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Heat Shock Response and Heat Shock Protein Antigens of *Vibrio cholerae*

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Sixteen heat shock proteins (Hsps) have been identified in the hypertoxinogenic strain 569B of *Vibrio cholerae* which are synthesized in response to small and large elevations of temperature. The induction of the Hsps is necessary for the cells to survive the deleterious effects of heat. There is no difference in the pattern of induction of the Hsps in *V. cholerae* strains varying in levels of toxinogenicity. One of the major low-molecular-mass Hsps, a 16-kDa protein, is preferentially degraded following shift down of temperature. This protein is induced at a much lower level at high temperatures in cells maintained in the laboratory for a prolonged period. The only Hsp located in the outer membrane of *V. cholerae* cells is a 23-kDa protein. Western immunoblot analysis with human immune sera collected from convalescent cholera patients revealed that this protein is markedly immunogenic. The human immune serum also reacted with the 69- and 16-kDa major Hsps and the 88-, 66-, and 46-kDa Hsps but not with the 61-kDa major Hsp identified as the *groEL* gene product. All major Hsps reacted with rabbit anti-*V. cholerae* sera. Ethanol stress leads to the induction of four of the major Hsps and three additional proteins.

Vibrio cholerae, a gram-negative noninvasive enteric bacterium, is the causative agent of the diarrheal disease cholera (5). Cholera still remains a major cause of human mortality, not only in the developing countries where it exists almost in endemic form but also in certain developed countries where it frequently assumes epidemic proportions. For successful infection of their human host, *V. cholerae* cells must colonize the mucosal cells of the small intestine (35). In the course of the transition from a typical environmental source such as water to the human intestine, *V. cholerae* cells are exposed to a series of environmental changes, some of which are known to be stressful for bacteria, such as sudden increase in temperature, low pH of the stomach, bile salts, and perhaps anaerobiosis and starvation (18). Several of these stressful stimuli trigger the enhanced synthesis of a set of conserved proteins known as the heat shock proteins (Hsps). The Hsps are now known to be induced by a variety of stresses besides heat. These include ethanol, changes in pH, heavy-metal ions, nutrient deprivation, anaerobiosis, and viral infection (18). Elevated levels of the Hsps are believed to be correlated with an increased tolerance to thermal and some other forms of stress (11, 14), although the specific physiological significance of the induction of Hsps is still obscure (29, 32). In several pathogenic bacteria, Hsps have been shown to be dominant antigens. In infection by *Mycobacterium leprae*, *Mycobacterium tuberculosis* (36), *Legionella pneumophila* (7), *Coxiella burnetii* (33), and *Borrelia burgdorferi* (2), Hsps synthesized by the bacteria are strongly antigenic.

Of particular interest in the study of microbial pathogenesis is evidence which suggests a link between the Hsps and survival of bacterial pathogens in their host and pathogenicity in vivo (8, 30). Although *V. cholerae* cells are subjected to a series of stressful stimuli following infection of their host, the role of stress proteins in the in vivo survival and pathogenicity of these cells is yet to be investigated. However, several lines of evidence indicate that environmental signals coordinately regulate the expression of virulence determinants in *V. cholerae*. While stress at the level of acidic pH (16) and anaerobiosis (28) increases the expression of virulence genes and enterotoxin production, temperature elevations reduce the levels of virulence determinants (22). It has been reported (22) that cholera toxin production in cells grown at 22°C is about fivefold higher than in cells grown at 37°C, the optimum growth temperature. It is in this context that the present report describes studies that analyze the response of *V. cholerae* cells to heat shock, the most well studied of the stress responses and one experienced by all mammalian parasites immediately upon entering their hosts. About 16 Hsps have been identified in the present study; several react with human immune sera collected from convalescent cholera patients.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *V. cholerae* strains used in this study are listed in Table 1. All strains were obtained from the National Institute of Cholera and Enteric Diseases, Calcutta, India. The strains were maintained on nutrient agar slants, and cells were grown in nutrient broth medium as described previously (12, 26). For animal passage, the cells were injected into the intraperitoneal cavity of guinea pigs. The animals were killed after 24 h, and stock cultures were prepared from single-colony subcultures after proper identification (17). Cell viability was assayed as CFU on nutrient agar plates.

Heat shock and in vivo labeling of cell proteins. Cultures used for heat shock experiments were grown to logarithmic phase at 30 or 37°C, and a part of the culture was shifted immediately to higher temperatures. The viability of the culture at high temperatures was assayed at different times after the temperature shift. To analyze the Hsps, 1 ml of cells was labeled with 15 μCi of [^{35}S]methionine (specific activity, 200 Ci/mmol; Bhabha Atomic Research Centre, Trombay, India) for the desired length of time. The labeled cells were harvested and washed, and whole-cell lysates were analyzed by

