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Stress- and Growth Phase-Associated Proteins of *Clostridium acetobutylicum*

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The response of *Clostridium acetobutylicum* ATCC 4259 to the stresses produced by a temperature upshift from 28°C to 45°C and by exposure of the organisms to 0.1% *n*-butanol or to air was examined by analysis of pulse-labeled proteins. The stress response was the induction of the synthesis of a number of proteins, some of which were elicited by the three forms of stress. Eleven heat shock proteins were identified by two-dimensional electrophoresis, as were two proteins whose synthesis was heat sensitive. In the absence of applied stress, the synthesis of four proteins was found to be associated with the growth phase in batch culture; three of these proteins had a higher rate of de novo synthesis when the cells entered the solvent production phase. One of the stress-induced proteins, hsp74, was partially purified an found to be immunologically related to *Escherichia coli* heat shock protein DnaK. The similarities of the proteins induced at the onset of solventogenesis and by stress suggest a relationship between the two processes.

Clostridium acetobutylicum cells growing in batch culture ferment sugars to butyric and acetic acids, which decrease the external pH to below 5 at the end of exponential phase (for reviews, see references 17 and 37). During stationary phase, the sugar and the preformed acids are converted to solvents (butanol, acetone, and ethanol) and traces of acetoin; H_2 and CO_2 are also produced. The switch of catabolism to solventogenesis has the effect of raising the medium pH. As stationary phase progresses, the cells undergo a series of morphological and physiological changes in motility, shape, and granulose content, culminating in the formation of endospores (16). The ability to produce solvents is associated with an early step in sporulation (17, 37). The accumulation of solvents causes the cell membranes to become progressively more permeable to small molecules, as shown by decreased rates of glucose uptake and glycolysis, dissipation of the H^+ gradient across the cell membrane (42), loss of internal glycolytic intermediates (15), and decrease in the viability of the vegetative cells (24). Thus, it is likely that solvents, endogenously produced membrane-chaotropic agents, are stressful to the cells.

The response of cells to stress has been studied intensively in recent years (for reviews, see references 9, 10, 18, 23, 26, 31, and 32). The most-examined form of stress, heat shock, has been shown to result in the repression of the synthesis of most cellular proteins, while a specific set of ca. 20 heat shock proteins (hsps) are induced in response to temperature upshifts. These proteins are thought to participate in the cellular response (or adaptation) to stress, as many of them are also induced by exposure of bacterial cells to a variety of stresses, including alcohols (27, 48), oxygen or H_2O_2 (11, 14, 30, 36), toxic metals (46), alkaline shift (40), and other stresses (38). Some of the hsps are highly conserved among archaebacteria (12), eubacteria, and eucaryotes (4, 5). Moreover, the stress response has been shown to have a developmental role or to be regulated developmentally in a number of higher organisms (7, 8, 19, 20, 22, 25, 47, 50), as well as in procaryotes (2, 33, 35, 36, 39, 44), and to play a role in cell division of procaryotes (45).

In this study, we report the induction in *C. acetobutylicum* cells of a set of hsps after exposure of the cells to heat, butanol, and oxygen. One of the hsps is antigenically related to the *Escherichia coli* hsp, DnaK. Moreover, specific proteins related to hsps are associated with the clostridial growth phase in batch culture.

MATERIALS AND METHODS

Growth of cells. *C. acetobutylicum* ATCC 4259 cells, supplied by CPC International, Inc., Moffett Technical Center, Summit-Argo, Ill., were grown in a defined medium (1), except that sodium dithionite was omitted and sodium acetate was added (5 g/liter). Separately deoxygenated and autoclaved glucose was added to a final concentration of 6% (wt/vol). Anaerobic growth methods, preparation of medium, and inoculation conditions have been described previously (6, 15). The fermentation end products were measured by gas chromatography to determine the growth phase of the batch cultures (42). During the exponential phase, the medium contained the 13 mM butanol contributed by the inoculum (42). After exponential growth ceased, the cells switched to butanol production, to a final butanol concentration of 164 mM.

