objective using a Leitz Aristoplan microscope with epifluorescence coupled to an Image Analyzer Visiolab 1000 (Biocom).

Fractionation. Bacterial proteins were separated into four compartments: supernatant, cell wall, membrane and cytoplasm, using a protocol described for *Listeria monocytogenes* (Jonquière *et al.*, 1999). Equivalent amounts of each fraction, corresponding to 20 ml bacterial culture, were separated by SDS-PAGE (Laemmli, 1970) using a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose and submitted to immunodetection as described below. The purity of the fractions was verified by studying two proteins with known cellular locations. Cwp66-C was used as a marker for the cell wall fraction (a peptidoglycan-attached protein, our unpublished data); PepC, a cytoplasmic peptidase reported to be adsorbed to the inner face of the cytoplasmic membrane in *Lactococcus lactis* (Jonquière *et al.*, 1999), was used as a marker for the cytoplasmic and membrane fractions.

The presence of GroEL in the supernatant of exponential-phase bacteria was investigated by precipitating proteins by 70% trichloroacetic acid as described by Schubert *et al.* (2000) and by detecting the presence of GroEL by immunoblotting as described below.

Immunoblotting. Proteins separated by SDS-PAGE were transferred electrically onto nitrocellulose membrane for immunoblotting. The nitrocellulose membrane was incubated for 1 h at room temperature in blocking buffer (5% skimmed milk in TNT [10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.05% Tween]) and then 1 h in the appropriate dilution of the specific antibody. The membranes were washed in TNT and bound antibodies were detected with goat anti-rabbit IgG-alkaline phosphatase conjugate (1:2500 dilution; Sigma) with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Life Technologies) as substrates.

Cell-adherence and adherence-inhibition assays. Cell-adherence assays were performed as previously described using the Vero cell line (Karjalainen *et al.*, 1994). For adherence inhibition with antibodies, bacteria that had been washed twice in PBS and heat shocked at 48 °C or 60 °C for 20 min were preincubated with antibodies (1:10, 1:100 or 1:1000 dilution in TNT + 5% skimmed milk) for 30 min before contact with cells (1 h at 37 °C under anaerobic conditions). When inhibitions were carried out with the purified GroEL protein or BSA (non-adhesive protein control), cells were preincubated for 15 min at 37 °C under a 5% CO₂ atmosphere with 10, 50 and 75 µg protein ml⁻¹ in Modified Eagle's Medium

(Life Technologies) before bacteria were put into contact with cells for 1 h. Non-adherent bacteria were eliminated by five washings in PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.0) and the cells were fixed and stained with May-Grünwald-Giemsa (Sigma). The adhesion index is given as the mean number of adhering bacteria per cell $\pm$ SD (counted at a magnification of 1000 $\times$) from at least three different assays. The significance of differences between various treatments was assessed by a Student's *t*-test.

Expression of GroEL induced by contact with target cells.

Exponential-phase *C. difficile* 79-685 (anaerobic culture in 100 ml TGY at 37 °C for 6 h) were collected by centrifugation and washed twice in PBS, pH 6.8, prior to application to the Vero cell monolayer. After 1 h adherence under anaerobic conditions, bacteria and cells were scraped off the tissue culture flasks with a rubber policeman and collected by centrifugation (10 min, 5000 g). After washing twice in PBS, the pellet was resuspended in 1.5 ml 0.1 M Tris/HCl, pH 8.6, and proteins were extracted by three successive freeze-thaw cycles. After centrifugation at 15000 g for 15 min, the supernatant was recovered and proteins present in it were separated by SDS-PAGE. GroEL was detected by immunoblotting as described above. The induction of expression of GroEL by contact with eukaryotic cells was evaluated by densitometric scanning of the bands. The *C. difficile* gelatinase (Poilane *et al.*, 1998), a non-adhesive protein, served as a negative control.

DNA manipulations. Plasmid isolations were performed by the alkaline lysis procedure using a kit from Qiagen. Ligations and restriction endonuclease digestions were done according to Sambrook *et al.* (1989) and protocols provided by vendors. The TSB method was used for transformation of *E. coli* (Chung *et al.*, 1989).