TABLE 1. *V. cholerae* strains

V. cholerae strain	Description	Source or reference
569B	Prototroph, highly toxinogenic, biotype classical, serotype Inaba	17
154	Prototroph, mildly toxinogenic, biotype classical, serotype Ogawa	5
MAK757	Prototroph, mildly toxinogenic, biotype El Tor, serotype Ogawa	5
EW6	Prototroph, nontoxinogenic, biotype El Tor, serotype Ogawa	5
GP5	Prototroph, nontoxinogenic, biotype El Tor, serotype Inaba	Y. Takeda, Kyoto University
GP7	Prototroph, nontoxinogenic, biotype El Tor, serotype Ogawa	

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Subcellular fractionation of cell lysates. *V. cholerae* 569B cells were labeled with 15 μCi of [^{35}S]methionine per ml for 10 min, sedimented (10,000 × *g*, 5 min, 4°C), suspended in buffer containing 10 mM Tris (pH 8), 20 mM NaCl, and 25 mM EDTA, and lysed by sonication. The crude cell envelope was prepared by centrifuging the cell lysate in a Beckman L7-55 centrifuge with a type 50 rotor at 45,000 rpm for 30 min. The membrane fraction was treated with 1% Sarkosyl for 60 min at 25°C to solubilize inner membrane proteins and centrifuged again at 45,000 rpm for 60 min to remove the outer membrane (4). Each fraction was analyzed by SDS-PAGE and autoradiography.

Electrophoretic methods. One-dimensional SDS-PAGE was performed as described by Laemmli (9) except that a slab gel apparatus was used. The stacking and separating gels were 5 and 12.5% acrylamide, respectively. Two-dimensional gel electrophoresis was performed essentially as described by O'Farell (19). Proteins were separated in the first dimension by isoelectric focusing followed by SDS-PAGE in 14% polyacrylamide slab gels and visualized by autoradiography.

Western blot (immunoblot) procedure. Cultures of *V. cholerae* 569B cells were grown and exposed to higher temperatures as described above. Either the total cellular proteins or the solubilized membrane proteins were separated by SDS-PAGE and transferred to nitrocellulose in a transblot apparatus (Bio-Rad) as described by Towbin et al. (31). The blots were incubated for 2 h with either rabbit or human anti-*V. cholerae* serum followed by alkaline phosphatase-conjugated anti-rabbit immunoglobulin G or peroxidase-conjugated anti-human immunoglobulin G. Antigens were detected by the Proto Blot system (Promega). The blots were used for autoradiography, and antigenic Hsps were identified by overlay of the autoradiograms on the blots.

Antiserum against *V. cholerae* 569B cells was raised in rabbits by injecting subcutaneously 10^{10} live bacteria in incomplete Freund's adjuvant at monthly intervals for 3 months, and it was used at a dilution of 1:200 in the Western blot experiment. Human immune sera were obtained 1 and 21 days after infection from patients diagnosed as having cholera and were generously provided by S. K. Bhattacharya, National Institute of Cholera and Enteric Diseases, Calcutta, India. Sera were employed at a dilution of 1:40. Rabbit antisera against purified Hsp60 of *Heliothis urescens* were provided by S. C. Lakhotia of Banaras Hindu University, Varanasi, India, and used at a dilution of 1:1,000.

Transformation. *V. cholerae* cells were transformed by plasmid pKV7 carrying the *Escherichia coli rpoH* gene (obtained from T. Yura, Kyoto University, Kyoto, Japan) by following the method of Panda et al. (21).

Maxicell analysis of plasmid-encoded proteins. *V. cholerae* 569B cells carrying plasmid pBR322 or pKV7 were grown to the logarithmic phase in Tris-Casamino Acids-glucose medium (24) and irradiated with UV light (100 J/m^2), and plasmid-encoded proteins were detected by the maxicell method (27).

RESULTS

Hsps. The viability of *V. cholerae* cells grown at 30°C was reduced to 0.5% after 10 min at 47°C. If, however, cells were incubated at 42°C for 15 min prior to shifting to 47°C, the survival was more than 5% 10 min after the temperature shift (Fig. 1a). Thus, prior heat shock at an intermediate temperature induces thermotolerance during subsequent exposure of the cells to lethal temperatures. This prompted an examination of stress proteins induced by heat shock in *V. cholerae*.

Analysis of newly synthesized proteins by SDS-PAGE and autoradiography following a shift of 30°C-grown cells to higher temperatures ranging between 37 and 47°C demonstrated the induction of six Hsps with molecular masses of 69, 66, 61, 55,