Pulse-labeling of clostridial proteins. Cells were grown at 28°C to the desired growth phase, as determined by gas chromatography of the fermentation end products, and subjected to the indicated stress. [^{3}H]leucine (20 μCi for one-dimensional gels, 40 μCi for two-dimensional gels; 40 to 60 Ci/mol) (ICN Radiochemicals, Irvine, Calif.) was added to 0.5-ml portions of the cultures. For heat shock, 3 ml of the culture was placed with [^{3}H]leucine in a 45°C water bath for 10 min, and then 0.5-ml portions were pulse-labeled for an additional 15 min at 45°C. For butanol shock, cells growing at 28°C were incubated for 15 min with 0.1% (vol/vol; 11 mM) *n*-butanol, and then 0.5-ml portions were pulse-labeled for 30 min. Control cells were labeled for 30 min at 28°C to allow sufficient incorporation of the label. After being pulse-labeled, the samples were placed on ice, chased with nonradioactive leucine (final concentration, 0.5 mM), incubated on

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ice for another 5 min, and then centrifuged at 13,000 × *g* for 3 min. The supernatants were decanted, and the cell pellets were washed once with 50 mM citrate–Tris buffer, pH 5.5. The pellets were then suspended in sodium dodecyl sulfate (SDS) sample buffer for one-dimensional gel electrophoresis (41) or in sonication buffer for two-dimensional gel analysis (34). The samples, kept cold in an ice bath, were sonicated with six 15-s pulses at 50 W (model W185 sonifier; Branson Sonic Power Co., Danbury, Conn.).

Gel electrophoresis. One-dimensional SDS–12% polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (21). The SDS-PAGE samples were boiled for 3 min, and the gels were loaded with 3×10^5 cpm per lane. Virtually all the radioactive counts in the samples were in trichloroacetic acid-precipitable material. Two-dimensional analysis was performed by the method of O'Farrell (34) on SDS–12% polyacrylamide gels. The samples were prepared as described previously (34), and each gel was loaded with 10^6 cpm. The ampholytes (Bio-Rad Laboratories, Richmond, Calif.) were used at a final concentration of 2% to provide a pH range of 5 to 8 (ampholytes 3/10 and 5/7 in a ratio of 1:4, respectively). The first-dimension gels were run at 7,800 V/h. After electrophoresis, the gels were stained with Coomassie blue, destained, fluorographed with En^{3}Hance (Dupont-New England Nuclear Corp., Boston, Mass.), dried, and exposed to X-ray film at −80°C. The term hsp is used here for both heat shock protein and heat-inducible protein.

Separation of soluble and membrane-bound proteins. Cells were heat shocked and labeled as described above. After sonication, the samples were centrifuged at 5,000 × *g* for 15 min at 4°C to remove unbroken cells and cell debris. The supernatants were centrifuged at 100,000 × *g* for 1 h at 4°C to pellet the membranes. The resulting supernatants and pellets were prepared for one-dimensional gel electrophoresis as described above.

Column chromatography and protein purification. Cultures were grown at 28°C as described above, and 50 ml of cells was labeled with 47 μCi (1.2 mCi total) of [^{3}H]leucine per ml, chased with nonradioactive leucine, washed, and sonicated, as described above, in buffer B (49) containing 1% Triton X-100 and 0.5% sodium deoxycholate. The sonicated material was applied to a DEAE-cellulose column (DE52; Whatman, Inc., Clifton, N.J.), the column was washed with buffer B, and proteins were eluted with a 20 to 350 mM linear gradient of NaCl in buffer B. Fractions were collected and prepared for one-dimensional gel electrophoresis as described above.

Immunoprecipitation. *C. acetobutylicum* was grown at 28°C as described above to mid-acid phase, and *Escherichia coli* DH5Aα was grown at 28°C in medium M9 (28) to an optical density at 625 nm of 1.0. Cells were labeled with 250 μCi of [^{3}H]leucine. *C. acetobutylicum* (3 ml) was heat shocked as described above. As a control, *E. coli* (5 ml) was labeled for 7 min at 28°C and heat shocked by shifting to 42°C for 3 min and incubating with label for 5 min at 42°C. The cells were chased with nonradioactive leucine (0.5 mM), washed, and sonicated, as described above. Sonic extracts were then solubilized in 1% (vol/vol) Triton X-100 and 0.1% (vol/vol) SDS at 90°C for 5 min (*E. coli*) or 10 min (*C. acetobutylicum*). Insoluble material was removed by centrifugation in a microcentrifuge for 15 min. The volume of the solubilized extract containing 2×10^6 cpm of radioactivity was adjusted to 100 μl with incubation buffer (2), and anti-DnaK antiserum (51) was added. The mixture was incubated at 4°C overnight, samples were prepared (2), and the precipitated proteins were electrophoresed as described above.