Computer analyses. Nucleotide and amino acid sequence alignments were performed with the CLUSTALX program (Thompson *et al.*, 1997). Homology searches were conducted with FASTA3 (European Bioinformatics Institute) or BLAST (National Institute for Biotechnology Information, Washington, DC). Hairpin loops in DNA were searched for using RNAdraw (Matzura & Wennborg, 1996).

RESULTS AND DISCUSSION

HSPs are considered to be major antigens in a number of bacterial pathogens and several micro-organisms express immunogenic GroEL homologues, giving rise to an immunogenic host response (Zugel & Kaufmann, 1999). Infection with bacterial pathogens is accompanied by upregulation of expression of bacterial HSPs in response to various stress stimuli. However, limited information concerning direct interaction between HSPs and the host or about the function of stress proteins in disease pathogenicity is available. An example of an Hsp60 with a defined biological function is the 66 kDa Hsp60 homologue of *Salmonella typhimurium*, which has been shown to be responsible for binding to intestinal mucus (Ensgraber & Loos, 1992). *Haemophilus ducreyi* produces a GroEL HSP which is associated with the cell surface and has the capacity to bind eukaryotic cells (Frisk *et al.*, 1998). Several years ago we demonstrated that *C. difficile* adherence to various tissue culture cells, including intestinal Caco-2 and mucus-secreting HT-29MTX cells, is increased by a heat shock (Eveillard *et al.*, 1993). We supposed that *C.*

difficile finds itself in very stressful surroundings after destruction of the normal flora by antibiotic treatment. Indeed, in a later study we presented evidence that *C. difficile* adherence to tissue culture cells is augmented in a microenvironment deviating from that found in the large intestine under equilibrium, i.e. high osmolarity, low pH and iron insufficiency (Waligora *et al.*, 1999). Under these conditions, *C. difficile* expresses three surface proteins that bind to radiolabelled cell membranes, one of which could be the GroEL protein (Waligora *et al.*, 1999).

Analysis of the *groESL* operon from *C. difficile*

The 1940 bp *groESL* operon of *C. difficile* 79-685, isolated by PCR, is composed of the 285 bp *groES* gene which has the capacity of encoding a 10198 Da protein (*pI* = 4.93) and the 1623 bp *groEL* gene with the capacity of encoding a 57677 Da protein (*pI* = 4.72) (Fig. 1). The genes are separated by a 32 bp non-coding sequence. The G + C content of *groES* is 34 mol% and that of *groEL* is 33 mol%. Analysis of the deduced protein sequence of GroEL revealed the presence of the peptide sequence AAVEEGIVAGGG at position 403–414 that is characteristic of the Hsp60 chaperone family [A-(AS)-X-(DEQ)-E-(X₄)-G-G-(GA)]. Neither GroES nor GroEL possesses a signal peptide. Secondary structure prediction using the Chou & Fasman (1978) algorithm revealed a predominantly α -helical conformation with one potential transmembrane domain in the GroEL protein (Fig. 1). The 540 aa protein carries 28% charged residues. *C. difficile* GroEL displays best homology with that of *C. acinetobutylicum* (75% identity), *C. thermocellum* (75%), *C. perfringens* (74%) and *Bacillus subtilis* (73%).

The nucleotide sequence upstream of *groES* carries two sequence motifs: (i) a 27 bp sequence resembling the CIRCE consensus sequence TTAGCACTC-N₉-GAG-TGCTAA, an inverted repeat that could constitute the operator consensus binding site for HcrA, the negative regulator of class I heat-shock genes (Hecker *et al.*, 1996; Mogk *et al.*, 1997); (ii) sequences resembling σ^{32} binding sites, the positive regulator of heat shock response genes (consensus sequence AAATC-N₁₅-TAXTGYTTZTA where X = A/T; Y = C/T; Z = T/G) (Haldenwang, 1995) (Fig. 1). Some *groESL* operons are both positively and negatively regulated, as appears to be the case for *C. difficile*, whilst others have either CIRCE or the σ^{32} consensus binding site. The translation-termination codon is followed by an imperfect inverted repeat that can form a stem-loop structure with a ΔG (37 °C) of -114.7 kJ; it could constitute a *p*-independent transcriptional terminator.