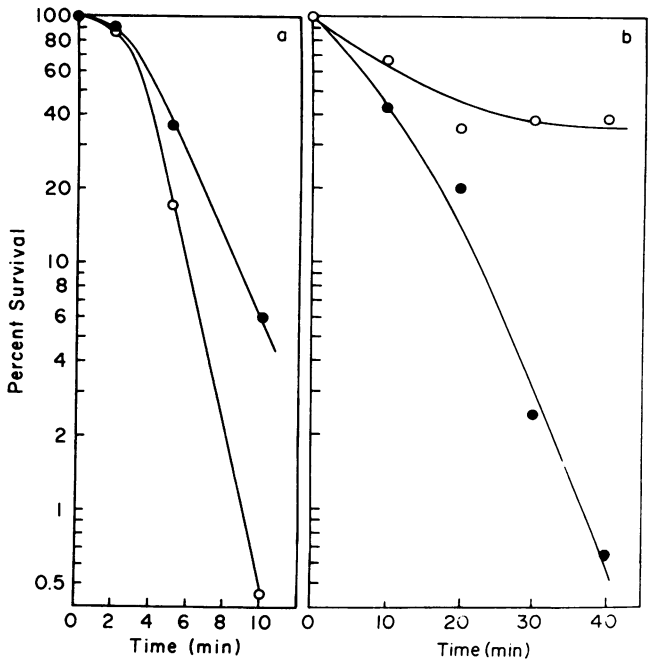

FIG. 1. (a) Effect of incubation at an intermediate temperature on survival of *V. cholerae* cells at a higher temperature. Survival of 30°C-grown *V. cholerae* 569B cells was measured at 47°C when the cells were shifted directly to 47°C (○) or shifted to 47°C after a 15-min preincubation at 42°C (●). (b) Effect of chloramphenicol on heat shock response. ●, survival of 30°C-grown *V. cholerae* 569B cells at 44°C in the presence of 4 μg of chloramphenicol per ml; ○, survival of 30°C-grown cells at 44°C when chloramphenicol was added 10 min after the temperature shift. In each case the time of addition of chloramphenicol was taken as 0 min.

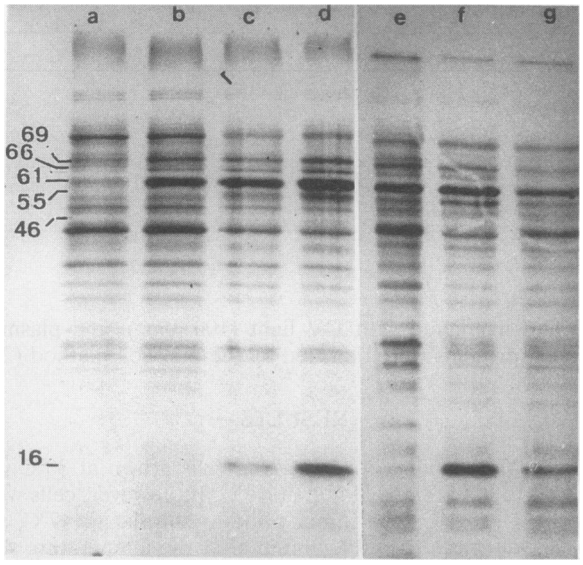

FIG. 2. Autoradiogram of *V. cholerae* 569B Hsps. Cells were labeled with [^{35}S]methionine for 10 min at elevated temperatures, and the proteins were analyzed by SDS-PAGE. Cells grown at 30°C (a) were shifted to 37°C (b), 42°C (c), and 44°C (d). Cells grown at 37°C (e) were shifted to 47°C (f) and 50°C (g). Lanes a, b, c, and d and lanes e, f, and g were from different gels. Equal counts per minute were loaded onto each lane for each gel. Numbers in the margin indicate the sizes of the proteins in kilodaltons.

46, and 16 kDa (Fig. 2, lanes a to d). The most abundant Hsps were the 61- and 16-kDa proteins. The 61-kDa Hsp has been identified as GroEL by overlay of autoradiographs on Western blots of labeled *V. cholerae* cell lysates probed with antisera against purified Hsp60. The amount of Hsps increased with temperature up to 47°C. When the cells were shifted to 50°C, there was very little uptake of radioactivity in the 10-min pulse time and none of the Hsps could be detected on the autoradiograph. The cell viability also declined drastically following the shift to 50°C. When cells grown at 37°C were shifted to higher temperatures, all six Hsps were induced (Fig. 2, lanes e to g), although there was a reduction in the amount of Hsps synthesized at temperatures above 47°C (Fig. 2, lane g). When cells grown at 30°C were shifted to 44°C in the presence of rifampin, no induction of Hsps was observed, suggesting that the synthesis of Hsps is controlled at the level of transcription as reported for other organisms (18).

For a more detailed analysis of the Hsps induced in *V. cholerae*, cells grown at 30°C were pulse-labeled for 10 min with [^{35}S]methionine at 30 and 44°C and proteins were analyzed by two-dimensional gel electrophoresis and autoradiography (Fig. 3). Sixteen proteins whose expressions were enhanced following heat shock were identified. The molecular sizes of these proteins ranged between 88 and 13 kDa. The molecular weights of the major Hsps detected on two-dimensional gels corresponded to those identified in one dimension. One of the major Hsps with a molecular mass of 16 kDa produced three spots in two-dimensional gels, two of which corresponded to a size of 16 kDa but differed in their isoelectric point; the third had a molecular mass of 17 kDa. In some two-dimensional gels, the 61-kDa protein could also be resolved into two spots of the same molecular weight. The 23-kDa Hsp consisted of two proteins of the same size (Fig. 3b).