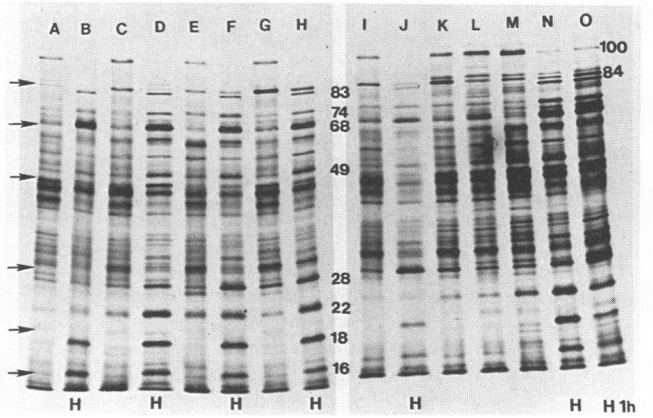

FIG. 1. hsps synthesized by *C. acetobutylicum* cells at various phases of growth. Cells were pulse-labeled with [^{3}H]leucine and analyzed by SDS-PAGE after temperature upshift from 28°C to 45°C (marked with H under the lanes) for 0.5 h (lanes B, D, F, H, J, and N) or for 1 h (lane O). The growth phase was determined on the basis of the fermentation end products revealed by gas chromatography. Cells were sampled during consecutive stages of the acidogenic phase (lanes A and B, early acid phase; lanes C and D, mid-acid phase; lanes E and F, late acid phase); at the time that the switch to solvent production occurred (lanes G and H); and during the solventogenic phase (lanes I and J [35 mM butanol], lane K [50 mM butanol], and lane L (70 mM butanol]). Lanes M to O were obtained with acid-phase cells from a different culture. The arrows at the left indicate molecular mass markers at 92, 66, 45, 31, 21, and 14 kDa. The most-prominent protein bands elicited by heat stress are indicated by their apparent molecular masses (in kilodaltons; numbers between lanes H and I). The most-prominent heat-sensitive protein bands are indicated at the right of lane O.

RESULTS AND DISCUSSION

Heat shock response in *C. acetobutylicum*. Pulse-labeling of *C. acetobutylicum* cells growing at 28°C for 0.5 h with [^{3}H]leucine resulted in a large number of newly synthesized proteins demonstrable as bands in one-dimensional gels (Fig. 1). Radioactive protein bands were seen in extracts from cells assayed at various phases of the growth cycle from the acid-producing phase well into the solvent-producing phase, when the cultures had accumulated 70 mM butanol. Later in the solvent-producing phase, the rate of incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material decreased (data not shown), showing decreased protein synthesis ability. When the temperature of the culture was raised to 45°C for 10 min and the cells were pulse-labeled subsequently for 15 min at 45°C, a number of hsps were induced (Fig. 1). The hsps were seen in extracts of cells throughout the acid-producing phase and in the solvent-producing phase up to the point when 35 mM butanol was present, somewhat before the midpoint of the solventogenic phase. The following eight of the most-prominent protein bands (designated by their apparent molecular masses) were found to be hsps of 83, 74, 68, 49, 28, 22, 18, and 16 kilodaltons (kDa) (Fig. 1, Table 1). The synthesis of most of these protein bands was increased as a result of heat stress compared with unstressed-control synthesis levels, whereas the 83-kDa hsp band was not visible without the temperature upshift. In contrast to the synthesis of hsps, the synthesis of at least two protein bands, at 100 and 84 kDa, was reduced

TABLE 1. Stress proteins in *C. acetobutylicum*[a]

Mass (kDa)	Relative band intensity of cells stressed with:			Cellular location[b]	Duration (h) of response[c]
	Heat	Butanol	Air		
83	++	+		M	>4
74	++	+		M	1
68	+++	++	+	M and C	<1
49	+			M	<1
28	+			M and C	<4
22	+++	+++	+++	C	<4
18	++	+		M	<4
16	++	+		C	1