groEL copy number

In some bacteria *groEL* is found as multiple copies in the genome, so a genomic Southern blot was performed to determine if the *C. difficile* *groEL* gene is single or multi-

Fig. 2. (a) Purification of *C. difficile* GroEL overexpressed in *E. coli* BL21 and immunoblot analysis. Lanes: 1, total cell lysate from 4 l culture analysed by SDS-PAGE; 2, soluble protein fraction (supernatant of lysed bacteria); 3, insoluble protein fraction (bacterial pellet); 4–6, successive washes of soluble protein fraction incubated with glutathione–Sepharose beads; 7, final eluate after cleavage with Prescission protease showing a 58 kDa protein corresponding to *C. difficile* GroEL. After SDS-PAGE, the purified protein was transferred to a membrane and incubated with polyclonal serum raised against *C. difficile* GroEL (dilution 1:2000) (lane 8) or preimmune rabbit serum at the same dilution (lane 9). Molecular mass markers (in kDa) are indicated on the right (low-range molecular mass marker from Bio-Rad). (b) Localization of GroEL by fractionation experiments. Clostridial proteins were fractionated into four compartments: supernatant (SN), cell wall (CW), cytoplasm (CY) and membrane (MB). Equivalent amounts of each fraction were separated by PAGE, transferred to nitrocellulose and submitted to immunodetection using GroEL antibody, PepC antibody (control for cytoplasmic and membrane fractions) and Cwp66-C antibody (cell wall protein in *C. difficile*, thus serving as control for the CW fraction). NS, non-shocked; HS, heat shocked.

1). All strains yielded an amplified product of 1.45 kb. The fragments were digested with *AccI*, *AluI*, *DraI*, *EcoRV*, *HindIII*, *PvuII*, *ScaI* and *RsaI*. All profiles obtained were identical except that of strain ATCC 53603 which with *RsaI* digestion exhibited a different pattern from the others (not shown). Sequencing of the gene from this strain showed an alteration of a nucleotide in an *RsaI* site but the gene was more than 99% homologous to the one isolated from the strain 79-685 at the nucleotide and amino acid level (data not shown). Thus GroEL is highly conserved between strains and could be used as a candidate in vaccine preparations.

Expression, purification, immunological detection and localization of GroEL

We decided to purify the GroEL protein in order to obtain a polyclonal, monospecific antiserum for GroEL permitting functional studies. *groEL* was expressed as a

fusion with GST in *E. coli* and purified by affinity chromatography. As shown in Fig. 2(a), lane 7, a 58 kDa band corresponding to GroEL was observed in the eluate by SDS-PAGE. N-terminal sequencing of 21 aa of the purified protein revealed the sequence **GPLGSP-EFEGFKMAKEIKFSE**, which is identical to the N-terminal amino acid sequence deduced from the *groEL* nucleotide sequence (residues in bold are the remaining GST residues encoded by pGEX-6P-1).

The 58 kDa band was cut out of the gel and injected into rabbits to obtain polyclonal antibodies. The monospecific antiserum thus obtained reacted with the purified 58 kDa GroEL protein (Fig. 2a, lane 8) in Western blots. Furthermore, a monoclonal antiserum raised against *M. tuberculosis* Hsp60 recognized *C. difficile* GroEL in Western blots (not shown).