To examine whether the Hsps offer protection to cells at higher temperatures, cells grown at 30°C were incubated at 44°C for 10 min to allow synthesis of the Hsps. Chloramphenicol (4 μg/ml) was then added to the cells at 44°C, and cell survival at different times was compared with the survival of cells to which chloramphenicol was added at the time of temperature shift (Fig. 1b). While about 40% of cells were viable after 40 min of incubation at 44°C when chloramphenicol was added 10 min after the temperature shift, only about 0.5% of cells were viable when chloramphenicol was added at the time of the heat shock. This observation showed that, as expected, the induction of the Hsps was necessary for survival of *V. cholerae* cells at higher temperatures.

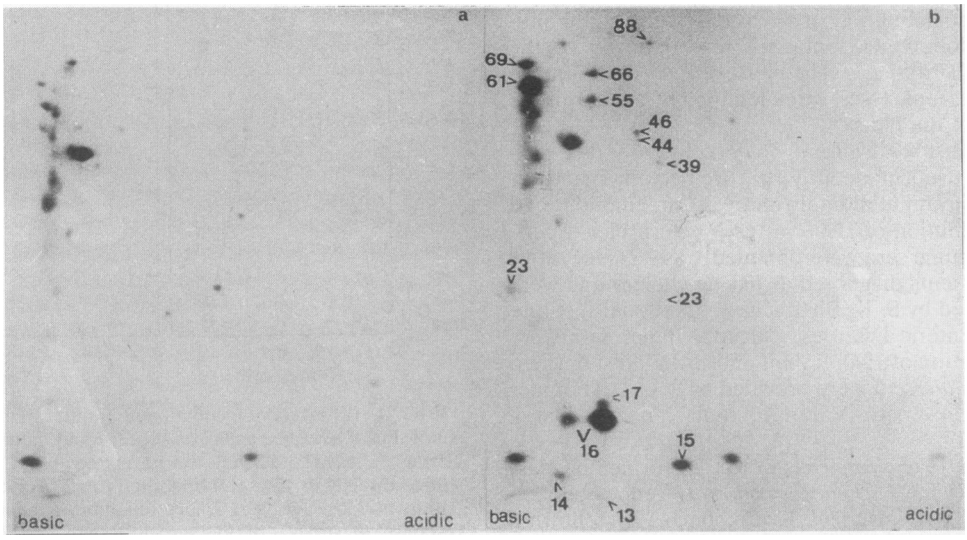

FIG. 3. Autoradiograms of two-dimensional gels showing the Hsps of *V. cholerae* 569B. Cells labeled with [^{35}S]methionine at 30°C (a) and 44°C (b) were analyzed by two-dimensional SDS-PAGE and autoradiography. Total cellular proteins were separated by isoelectric focusing in the first dimension and by SDS-PAGE in the second dimension as described in Materials and Methods. Equal counts per minute were loaded onto each gel. Hsps are indicated by arrowheads in panel b. The numbers indicate the molecular masses of the proteins in kilodaltons.

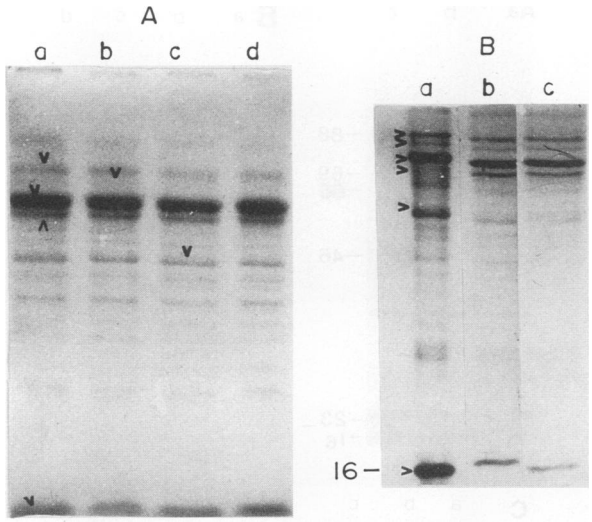

FIG. 4. Stability of Hsps at elevated and normal growth temperatures. *V. cholerae* cells were labeled at 42°C for 10 min with [^{35}S]methionine, and the pulse-labeled proteins were chased at 42°C (A) after suspension in cold medium. Lanes: a, 0 min; b, 20 min; c, 40 min; d, 60 min. (B) Pulse-labeled proteins at 42°C were chased following suspension in cold medium at 37°C. Lanes: a, 0 min; b, 20 min; c, 40 min. Arrowheads indicate the positions of the Hsps.

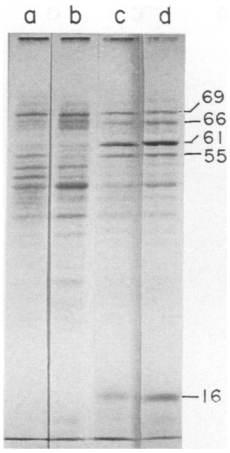

FIG. 5. Induction of *V. cholerae* Hsps in the presence of *E. coli* σ^{32}. *V. cholerae* 569B cells transformed with plasmid pBR322 or pKV7 were grown at 30°C, heat shocked at 42°C, and labeled with [^{35}S]methionine. The pulse-labeled proteins were analyzed by SDS-PAGE and autoradiography. Lanes a and c, *V. cholerae* 569B cells containing plasmid pBR322 labeled for 15 min at 30°C and 5 min at 42°C. Lanes b and d, *V. cholerae* 569B cells containing plasmid pKV7 labeled for 15 min at 30°C and 5 min at 42°C. Equal counts per minute were loaded onto lanes c and d. The numbers indicate molecular masses of proteins in kilodaltons.