[a] One-dimensional electrophoresis was carried out as described in text.
[b] M, Membrane fraction; C, cytoplasmic fraction.
[c] Heat stress was applied as described in the legend to Fig. 1, and then the temperature was lowered to 28°C. The cells were labeled for 0.5 h at 1, 2, 3, and 4 h after the temperature downshift.

by heat treatment. When cells were pulse-labeled at 45°C for 1 h instead of for 0.5 h, differences were noted among the hsps (compare lanes O and N, Fig. 1). The 49-kDa protein band, as well as the 22-, 18-, and 16-kDa protein bands, was less prominently labeled after 1 h, and a novel 35-kDa hsp appeared, suggesting that there were differences in the turnover of the hsps or in the duration of the synthesis of the hsps.

Variability in hsp metabolism was also suggested by the differences in the time required for return of hsp synthesis to pre-heat shock rates (Table 1). In these experiments, cells were temperature shifted to 45°C for 0.5 h and then returned to 28°C and pulse-labeled for 30 min at 1-h intervals. The increased synthesis of the 68- and 49-kDa protein bands was seen for less than 1 h after return to 28°C and for approximately 1 h for the 74- and 16-kDa protein bands. For the 28-, 22-, and 18-kDa proteins, the induction of protein synthesis was no longer noticeable 4 h after removal of the heat stress, whereas the 83-kDa protein was still synthesized at an accelerated rate even 4 h after removal of the stress.

Butanol- and air-induced-stress response. Several of the hsps were clearly stress proteins, because they were also elicited upon exposure of the cells to 0.1% (11 mM) butanol for 30 min. The protein bands at 83, 74, 68, 22, 18, and 16 kDa were found to be butanol shock proteins (Fig. 2, Table 1). The synthesis of the 100-kDa protein was butanol sensitive as well as heat sensitive. The stress response to addition of 11 mM butanol was less pronounced than the response elicited by a temperature upshift of 17°C. However, stress proteins were induced by increasing the butanol concentration by as little as 11 mM throughout the growth cycle (about 6% of the total butanol produced by the cells), even in the later stages of the solvent-producing phase, when 48 mM butanol was present in the culture (Fig. 2, lanes I and J). In other experiments (data not shown), protein synthesis, as determined by the incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material, was decreased by approximately 70% by raising the butanol concentration from 48 to 59 mM. The synthesis of adenylylated nucleotides, another response to stress, has also been found to be less pronounced after addition of butanol than after temperature upshift (3). Thus, the butanol inhibited total protein synthesis but stimulated synthesis of specific stress proteins.

The third stress tested on these obligate anaerobes was exposure to air. Exposure of *C. acetobutylicum* cells to air induced at least two of the stress proteins, the 68- and 22-kDa bands (Table 1). The 100-kDA protein was air sensitive (data not shown).

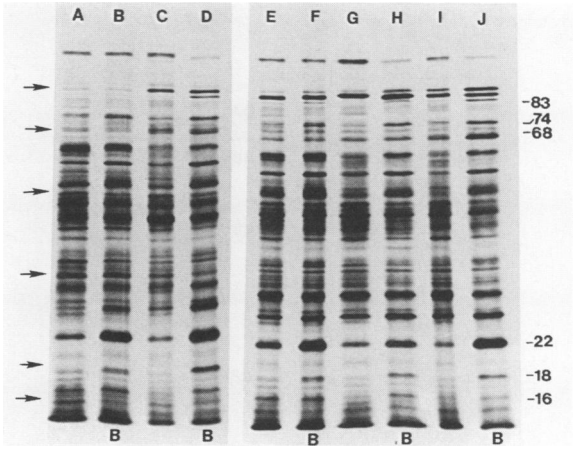

FIG. 2. Butanol shock proteins. The experiment was similar to that described in the legend to Fig. 1, except that the cells were stressed with 0.1% (vol/vol) butanol (11 mM) (lanes marked with B at the bottom) instead of being stressed with a temperature upshift. Consecutive samplings of acid-phase cells (lanes A and B [early in acid phase], lanes C and D [mid-acid phase], lanes E and F [during switch to solvent production], lanes G and H [early in solvent phase, 25 mM butanol], and lanes I and J [mid-solvent phase, 48 mM butanol]) are shown.

