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Heat Shock- and Alkaline pH-Induced Proteins of *Campylobacter jejuni*: Characterization and Immunological Properties

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The protein response to physiological stress was characterized in *Campylobacter jejuni* 81176 after exposure to heat and pH shock and following periods of recovery. Immunoreactivities of major stress-related proteins were determined with anti-*Campylobacter* immune rabbit serum and intestinal lavage fluid. Distinct proteins with molecular masses ranging from 10 to 120 kDa were induced and/or released by selective heat or pH treatments. The most notable responses were those of two proteins with apparent molecular masses of 45 and 64 kDa that were induced and two other proteins of 10 and 12 kDa that were released by selective heat shock, alkaline pH treatment, or both. On the basis of N-terminal sequence analysis and immunological cross-reactivity data, the 64- and 10-kDa proteins were the *C. jejuni* homologs of *Escherichia coli* GroEL and GroES proteins, respectively. Enhanced chemiluminescence Western blotting (immunoblotting) revealed that all four proteins were among the major protein antigens recognized by anti-*Campylobacter* rabbit serum immunoglobulin G (IgG) and immune rabbit intestinal lavage IgA (secretory IgA). The results of this investigation suggest that the *C. jejuni* 10-, 12-, 45-, and 64-kDa proteins and a number of minor stress-related proteins deserve further evaluation of their respective roles in *Campylobacter* pathogenesis and immunity.

Prokaryotic and eukaryotic cells are known to react to chemical and/or physiological assaults by the induction of stress or heat shock proteins (19, 35, 36, 37). Studies of stress proteins have shown that these proteins play important roles in vivo as molecular chaperones (8, 15, 22) and as immunodominant antigens during bacterial infections (5, 12, 21, 28). During bacterial infections, the interaction between a bacterial pathogen and its mammalian host exposes both parties to multiple physiological and biological stresses (19). A variety of different stressors are known to induce distinctly similar sets of stress proteins, among which homologs of *Escherichia coli* heat shock GroEL and GroES proteins have been the most prominent and well characterized (40).

With the emergence of *Campylobacter jejuni* infections as one of the leading causes of human gastroenteritis worldwide (9, 30, 31), development of molecular vaccines and new diagnostic techniques is highly desirable (10, 24, 25). Bacterial GroELs homologs are known to be immunodominant antigens for B cells and T cells during bacterial infections (13, 18, 28). Because of their highly conserved nature, they are also called common antigens and can cause significant specificity problems in serological immunodiagnostic tests because they induce strongly cross-reactive antibodies (1). The importance of GroEL homologs in the pathogenesis of enteric pathogens and host immunity is suggested by a recent publication in which a GroEL homolog of *Helicobacter pylori* was found to be bound to urease, a recognized virulence factor of the bacterium (7). It was speculated that the protein functions in the export, extracellular assembly, and/or protection of the urease against inactivation in vivo (7). In another report, the GroEL homolog of *Salmonella typhimurium* is found to be responsible for

binding the bacterium to intestinal mucus (6). There has been no reported study of similar proteins in *C. jejuni*.

Since the majority of infectious microorganisms are encountered through mucosal surfaces, the importance of the mucosal immune system in the prevention and control of infectious diseases is being increasingly appreciated (17, 33). Secretory IgA (sIgA) is an important mediator of humoral immunity in the mucosal environment (17). A number of studies have demonstrated its importance in protective immunity against pathogens, including *Campylobacter* spp. (4, 14, 34). To our knowledge, there have been no published investigations of mucosal sIgA's response to bacterial stress proteins. Heat shock- and pH shock-induced proteins of *C. jejuni* may play a role in pathogenesis and host immunity.

Systemic immune responses to well-known stress proteins have been investigated extensively (5). In this paper, however, we report on characterization of *C. jejuni* stress-related proteins and their reactivities with both systemic and mucosal antibodies. We documented strong reactivity of these proteins to sIgA from experimentally infected rabbits, which indicates that these proteins are expressed in vivo and are potentially important immunogens.