Earlier studies had indicated that Hsp60 and Hsp70 (DnaK) are in general cytoplasmic proteins, but more recent studies suggest that they are membrane associated in some micro-organisms or secreted from the cells. If a protein is to serve as an adhesin, surface localization is required. GroEL of numerous bacteria, such as *Legionella pneumophila* (Garduno *et al.*, 1998a, b; Hoffman *et al.*, 1990), *Helicobacter pylori* (Cao *et al.*, 1998; Dunn *et al.*, 1997; Eschweiler *et al.*, 1993; Huesca *et al.*, 1996, 1998; Kamiya *et al.*, 1998; Phadnis *et al.*, 1996; Vanet & Labigne, 1998; Yamaguchi *et al.*, 1996a, b, 1997, 1998), *Haemophilus ducreyi* (Frisk *et al.*, 1998; Parsons *et al.*, 1997), *M. avium* (Rao *et al.*, 1994), *S. typhimurium* (Ensgraber & Loos, 1992), *Actinobacillus actinomycetemcomitans* (Goulhen *et al.*, 1998) and *Borrelia burgdorferi* (Kaneda *et al.*, 1997), has been shown to be involved in adhesion or invasion of various target cells or tissues and can be surface localized. *C. difficile* grown at 37 °C and heat shocked at 48 °C was fractionated to determine the localization of GroEL. The proteins in each fraction were separated by SDS-PAGE and analysed for GroEL by Western immunoblotting (Fig. 2b). The GroEL protein was found mostly in the cytoplasmic and membrane fractions as well as extracellularly. A small amount of protein was found in the cell wall fraction.

Localization of GroEL on whole bacteria was examined by immunoelectron microscopy and immunofluorescence. In the non-heat-shocked condition (Fig. 3a), little immunolabelling of bacteria was observed [mean 1.2 grains (cm bacterial surface)⁻²]. More than twofold increase of grain density cm⁻² was observed in bacteria shocked at 42 °C (mean 2.7 grains cm⁻²) (Fig. 3b) and a more than sixfold increase at 48 °C (7.7 grains cm⁻²) (Fig. 3c) compared with non-heat-shocked bacteria. The ratio of density of bacterial associated grains compared to the background labelling increased at each temperature. After heat shock at 48 °C the protein was associated with amorphous electron-dense, cell-surface associated material and was distributed in a relatively uniform fashion over the bacterial surface. After a heat shock, especially at 48 °C, the GroEL protein was also found to be localized within the extracellular space.

Fig. 3. Localization of *C. difficile* GroEL investigated by immunoelectron microscopy. (a) Representative electron micrograph of *C. difficile* 79-685 showing little labelling on whole bacterial cells which were grown at 37 °C and not heat shocked. (b) A slight increase in labelling is observed after a heat shock at 42 °C. (c) Profuse labelling on the surface of an intact, whole bacterial cell heat shocked at 48 °C. The arrows indicate the filamentous material associated with the cell. Note the abundance of extracellular grains. (d) Whole bacterial cells incubated with the gold-labelled protein A-conjugated second antibody only served as a negative control. Bars, 0.3 µm.

Immunoblot analysis of proteins precipitated from the supernatant of heat shocked but intact and viable bacteria confirmed the presence of Hsp60 in this localization (data not shown).

The results obtained by immunoelectron microscopy were further corroborated by indirect immunofluorescent investigations of whole bacteria, using antibodies directed against GroEL, whole *C. difficile* or PepC, an intracellular protein. As shown in Fig. 4, heat shocked whole *C. difficile* bacteria showed strong

Fig. 4. Localization of *C. difficile* GroEL by indirect immunofluorescence. Photomicrographs of heat shocked *C. difficile* 79-685 visualized by immunofluorescence, with GroEL antiserum (a) or *C. difficile* antiserum (b) as the primary antibody and TRITC (tetramethylrhodamine isothiocyanate)-labelled anti-mouse immunoglobulin as the secondary antibody. No fluorescent staining was observed when PepC antiserum (c) was used or when the primary antibody was omitted (d).

fluorescence with the first two antisera, whereas antibodies against the intracellular PepC did not react with these cells. These results confirm the surface association of GroEL on heat shocked bacteria.