Not all stresses elicit the de novo synthesis of the same set of stress proteins in a number of bacteria studied, e.g., *Salmonella typhimurium* (38), *Bacteroides fragilis* (14), and *Zymomonas mobilis* (27), where more than one independent group of stress responses occur. In *C. acetobutylicum*, however, the three stresses tested induced similar proteins but to different degrees. The shift from 28°C to 42°C was more effective in eliciting hsp synthesis than was the addition of 0.1% butanol, suggesting that the former stress was greater.

Cellular location of stress proteins. Acid-phase cells were separated into membrane and soluble fractions, and extracts from each fraction were examined on one-dimensional gels (Fig. 3, Table 1). The 83-, 74-, 49-, and 18-kDa hsps were membrane associated (Fig. 3, lane F). The 22- and 16-kDa hsp bands were seen primarily in the cytoplasmic fraction (lane E), and the 68- and 28-kDa protein bands were found in both membrane and cytoplasmic fractions.

Two-dimensional gel electrophoresis patterns of hsps. The protein bands demonstrating the heat shock response were resolved into individual proteins with the O'Farrell two-dimensional gel system (34) (Fig. 4). Eleven hsps were discerned, at 83, 74, 68, 62, 49a, 49b, 36, 22, 18a, 18b, and 16 kDa (compare Fig. 4b with Fig. 4a). The synthesis of the 100- and 84-kDa proteins was heat sensitive.

Cell cycle-associated proteins. In the absence of heat or butanol stress, a number of protein bands seen in the one-dimensional system were found to vary with the *C. acetobutylicum* growth phase (Fig. 1). These include the bands at 84, 74, 68, and 22 kDa. The 84-kDa band was most prominent during the switch phase (Fig. 1, lane G), and the 68-kDa protein was most prominent in the solvent-producing phase (Fig. 1, lanes I, K, and L). The 74- and 22-kDa protein bands were most prominent in late acid-producing phase (Fig. 1, lanes E and M).

The induction of proteins related to stress proteins during the course of batch culture and cellular differentiation in the absence of an imposed stress has been reported in *Bacillus* spp. (2, 36, 39), as well as in *Myxococcus xanthus* (33),

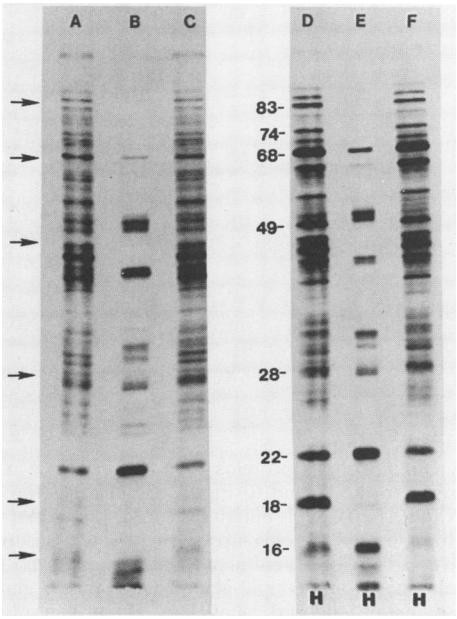

FIG. 3. hsps in membrane and soluble fractions. Cells in the acid phase were pulse-labeled, separated into membrane and soluble fractions, and analyzed by SDS-PAGE as described in the text. Lanes A to C, without temperature shift; lanes D to F, with temperature upshift. Lanes A and D, unfractionated cell sonic extracts; lanes C and F, membrane fractions; lanes B and E, soluble fractions. The most prominent hsp bands are indicated.

Caulobacter crescentus (13, 35), *Zymomonas mobilis* (27), *Methylophilus methylotrophus* (48), and *Bacteroides fragilis* (14). During the sporulation stages of *B. subtilis*, major hsps at 84 and 69 kDa have been found to be analogous to the products of the *E. coli dnaK* and the *groEL* genes, respectively (2). In *Caulobacter crescentus*, cell cycle-regulated hsps have been shown to be synthesized in the absence of heat shock (13) and partitioned specifically to daughter stalked and swarmer cells (35).