MATERIALS AND METHODS

Preparation of bacterial cells. *C. jejuni* 81176 (Penner serotype 23/26, Lior serotype 5) was isolated from an ill 9-year-old girl in an outbreak in Minnesota in which 52% of those consuming the implicated raw milk developed symptoms such as diarrhea (100%), abdominal cramps (84%), and fever (72%) but no grossly bloody stool. Motile bacteria were grown from frozen stocks. Sheep blood agar plates were inoculated with thawed cells and incubated for 18 to 24 h at 37°C in a microaerophilic environment (5% O₂, 10% CO₂, 85% N₂). Bacterial cells were harvested and transferred to a biphasic culture system for homogeneous broth growth (26). The

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resulting growth was subcultured to brain heart infusion broth supplemented with 1% yeast extract (BHIYE) and incubated for an additional 18 to 24 h at 37°C in a shaking incubator at 100 rpm. Motilities of preparative cultures for heat and pH shock studies were monitored microscopically and by growth patterns on motility agar. Mid-log phase cultures were harvested and pelleted at 12,000 × g. Bacterial pellets were resuspended in phosphate buffered saline (PBS) to 1/20 of the original volume. This resuspension was used for subsequent stress studies.

Immune rabbit sera and intestinal lavage fluid. Experiments reported in this paper were conducted according to the *Guide for the Care and Use of Laboratory Animals* (106). Immune rabbit serum was kindly provided by Paul Cohen (University of Rhode Island) and was prepared by immunizing rabbits with formalin-inactivated *C. jejuni* 81176. The animals were orally infected with live *C. jejuni* and evaluated for protection 30 days later by intraintestinal challenge in the removable intestinal ile adult rabbit diarrhea model (3a). Intestinal lavage fluids were collected from both control and immune rabbits (live infected and colonization resistant in the removable intestinal ile adult rabbit diarrhea challenge model) by the methods of Burr et al. (3) and Caldwell et al. (3a).

Heat and pH shock treatment of bacterial cells. For the analysis of the effects of heat shock treatment, a bacterial suspension (5 ml) was mixed with 20 ml of prewarmed PBS or BHIYE in water baths set at preselected heat shock or control (37°C) temperatures. Samples were removed from heat shock treatment after various shock times or after shock plus recovery times. For studies of pH effects, four bacterial suspensions, each of 6.75 ml, were mixed with 60.75-ml volumes of pH-buffered saline to give 1:10 dilutions and final pHs of 3.0, 4.0, 5.0, and 8.6. Mixed suspensions were incubated at 37°C. Samples were taken for analysis after selected times at acidic or alkaline pHs and also after recovery periods, which were carried out by pelleting bacterial cells and resuspending them in a medium of normal growth pH. Recovery after heat shock and pH treatments was performed in PBS or BHIYE at 37°C. Finally, all samples were pelleted promptly by centrifugation, and whole-cell pellets and supernatants were stored separately at -70°C until needed for further characterization.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and enhanced chemiluminescence (ECL) Western blotting (immunoblotting). Tricine SDS-PAGE was performed on the basis of the work of Schagger and Jagow (27). A 10 to 20% linear gradient polyacrylamide separating gel (0.75 mm by 18 cm by 20 cm) was overlaid with a 4.5% polyacrylamide stacking gel. Whole cells were solubilized with a solution of 2% SDS and 0.1% Triton X-100 by repeated ultrasonication and heating in a boiling water bath. Supernatants were washed free of medium components with Centricon P-10 (Amicon) and concentrated. Samples were then mixed with the sample buffer, heated at 60°C for 10 min, and loaded onto gels. Loaded gels were run at 30 V for 1 h or until loadings had completely left the sample pockets. Electrophoresis was then performed at 185 V for approximately 3,200 V · h. Gels were stained with a silver stain kit (Bio-Rad) or transferred onto membranes in a phosphate buffer system (25 mM, pH 7.25) in a Trans-Blot Cell (Bio-Rad) for 4 h at 420 mA. Precast SDS-PAGE minigels of both the Tricine and Laemmli systems were used according to the instructions of the manufacturer (NOVEX).

ECL Western blotting was performed according to the instructions of the manufacturer (Amersham) with minor modifications. Proteins were blotted onto high-bond nitrocellulose membranes (Amersham). Membranes were then blocked

with 5% nonfat dry milk in TBST (0.05 M Tris, 0.25 M NaCl, 0.01% sodium merthiolate, and 0.1% Tween 20). Primary and secondary antibodies were used with 5% nonfat dry milk in TBST. Serum was typically used at a 1:10,000 dilution. Lavage fluid was used at 1:100 dilution or higher. Horseradish peroxidase-labelled goat anti-rabbit IgG (Bio-Rad) was used at 1:5,000. Horseradish peroxidase labelled goat anti-rabbit IgA (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was used at 1:2,500 from a stock of 0.1 mg of protein per ml. Blocked membranes were incubated with lavage samples for 2 h at room temperature plus overnight at 4°C or with serum samples for 1 h at room temperature. Blots were washed extensively in TBST before incubation with secondary antibodies for 1 h at room temperature. They were extensively washed in TBST and developed in a darkroom according to standard procedures. X-ray films were exposed for times ranging from seconds (mostly for serum IgG) to a few minutes (for lavage IgA). In control studies, films were exposed for 20 min.