How Hsp60 of *C. difficile* ends up on the bacterial surface is unclear at the moment as the protein carries no classical signal peptide. Several hypotheses can be proposed: (i) the protein is released by autolysis in the extracellular space and then adsorbed to adjacent bacteria as described for *H. ducreyi* and *H. pylori* (Frisk *et al.*, 1998; Phadnis *et al.*, 1996), although this finding concerning *H. pylori* has been contested (Vanet & Labigne, 1998). We did not observe any lysis of bacteria after heat shock and bacteria remained viable (data not shown); thus this hypothesis does not appear to be valid for *C. difficile*; (ii) the protein is secreted involving type III secretion machinery or a simpler system such as an ABC transporter; however, so far this has not been shown in *C. difficile*; (iii) the protein is artefactually associated with the membrane because of its chaperone function after release (Bukau *et al.*, 1993). The hydrophobic domains, located in the C-terminal portion of GroEL, could facilitate its interaction with hydrophobic regions of other proteins as well as with hydrophobic regions of molecules such as phospholipids or lipopolysaccharides. The results presented here seem to favour extracellular release followed by surface adsorption. This hypothesis is supported by the fact that GroEL was detected extracellularly after precipitation of proteins from culture supernatant, even when heat shocked bacteria remained intact and viable (data not shown).

Fig. 5. Inhibition of *C. difficile* adherence to tissue culture cells by antibodies and purified GroEL. Adherence was determined in the presence of PBS, preimmune serum (negative controls) and various concentrations of GroEL antibodies, purified GroEL ($10 \mu\text{g ml}^{-1}$ shown; at higher concentrations cells were damaged and counting of adherent bacteria was therefore not accurate) or BSA (negative control; a non-adhesive protein). Values shown are the mean $\pm$ SD of at least four separate experiments. *, Statistically significant difference (Student's *t* test; $P < 0.05$) compared to controls.

Fig. 6. Increase in GroEL expression after contact with target cells. GroEL was detected by immunoblotting (1:2000 dilution of antibodies). Lane 1, proteins isolated from bacteria that had not been attached to eukaryotic cells, separated by SDS-PAGE and blotted onto a membrane. Lane 2, proteins isolated from bacteria after 1 h contact with eukaryotic cells. Each lane contains proteins from equivalent numbers of bacteria. Densitometric scanning of bands revealed a 2.5-fold induction in the presence of eukaryotic cells.

GroEL plays a role in cell adherence

The fact that *C. difficile* cell attachment is increased by stress and the presence of GroEL on the bacterial cell surface suggests a possible role for this protein in cell adherence. Involvement of the Hsp60 in adhesion of *C. difficile* to eukaryotic cells was investigated in competitive inhibition assays using anti-GroEL antibodies or the purified protein. As shown in Fig. 5, co-incubation of bacteria with antibodies at a dilution of 1:10 demonstrated a relative adherence of 55% compared with the control (incubation with preimmune serum at the same dilution), indicating that Hsp60 may indeed be involved in the adherence process. A significant reduction of adherence was also observed at 1:100 dilution

of antibodies, whereas at 1:1000 dilution the decrease was not significant. Furthermore, the purified protein inhibited cell adherence by 50% at a concentration of $10 \mu\text{g ml}^{-1}$. In contrast, no inhibition was observed when competitive inhibition was carried out with the non-adhesive protein BSA. As in *H. ducreyi* (Frisk *et al.*, 1998), the partial inhibition can be explained by the likelihood of having multiple adhesins and mechanisms involved in adherence of *C. difficile*. We are in the process of characterizing some of the other adhesins.

Indirect evidence for the role of GroEL in cell adherence is presented in Fig. 6. The expression of GroEL was induced when the bacteria were in contact with target cells under conditions that do not involve thermal stress. Densitometric scanning of the bands revealed a 2.5-fold increase in expression of GroEL following cell contact. Expression of the non-adhesive protein gelatinase, used as a negative control, was not induced (data not shown). We speculate that cell contact could send a signal to *C. difficile*, the result of which is increased expression of the GroEL protein and its subsequent surface display. Others (Qoronfleh *et al.*, 1998) have shown a *de novo* synthesis of proteins involved in cell adherence or invasion upon epithelial cell contact.

In conclusion, we provide evidence in this communication that GroEL of *C. difficile* serves an adhesive function in this bacterium. As in other bacteria, *C. difficile* is likely to possess multiple adhesins and we are pursuing studies to elucidate the role of each. Furthermore, investigations are underway in our laboratory to evaluate the capacity of GroEL to trigger a protective immune response.

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