Purification of hsps. Because some of the hsps are highly conserved throughout the biological kingdoms, we used methods reported previously to be successful for the 72-kDa mammalian hsps of the 70-kDa-protein family (49) to purify and characterize further the clostridial hsps. Clostridia were pulse-labeled with [^{3}H]leucine, and cell extracts were chromatographed on the anion exchanger DEAE-cellulose. Fractions eluted with a linear gradient of NaCl from acid-phase cell preparations (Fig. 5A) and mid-solvent-phase cell preparations (Fig. 5B) were examined by one-dimensional electrophoresis. The fractions eluted from DEAE-cellulose containing the 74- and 68-kDa proteins (fractions 33 to 45) were pooled and concentrated by lyophilization. Fractions were prepared in the same manner from cells collected during various growth phases. Portions containing the same number of counts per minute were reelectrophoresed on one-dimensional gels (Fig. 6, lanes A to D). The developmental protein bands at 74 and 68 kDa are predominant in gels from cells collected during the switch phase (Fig. 6, lane C), when growth has ceased and solvent production has begun.

To investigate the stability of the proteins, in particular to determine whether they undergo proteolysis as is reported for the mammalian 70-kDa hsp family (29), pooled fractions were electrophoresed in a similar manner; these fractions were kept at 23°C for 48 and 72 h (Fig. 6) and compared with fractions kept at −80°C. On the basis of the relative intensities of bands at 74 and 68 kDa, the bands are less pronounced after 48 h (lanes E to H) and 72 h (lanes I to L) than are bands from control cells (lanes A to D). The clostridial 74- and 68-kDa hsps, like their eucaryotic homologs, are thus unstable at room temperature.

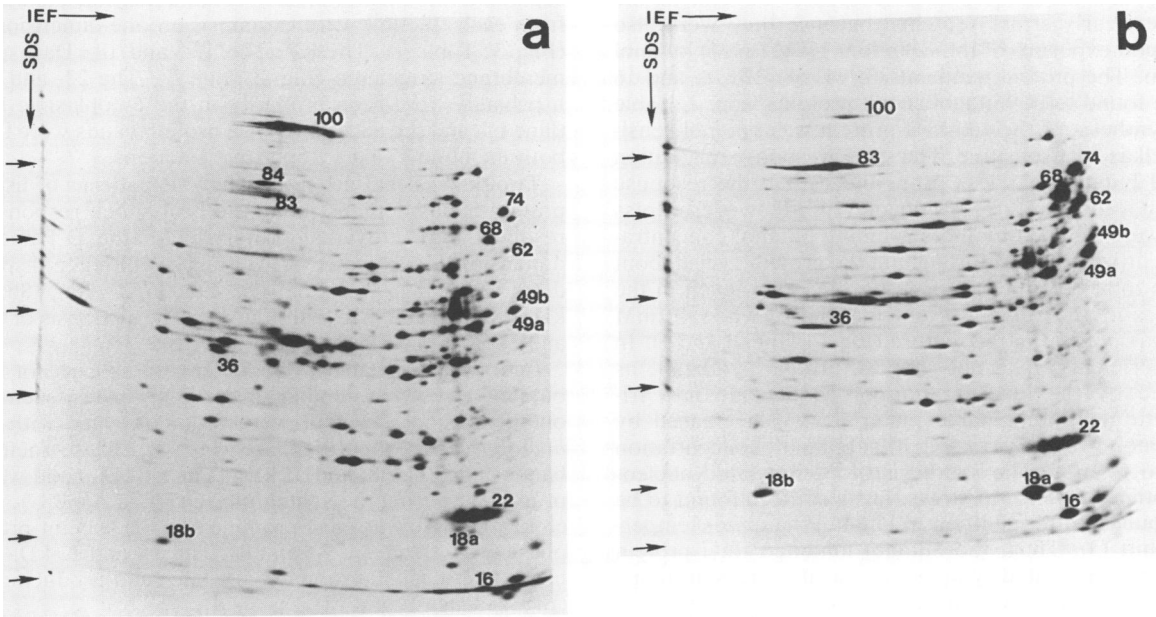

FIG. 4. Two-dimensional analysis of proteins of cell extracts from switch-phase cells (a) and switch-phase cells after heat shock (b). The experiment was carried out as described in the text. The most-prominent hsps and the heat-sensitive proteins are indicated. The numbers indicate molecular sizes (in kilodaltons). The arrows at the left indicate molecular mass markers, as described in the legend to Fig. 1. The isoelectric focusing acidic end is to the right.