Protein N-terminal sequencing. Protein N-terminal sequencing was done with an Applied Biosystems, Inc. (Foster City, Calif.) ABI 477A protein sequencer with an on-line 120A high-pressure liquid chromatography amino acid analyzer. Following SDS-PAGE, proteins were transferred onto polyvinylidene difluoride membranes. Blotted proteins were stained briefly with Coomassie blue and destained. Protein bands were located by alignment with purified homologous proteins and/or by Western blotting. They were cut out and sequenced by running a standard operating program with a Blott-2 reaction cartridge.

RESULTS

Stress responses of C. jejuni following heat or pH shock.

This study was designed to characterize the induction and/or release of protein antigens following physiological stress. Induced and/or released proteins were visualized by silver staining and ECL Western blotting.

Induction of distinct proteins by heat shock at 60°C in the culture medium (Fig. 1A, lanes A to C) or by alkaline pH shock at pH 8.6 (Fig. 1B, lanes B to D) is evident. Upregulation of a 64- and a 45-kDa polypeptide by heat shock at 60°C for 15 min to 2 h or alkaline pH shock at pH 8.6 for 0.5 to 2 h continued for several hours after removal of stressors. In addition, four proteins of 22, 38, 53, and 66 kDa were induced by pH 8.6, while two proteins of 27 and 53 kDa were induced by heat shock. Acid shock at pH 3.0 caused significant reduction of an sIgA-reactive flagellar protein in the cell pellet, while the lost reactivity was detected in the supernatant (result not shown). Acid shock at pH 4.0 and 5.0 did not cause significant protein induction under conditions used in this work (Fig. 1B, lanes E to K).

Release of 10- and 12-kDa polypeptides is best seen with Western blotting (Fig. 2) because these two proteins stained much more poorly than large proteins with silver staining reagents. Heat shock in PBS, but not in BHIYE, at 50°C or above caused the release of the 10-kDa polypeptide (Fig. 2A, lanes A, C, and H). Both heat shock and alkaline pH treatment caused near-complete dissociation of an immunoreactive 12-kDa polypeptide from the cells, while acid treatment at pH 5.0 caused only partial dissociation (Fig. 2).

Identification of *C. jejuni* GroEL and GroES proteins. The stress-inducible 64-kDa protein of *C. jejuni* was found to cross-react with several monoclonal antibodies against a GroEL homolog of *Rickettsia typhi*. This protein was located on a Western blot, and its N-terminal sequence was determined (Fig. 3A). This 64-kDa protein is more than 80%