Kinetics of the heat shock response. To examine the kinetics of induction of the major Hsps, cells were pulse-labeled for 5 min with [^{35}S]methionine at different times after the transfer of the culture from 30 to 42°C and proteins were analyzed by SDS-PAGE and autoradiography. All of the Hsps could be resolved in the gel within the first 10 min of heat shock, and the maximum induction of all of the proteins occurred by 20 min after transfer. After 25 min, the rate of synthesis of Hsps declined to a steady-state value (data not shown).

Stability of Hsps. To determine the stability of *V. cholerae* Hsps at elevated temperatures as well as that after shift down, cells at 42°C were labeled with [^{35}S]methionine for 10 min and the labeled proteins were chased following suspension of cells in cold medium. Pulse-labeled cells were washed, resuspended in a fresh medium, and incubated at either 42 or 37°C. Samples were removed at different times during incubation, and proteins were analyzed by SDS-PAGE and autoradiography. There was no change in the amount of any of the Hsps up to 90 min for cells maintained at 42°C (Fig. 4A, lanes a to d).

When the fate of the pulse-labeled proteins synthesized at 42°C was examined after shift down to 37°C, the amount of high-molecular-weight Hsps was observed to remain unaltered (Fig. 4B). These proteins are normally synthesized at 37°C (Fig. 2, lanes b to e). However, the amount of the 16-kDa protein was drastically reduced within 40 min of temperature shift down (Fig. 4B, lane c). The reason for the preferential degradation of the 16-kDa Hsp at 37°C is not clear.

***E. coli* σ^{32} factor induces synthesis of the Hsps in *V. cholerae*.** To examine whether the *E. coli* σ^{32} protein, the positive regulator of the heat shock response in *E. coli*, can also induce heat shock genes in *V. cholerae*, plasmid pKV7 carrying the *E. coli rpoH* gene was cloned into *V. cholerae* 569B cells. The *E. coli rpoH* gene in plasmid pKV7 is expressed from its own promoter (37). It was confirmed by maxicell analysis that the *E. coli* σ^{32} protein is expressed in *V. cholerae* cells carrying plasmid pKV7. A 32-kDa protein was detected in the maxicells of *V. cholerae* carrying plasmid pKV7 but not in *V. cholerae* cells carrying the vector pBR322 (data not shown). *V. cholerae* 569B cells carrying either plasmid pBR322 or pKV7 were grown at 30°C and transferred to 42°C. The cells were labeled for 5 min after transfer with [^{35}S]methionine, and the amounts of Hsps synthesized were compared by analyzing the cell lysates by SDS-PAGE and autoradiography. The amount of Hsps synthesized in the cells transformed with plasmid pKV7 (Fig. 5, lane d) was more than that synthesized in *V. cholerae* 569B cells carrying plasmid pBR322 (Fig. 5, lane c). Densitometric scanning of the autoradiographs showed that the synthesis of the Hsps in *V. cholerae* cells transformed with plasmid pKV7 was two- to threefold higher. However, the amount of Hsps synthesized in the cells carrying pKV7 at 30°C (Fig. 5, lane b) was similar to that in *V. cholerae* cells transformed with plasmid pBR322 (Fig. 5, lane a). The increased synthesis of Hsps at higher temperatures in the presence of *E. coli* σ^{32} protein suggests that a σ^{32}-like factor might be responsible for the induction of the Hsps in *V. cholerae*.

Intracellular location of Hsps. The outer membrane, inner membrane, and cytoplasmic proteins were isolated from *V. cholerae* cells labeled with [^{35}S]methionine after transfer to 42°C and analyzed by SDS-PAGE. The 69-, 55-, 46-, and 16-kDa Hsps were detected exclusively in the inner membrane (Fig. 6, lanes c and d), while the 66- and 61-kDa proteins were observed in both the inner membrane and cytoplasmic fractions (Fig. 6, lanes a to d). The 23-kDa Hsp was detected in the outer membrane (Fig. 6, lanes e and f).

Interaction of Hsps with immune sera. To investigate whether any of the *V. cholerae* Hsps are antigenic, either whole-cell lysates or solubilized membrane proteins of *V. cholerae* 569B cells grown at 30°C and heat shocked at 37 or 42°C were separated by SDS-PAGE, blotted onto nitrocellulose, and reacted with human immune sera collected from cholera patients and rabbit antisera raised against subcutaneously administered live *V. cholerae* cells. With human immune sera obtained from cholera patients 21 days after infection, several immunoreactive bands were detected (Fig. 7A).