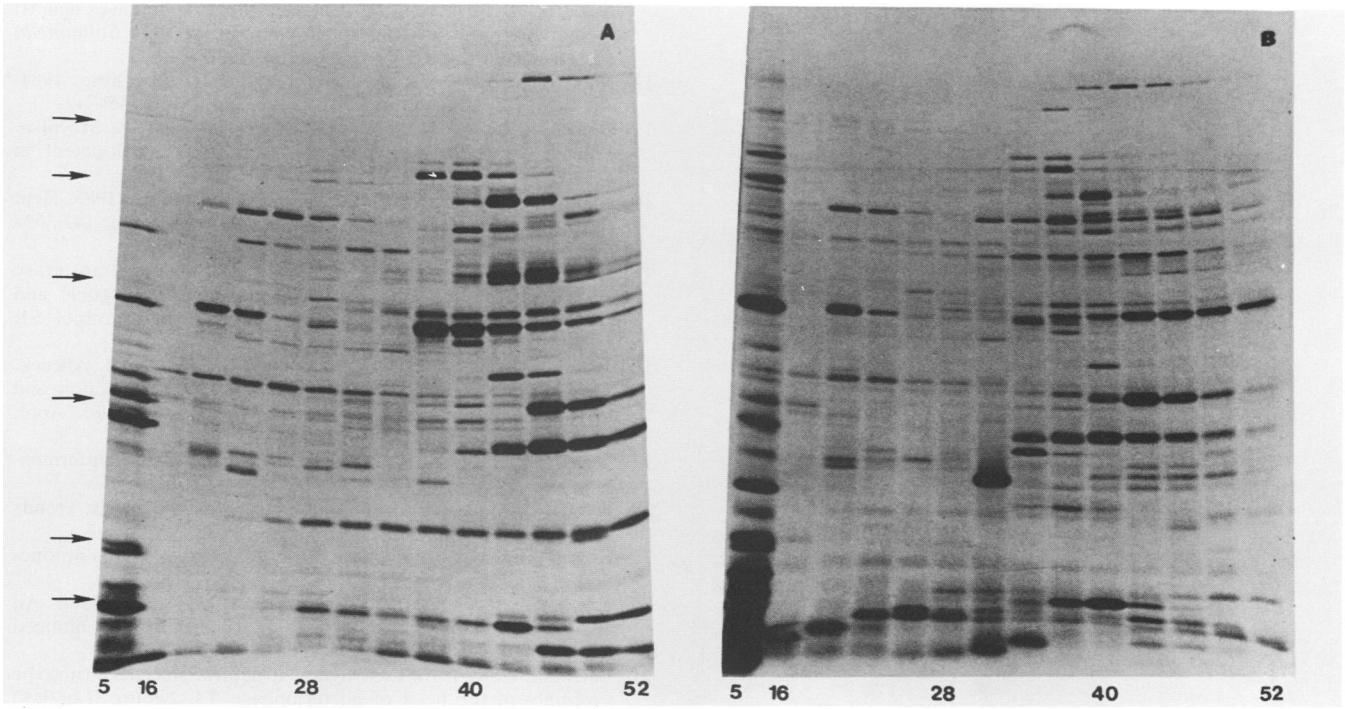

FIG. 5. SDS-PAGE of fractions from DEAE-cellulose chromatography of heat-shocked acid-phase cells (A) and solvent-phase cells (B). Sonic extracts were chromatographed on DEAE-cellulose, and the fractions eluted with NaCl were electrophoresed as described in the text. Fraction 5 and every third fraction thereafter are shown; the next 48 fractions collected are not shown.

Immunoprecipitation of the 74-kDa hsp with anti-DnaK antiserum. *Caulobacter crescentus* and *Bacillus subtilis* have been found to induce the synthesis of proteins antigenically related to the DnaK protein of *E. coli* (2, 13, 35, 43). It seemed likely that *C. acetobutylicum* also would induce a related protein, and indeed, the 74-kDa band (Fig. 7, lanes E and F) was found to cross-react with anti-DnaK antiserum. The clostridial 74-kDa protein band thus matches the 72-kDa DnaK band (lanes A and B) (50). A fainter band was observed at 68 kDa (lanes E and F). In *Caulobacter crescentus*, a similar band has been attributed to cross-reactivity of a GroEL homolog with the anti-DnaK antiserum (35).