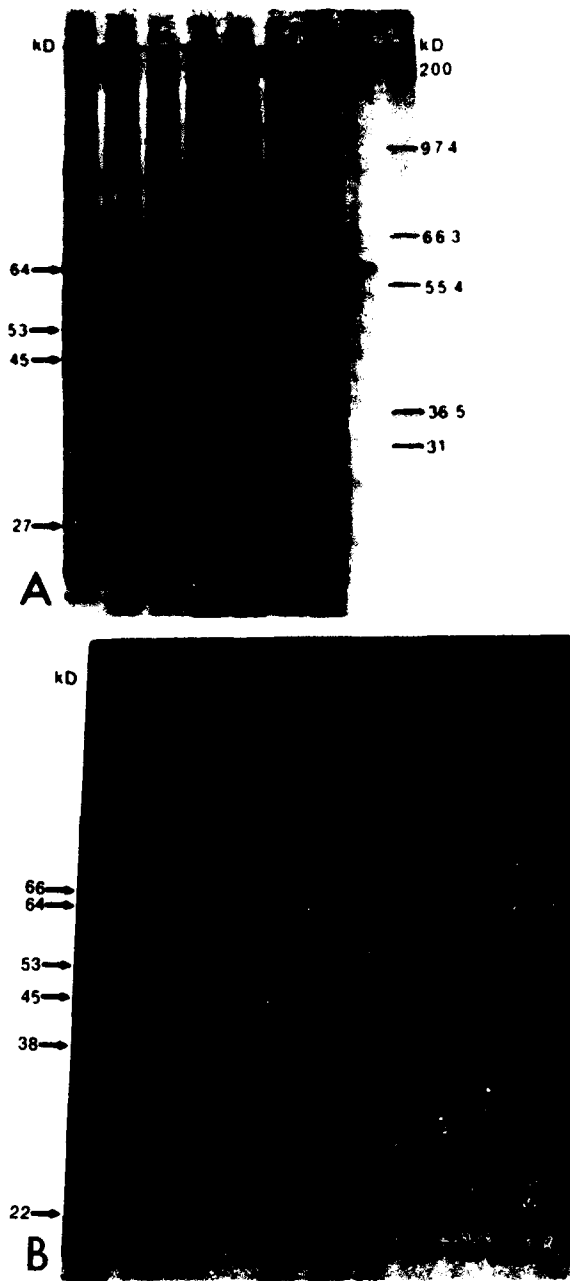


FIG. 1. (A) Silver stained Tricine SDS-10 to 20% PAGE profiles of heat-shocked *C. jejuni* whole cells. Lanes: I, molecular mass markers (wide-range protein standards from NOVEX [catalog no. LC5677]); H, purified *R. typhi* GroE1; G and F, control whole cells with no stress treatment; E and D, cells heat shocked for 1.5 h at 50°C in PBS (E) with a 2-h recovery (D); C, B, and A, cells heat shocked for 1 h at 60°C in BHIYE (C) with a 2-h recovery (B) or a 3.5-h recovery (A). (B) Silver-stained SDS-10 to 20% PAGE profiles of pH-shocked *C. jejuni* whole cells. Lanes: K, J, I, and H, cells, treated for 1 h at pH 4.0 (K) with a 2-h recovery in PBS (J and H) or a 2-h recovery in BHIYE (I); G, F, and E, cells treated for 1 h at pH 5.0 (G) with a 2-h recovery in PBS (F) or a 2-h recovery in BHIYE (E); D, C, and B, cells treated for 1 h at pH 8.6 (D) with a 2-h recovery in PBS (C) or a 2-h recovery in BHIYE (B); A, control cells with no stress treatment. Molecular mass markers are not shown.