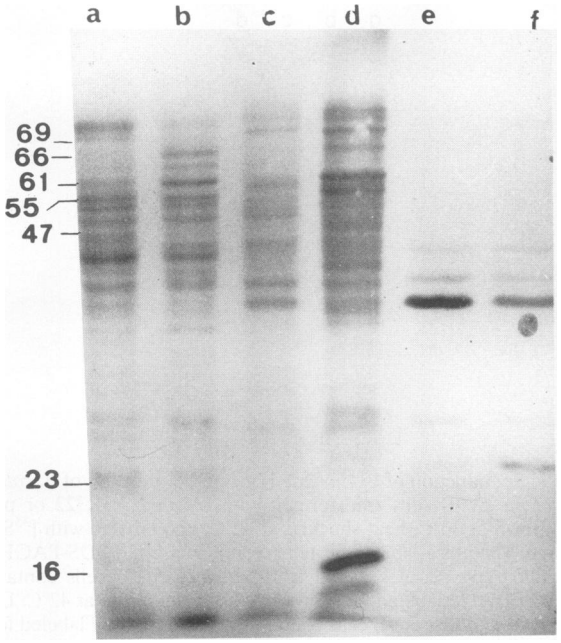

FIG. 6. Intracellular localization of the Hsps. *V. cholerae* cells grown at 30°C were transferred to 42°C and labeled with [^{35}S]methionine for 10 min. Cytoplasmic and inner and outer membrane proteins were isolated and analyzed by SDS-PAGE and autoradiography. Lanes a, c and e, cytoplasmic and inner and outer membrane proteins, respectively, of cells grown at 30°C. Lanes b, d, and f, cytoplasmic and inner and outer membrane proteins, respectively, from heat-shocked (42°C) cells. The numbers indicate the molecular masses of proteins in kilodaltons.

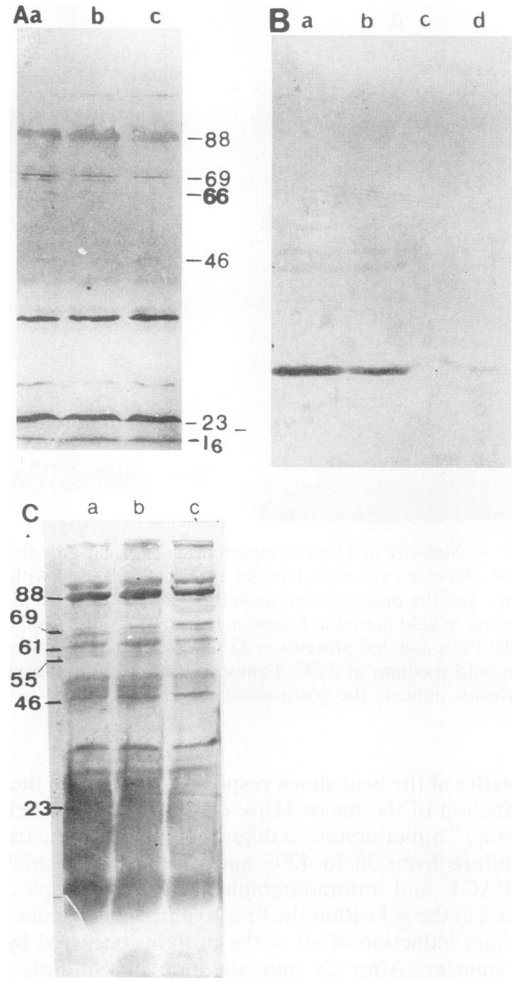

FIG. 7. Identification of *V. cholerae* Hsps recognized by human and rabbit immune sera. (A) *V. cholerae* 569B cells grown at 30°C (lane c) were exposed for 10 min to 37°C (lane b) or 42°C (lane a). Total cellular proteins were separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose. The blots were probed with human immune serum collected from cholera patients 21 days after infection. (B) Inner and outer membrane proteins from *V. cholerae* 569B cells grown at 30°C and heat shocked at 42°C were isolated, separated by SDS-PAGE (12.5% acrylamide), blotted on nitrocellulose, and reacted with serum obtained from cholera patients 1 day after infection. Lanes a and c, outer and inner membrane proteins from heat-shocked cells. Lanes b and d, outer and inner membrane proteins from 30°C-grown cells. (C) *V. cholerae* 569B cells were grown at 30°C (lane a) and heat shocked for 10 min at 37°C (lane b) or 42°C (lane c). Cell lysates were analyzed by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit antisera prepared as described in Materials and Methods. The immunoreactive bands were visualized with goat anti-human or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (A and B) or alkaline phosphatase (C). The numbers in the margins indicate molecular masses of the proteins in kilodaltons.

Among these, the 69-, 66-, 46-, 23-, and 16-kDa bands were identified as Hsps by overlay of the autoradiograph on Western blots. That the highly immunoreactive 23-kDa protein represents the 23-kDa outer membrane Hsp was confirmed by immunoblotting the outer membrane proteins. The 16-kDa major Hsp located in the inner membrane reacted with the immune sera in immunoblots of solubilized inner membrane proteins. The 88-kDa major immunoreactive band (Fig. 7A, lanes a to c) may correspond to the 88-kDa minor Hsp detected by 2-dimensional SDS-PAGE (Fig. 3). The human immune sera did not react with the 61-kDa GroEL Hsp of *V. cholerae*. In most pathogenic bacteria, the GroEL protein has been reported to be a major antigen (36). Human serum obtained from the same cholera patient 1 day after infection was used as a control in these experiments. None of the antigenic Hsps detected in the convalescent serum could be seen in these Western blots except the 23-kDa Hsp (Fig. 7B). However, this protein was not detected when the normal human serum was used as probe.