The induction of similar patterns of protein synthesis after heat stress and after butanol-induced stress, as well as the de novo synthesis of similar proteins during the solvent-producing phase in the absence of an added stress, suggests a connection between the stress response and solventogenesis in this clostridium. The stress response elicited by low concentrations of butanol produced by the cells may thus be a part of the triggering mechanism that switches metabolism from acidogenesis to solventogenesis and initiates sporulation.

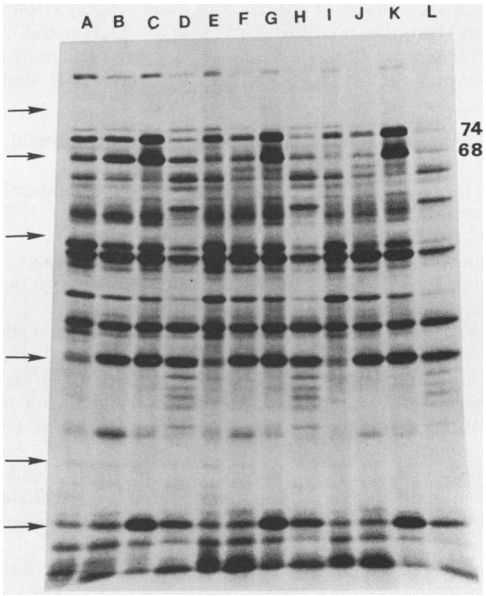

FIG. 6. Partially purified hsps from cells at various growth phases. Shown is the SDS-PAGE pattern of pooled fractions 33 to 45 obtained by DEAE-cellulose chromatography (Fig. 5) from cells early in acid phase (lanes A, E, and I), later in acid phase (lanes B, F, and J), in switch phase (lanes C, G, and K), and in mid-solvent phase (lanes D, H, and L). Control cell extracts kept at −80°C (lanes A to D) and extracts kept at 23°C for 48 h (lanes E to F) and 72 h (lanes I to L) were tested. The 74- and 68-kDa hsps are indicated on the right, and molecular mass markers are indicated by arrows on the left, as described in the legend to Fig. 1.

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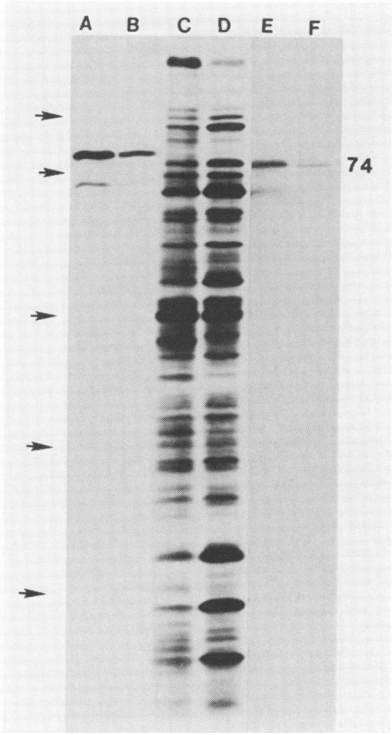

FIG. 7. Immunoprecipitation with anti-DnaK antiserum. Acid-phase *C. acetobutylicum* cells were heat shocked, and gels were prepared as described in the legend to Fig. 1 and in the text (lane C, total cell protein; lane D, total cell protein from heat-stressed cells). The clostridial 74-kDa hsp was immunoprecipitated with anti-DnaK antiserum diluted 1:20 (lane E) and 1:100 (lane F); the 72-kDa hsp (DnaK) from heat-shocked *E. coli* DH5 was immunoprecipitated with anti-DnaK antiserum diluted 1:20 (lane A) and 1:100 (lane B). Lanes A to D were exposed to X-ray film for 2 days, and lanes E to F were exposed for 15 days. The arrows on the left indicate, from the top down, molecular mass markers at 92, 66, 45, 31, and 21 kDa.

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