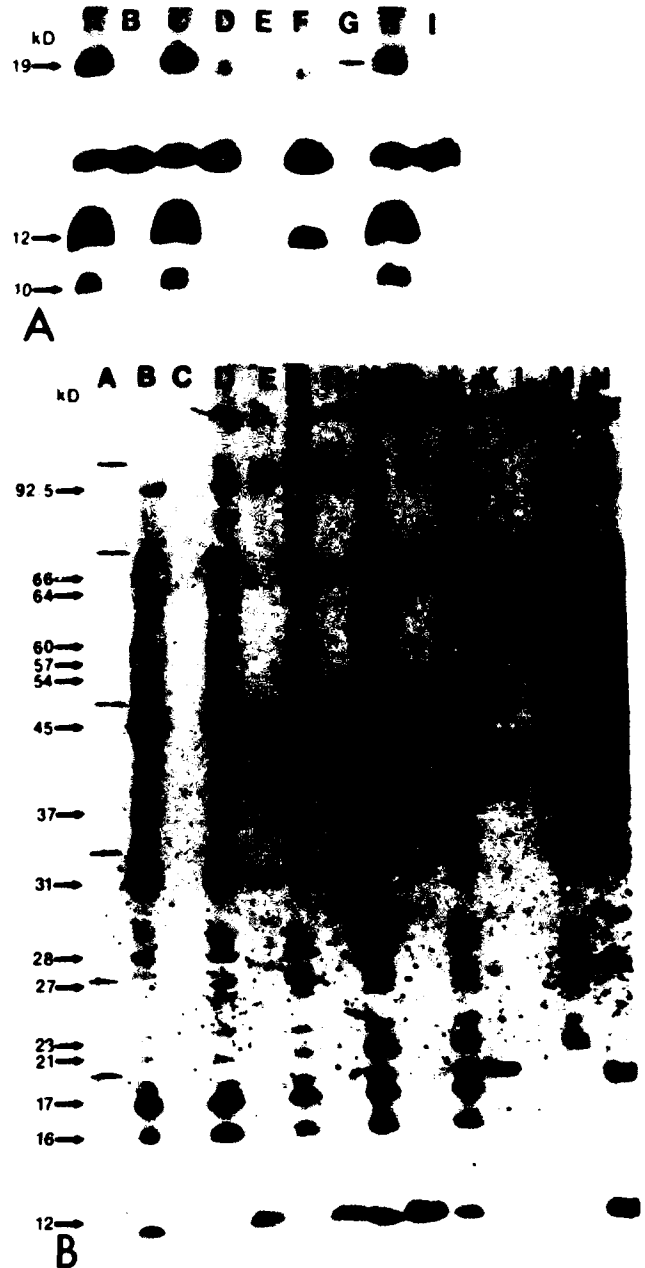


FIG. 2. (A) Immune rabbit serum IgG ECL-probed Western blot of whole cells and supernatants of heat-shocked *C. jejuni*. Lanes: I, whole cells heat shocked for 0.5 h at 60°C in PBS with a 1-h recovery; H, supernatant of lane I; G, molecular mass markers; F, control whole cells with no stress treatment; E, supernatant of lane F; D, lane I whole cells after an additional 1-h recovery; C, supernatant of lane D; B, lane I whole cells after 2.5-h recovery; A, supernatant of lane B. Note that a Tricine-10 to 20% polyacrylamide gel was used and only part of the blot is shown. (B) Immune rabbit sIgA ECL-probed Western blot of whole cells and supernatants of stressed *C. jejuni*. Lanes: A, molecular mass markers (prestained low-range standards of Bio-Rad, catalog no. 74177); B, control whole cells; C, supernatant of lane B; D, whole cells heat shocked for 1 h in PBS with a 2-h recovery; E, supernatant of lane D; F, lane D whole cells after an additional 1.5-h recovery; G, supernatant of lane F; H, whole cells treated for 2 h at pH 5.0; I, supernatant of lane H; J, lane H whole cells after a 2-h recovery; K, supernatant of lane J; L, purified *R. typhi* GroE1; M, whole cells treated for 2 h at pH 8.6; N, supernatant of lane M.

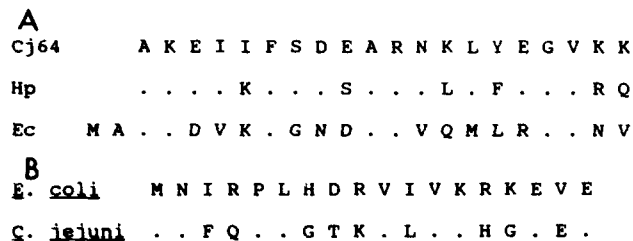


FIG. 3. (A) N-terminal amino acid sequences of the *C. jejuni* 64-kDa protein and several GroEL homologs. The single-letter code is used to denote amino acids. Dots indicate identities between corresponding amino acids. Abbreviations: Cj64, 64-kDa protein of *C. jejuni*; Hp, *H. pylori* HSP62 (7); Ec, *E. coli* GroEL heat shock protein (10a). (B) N-terminal amino acid sequences of *E. coli* GroES protein (10a) and the GroES-like protein of *C. jejuni*.

homologous (identities plus conservative substitutions) to a GroEL homolog of *H. pylori* and 67% homologous to the *E. coli* GroEL in 20 N-terminal amino acids. We were able to conclude that the 64-kDa protein is a GroEL homolog of *C. jejuni* on the basis of its N-terminal amino acid sequence and immunological cross-reactivity. A GroES homolog of *C. jejuni* was also identified by N-terminal sequence analysis of a 10-kDa protein located by alignment with a purified GroES homolog of *R. typhi* (Fig. 3B). The GroES homolog of *C. jejuni* cross-reacted with rabbit serum IgG against a purified homologous protein of *R. typhi* (blot not shown).

Identification of stress-released 12-kDa protein, stress-induced 45-kDa protein, and flagellin protein. A major immunoreactive protein that migrated slightly behind the GroEL homolog on high-resolution gels was suspected to be flagellin. N-terminal sequence analysis revealed that the protein band did contain a major N terminus identical to those published for flagellins in other *Campylobacter* spp. (10) and at least two more N-terminal species (data not shown). N-terminal amino acid sequences of the 12- and 45-kDa proteins were determined but are not reported here. A search of major data banks of protein and gene sequence did not result in finding homologous matches for either of these two proteins.