Rabbit antisera reacted with all of the major Hsps identified in this study (Fig. 7C). The 88-kDa major immunoreactive band was also detected in this case. With some rabbit antisera a strong but diffuse region of immunoreaction was detected in the region of 30 to 20 kDa. This may correspond to *V. cholerae* lipopolysaccharides, which are known to be immunogenic (13). The normal rabbit sera did not show any reactivity against *V. cholerae* proteins.

Hsps in animal-passaged and laboratory-maintained cells. It has been reported that during laboratory subculturing of *V. cholerae* cells several metabolic functions are altered along with a reduction in the toxinogenicity of the cells. All of these functions can be restored by a single passage of the cells in guinea pigs (26). It was intriguing to examine whether the induction of the Hsps was affected in laboratory-maintained cells. All six major Hsps were induced in freshly animal-passaged hypertoxinogenic strain 569B of *V. cholerae* (Fig. 2). When the animal-passaged *V. cholerae* cells were maintained in the laboratory for approximately 1 year, with subculturing each month, a substantial reduction in the amount of 16-kDa Hsp

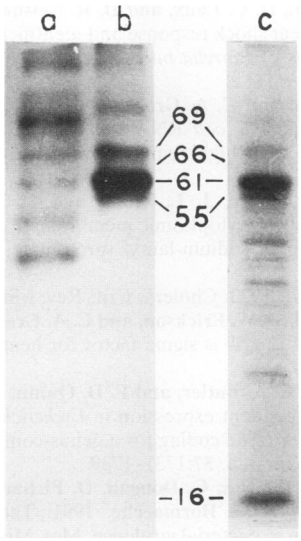

FIG. 8. Hsps of laboratory-maintained *V. cholerae* 569B cells. Cells at 30°C or after transfer to 42°C were labeled with [^{35}S]methionine for 10 min, and proteins were analyzed by SDS-PAGE and autoradiography. Lanes a and b, laboratory-maintained cells at 30 and 42°C. Lane c, animal-passaged cells at 42°C. Lane c is from a different gel. The numbers in the margin indicate molecular masses of the proteins in kilodaltons.

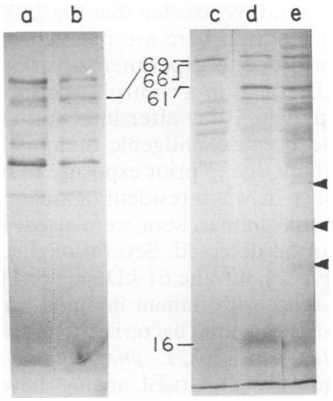

FIG. 9. SDS-PAGE analysis of proteins induced by other stress stimuli. *V. cholerae* 569B cells were grown at 30°C to the logarithmic phase and were labeled for 10 min with [^{35}S]methionine at 30°C (lane c) and after transfer to 42°C (lane d). The 30°C-grown cells were labeled for 20 min in the presence of 0.09 M NaCl (lane a), 0.35 M NaCl (lane b), or 6% ethanol (lane e). Arrowheads indicate ethanol-induced proteins other than Hsps. Numbers indicate molecular masses of proteins in kilodaltons.

was observed (Fig. 8, lane b), and this protein could only be detected at a very low level on overloaded gels and on longer exposure of the autoradiographs. The synthesis of 16-kDa Hsp resumed following a single passage in a guinea pig (Fig. 8, lane c). However, synthesis of the other Hsps was not affected during laboratory subculturing (Fig. 8, lane b). Parenthetically, these laboratory-maintained cells showed increased sensitivity to heat and low pH compared with the animal-passaged cells.

Hsps in strains with varying degrees of virulence. Physiological responses to varying external conditions have been shown to be related to the level of toxinogenicity of *V. cholerae* strains (5). The induction of Hsps has been investigated in several strains of *V. cholerae* belonging to the biotypes classical and El Tor and differing in degree of virulence and was compared with Hsps induced in the hypertoxinogenic strain 569B. The strains examined included the nontoxinogenic strains EW6, GP5, and GP7 and the mildly toxinogenic strains 154 and MAK757. No difference in the expression of the major Hsps could be detected in these strains (data not shown).

Other stress stimuli. Since many of the Hsps are known to be induced by stress conditions other than elevated temperatures, the induction of the Hsps by ethanol, low pH, and high salt concentration was examined. Exposure of *V. cholerae* cells to 0.2 to 0.35 M NaCl resulted in the induction of only the 69-kDa Hsp (Fig. 9, lanes a and b). Following treatment with 6% ethanol for 20 min, four of the major Hsps were induced (Fig. 9, lane e). Besides the Hsps, three proteins with molecular sizes of 37, 25, and 22 kDa were induced (Fig. 9, lane e, arrowheads). When the cells were exposed to pH 5 and 6, the 23-kDa protein, in addition to several other proteins, was induced (data not shown).

DISCUSSION

A set of 16 Hsps are induced in *V. cholerae* cells in response to small and large elevations of temperature either in the range of the normal growth temperature or to supraoptimal temperatures. The induction of the Hsps is necessary for *V. cholerae* cells to survive the deleterious effects of heat, similar to that observed in other organisms (18). Inhibition of synthesis of the Hsps at the transcriptional or translational level makes the cells considerably more sensitive to high temperatures (Fig. 1). In contrast to *E. coli*, in which the stimulation of Hsp synthesis is maximum around 5 min after temperature shift (10, 34), the synthesis of Hsps in *V. cholerae* is maximum 20 min after transition to higher temperatures. The delayed induction of the Hsps may account for the observed greater sensitivity of *V. cholerae* cells to sublethal temperatures compared with *E. coli* (25).

In *E. coli*, σ^{32} factor is required for the expression of the heat shock genes at higher temperatures, and its concentration regulates the cellular response to heat (6). The enhanced synthesis of the Hsps following temperature shift in *V. cholerae* cells carrying the *E. coli rpoH* gene, coding for the σ^{32} protein, in a multicopy plasmid suggests that an *E. coli* σ^{32}-like factor might be necessary for the induction of the Hsps in *V. cholerae*. The presence of the *E. coli rpoH* gene in a multicopy plasmid, however, failed to enhance the synthesis of the Hsps at 30°C in *V. cholerae*. This is not surprising in view of the fact that *E. coli* σ^{32} protein is not stable at 30°C (6), and hence the amount necessary to increase the synthesis of the Hsps above the basal level could not accumulate at this temperature.

Several *E. coli* heat shock genes whose products are necessary for synthesis of the Hsps at 50°C and above have been identified (3, 20, 23). The drastic reduction in survival of *V. cholerae* cells upon severe heat shock (30 to 50°C) and inability of the cells to synthesize Hsps under these conditions might be due to the absence of one or more of these genes. Whether an *E. coli* σ^{E}-like factor required for the transcription of the gene (*rpoH*) coding for σ^{32} at 50°C (3) is missing in *V. cholerae* is not known.

Among the outer membrane proteins of *V. cholerae*, only the 23-kDa protein is an Hsp. This protein is highly antigenic, and antibodies against it are produced in substantial amounts in the sera of convalescent cholera patients (Fig. 7A). A 25-kDa outer membrane protein of *V. cholerae* has been reported to be

a major antigen (13). It is possible that the 25-kDa protein and the 23-kDa Hsp reported here are the same protein and the difference in size is due to experimental variation. The observation of a 23-kDa band in a control experiment using serum from the same patient 1 day after infection (Fig. 7B, lanes a and b) might be because antigenic memory of this protein existed in the patient due to prior exposure to subclinical levels of *V. cholerae*, since he was a resident of an area of endemicity. When other normal human sera were used as controls, this protein could not be detected. Several of the other Hsps are also antigenic (Fig. 7), but the 61-kDa major Hsp identified as GroEL did not react with human immune sera from cholera patients. In most pathogenic bacteria examined so far, including *Mycobacterium* spp. (36), *L. pneumophila* (7), *C. burnetii* (33), and *B. burgdorferi* (2), GroEL analogs have been shown to be highly antigenic.

Although the 16-kDa protein is one of the most abundant Hsps, it is not synthesized following temperature upshift in cells maintained in the laboratory for an extended period of time (Fig. 8, lane b), and these cells are unusually sensitive to high temperature and some other forms of stress. When the laboratory-subcultured cells were passaged in guinea pigs, the synthesis of the 16-kDa protein resumed upon heat shock (Fig. 8, lane c), and the sensitivity of the cells to high temperatures and other stress conditions was reduced. These observations suggest that the 16-kDa protein is necessary for survival of cells at high temperatures. It has been reported that during laboratory subculturing the toxinogenicity of the cells is reduced; this could be restored following a single passage in guinea pigs (26). Whether the 16-kDa protein has any role in virulence is yet to be investigated. This protein is antigenic and antibodies against this inner membrane Hsp could be detected in human sera collected from convalescent cholera patients (Fig. 7A).

In many bacterial pathogens, including *V. cholerae*, proteins that play important roles in virulence are known to be regulated by environmental signals (15). It has been proposed that the divergent transcription of an *htpG*-like heat shock gene in *V. cholerae* leads to a proportionate decrease in the expression of *toxR* coding for a transmembrane DNA-binding protein that positively regulates transcription of the genes for cholera toxin and other virulence determinants. Hence, there is a decrease in enterotoxin production with increase in temperature (22). The decreased levels of ToxR have been postulated to result in an increased level of expression of several genes which may be important at the early stages of the infection cycle of the bacteria in the host intestine. A study of the heat shock response in *V. cholerae* is expected to contribute to an understanding of the fundamental aspects of host-parasite interaction leading to the disease.

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