Rabbit mucosal and systemic immune responses to *C. jejuni* stress-induced and other major protein antigens. The GroEL (Fig. 2B) and GroES (blots not shown) homologs of *C. jejuni* were strongly reactive in Western blots with sIgA from intestinal lavage fluids from immune rabbits. The same sIgA preparation cross-reacted with purified GroEL (Fig. 2B, lane L) and GroES (blots not shown) homologs of *R. typhi*. Similar results were obtained with immunoblots using immune rabbit serum IgG (blots not shown).

The stress-released 12-kDa protein was strongly reactive with both mucosal IgA (Fig. 2B) and serum IgG (Fig. 2A). The stress-inducible 45-kDa polypeptide was a major antigen of mucosal IgA (Fig. 2B). Its reactivity with serum IgG was consistently weaker (blots not shown). Several other major sIgA-reactive antigens are apparent in Fig. 2B. These antigens include polypeptides with apparent molecular masses of 16, 17, 21, 23, 27, 28, 31, 37, 54, 57, 60, 66, and 92.5 kDa but were not further studied.

In control studies, in ECL Western blots *C. jejuni* whole-cell antigens did not react with preimmune rabbit lavage fluids and yielded only a few weak reactions, mostly in the 45- and 60- to 66-kDa regions with preimmune rabbit serum (blots not shown).

DISCUSSION

Our results show for the first time that a stress-inducible GroEL homolog and a stress-released GroES-like protein of *C. jejuni* are among the targets of mucosal IgA response during *Campylobacter* infection in the rabbit model. External alkaline pH, but not acidic pH, and heat shock induced stress responses in *C. jejuni* that were comparable to those in studies with *E. coli* (29).

Only a single, intensely immunoreactive band was detected between 64 and 66 kDa on ECL Western blots, if electrophoresis was performed using precast SDS-12.5% PAGE minigels of the Laemmli system (blots not shown). This indicates that the GroEL homolog and the flagellin may not be differentiated on low-resolution SDS-PAGE minigels and in subsequent Western blotting. Using high-resolution SDS-PAGE gels we were able to find an alkaline-pH-inducible 66-kDa protein. In their recent publication, Panigrahi et al. (23) reported a protein with a similar molecular mass that was induced in vivo in rabbits. These two proteins may be identical since alkaline pH shock mimics the pathogen's in vivo environment, since the pH of the small intestine is alkaline (16, 17).

In *H. pylori*, a GroEL-like protein is bound to extracellular urease (7), and in *S. typhimurium*, an extracellular GroEL-like protein is responsible for binding the pathogen to mucus (6). However, we detected no extracellular GroEL homolog of *C. jejuni* in the supernatants of heat- and pH-shocked, as well as unshocked, cells. Given the fact that this GroEL homolog was strongly reactive with sIgA from infected animals, this protein may play a novel and as yet unknown role in *Campylobacter* infection.

It is interesting to note that a GroES-like protein was released upon heat shock in PBS, while the GroEL-like protein was not. As molecular chaperonins, bacterial GroES-like proteins are regarded to be functional partners to GroEL-like proteins (8, 10a, 40). Our observation may indicate that GroES can also function separately.

A 45-kDa major antigen described in this study is likely the major outer membrane porin protein discussed elsewhere (32). Although the N terminal sequence of this 45-kDa protein that we determined (data not reported herein) does not align well with amino acid sequences in major data banks, the N termini of mature porin proteins (i.e., after cleavage of the signal sequence) are known to have poor sequence conservation (11). The 45-kDa protein band was induced by both heat and alkaline pH shock, similarly to induction of a porin protein in *Pseudomonas aeruginosa* by anaerobiosis (39), and in a deep-sea photobacterium (SS9) by elevated hydrostatic pressure (2).

The immunogenic 12-kDa protein should not be a degraded product since it was detected comigrating with another 12-kDa polypeptide in unstressed whole cells (unpublished data). Two proteins with similar molecular masses, 12 and 14.5 kDa, have previously been shown to be recognized by serum IgG and sIgA present in the stools of patients recovering from naturally acquired *C. jejuni* enteritis (20, 35). Given that this 12-kDa protein dissociated from cells upon stress and was strongly antigenic, this protein may be an important surface-associated immunogen.

In summary, we characterized the protein response to heat and pH shock treatments and identified two major heat shock proteins of *C. jejuni*. The two identified heat shock proteins were strong in vivo antigens of rabbit mucosal sIgA response. These two proteins, as well as the strongly antigenic stress-released 12-kDa and stress-inducible 45-kDa proteins, deserve further investigation of their respective roles in *Campylobacter* infections.

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