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Stress-shock Response of a Methylotrophic Bacterium *Methylovorus* sp. strain SS1 DSM 11726

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***Methylovorus* sp. strain SS1 DSM 11726 was found to grow continuously when it was transferred from 30°C to 40°C and 43°C. A shift in growth temperature from 30°C to 45°C, 47°C and 50°C reduced the viability of the cell population by more than 10^2, 10^3 and 10^5 folds, respectively, after 1 h cultivation. Cells transferred to 47°C and 50°C after preincubation for 15 min at 43°C, however, exhibited 10-fold increase in viability. It was found that incubation for 15 min at 40°C of *Methylovorus* sp. strain SS1 grown at 30°C was sufficient to accelerate the synthesis of a specific subset of proteins. The major heat shock proteins had apparent molecular masses of 90, 70, 66, 60, and 58 kDa. The 60 and 58 kDa proteins were found to cross-react with the antiserum raised against GroEL protein. The heat shock response persisted for over 1 h. The shock proteins were stable for 90 min in the cell. Exposure of the cells to methanol induced proteins identical to the heat shock proteins. Addition of ethanol induced a unique protein with a molecular mass of about 40 kDa in addition to the heat-induced proteins. The proteins induced in paraquat-treated cells were different from the heat shock proteins, except the 70 and 60 kDa proteins.**

Key words: *Methylovorus* sp. strain SS1, stress response, heat shock proteins

The transient induction of a distinct subset of proteins, heat shock proteins (HSPs) or stress proteins, after heat or stress shock has been observed in a wide range of organisms (4, 7-10, 12, 18, 20, 32). The induction of HSPs indicates an important protective and homeostatic role of the proteins in coping with physiological and environmental stress at the cellular level. Most HSPs are also produced under nonstress conditions, even though at reduced rates, and play fundamental roles in normal cell physiology (7, 18, 20, 32). Among the shock systems described for bacteria, yeasts, and various higher organisms to date, the bacterial systems are of special interest since mutants affecting a wide variety of responses can be generated. The prokaryotic shock response has been studied in detail especially in *Escherichia coli* in which the synthesis of a set of at least 17 proteins was induced or increased after heat shock treatment (18, 20).

Methylotrophic bacteria are a group of bacteria which are able to grow aerobically at the expense of methanol as the sole source of carbon and energy (2). It has been reported that *Methylophilus methylotrophus*, a restricted facultative methylotroph, produces several shock proteins when exposed to heat or high concentrations of methanol and ethanol (30). Induction of shock proteins in this bacterium treated with methanol indicates that methanol is not only beneficial but is also toxic to methylotrophic bacteria.

Methylovorus sp. strain SS1 DSM 11726 is another restricted facultative methylotrophic bacterium isolated from soil samples from Malaysia (26). The bacterium was found to grow optimally at 40°C with a growth rate 1.3 times faster than at 30°C, the temperature used for usual cultivation of this bacterium (26), suggesting that the HSPs induced or increased upon a temperature shift from 30°C to 40°C may be directly or indirectly involved in the fast growth at 40°C.

We performed this experiment to study in detail the growth response of *Methylovorus* sp. strain SS1 at high temperatures. We also tried to elucidate the profile of HSPs and other shock proteins in this bacterium to learn more about shock responses in prokaryotic organisms.

Materials and Methods

Bacterial strain and cultivation

Primary cultures of *Methylovorus* sp. strain SS1 DSM

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11726 were prepared by cultivation at 30°C in 100-ml Erlenmeyer flasks containing 30 ml of standard mineral medium (SMM [13]) supplemented with 1.0% (vol/vol) methanol. Throughout this experiment, the cells were cultivated or incubated by shaking at an agitation speed of 200 rpm in an orbital shaker unless otherwise indicated. Growth was measured by turbidity determined at 600 nm using a spectrophotometer.

Shock treatment and protein labeling

To examine the effect of heat shock on the growth response of *Methylovorus* sp. strain SS1, 1.0-ml aliquots of the primary cultures (A_{600} = 0.7-0.8) were transferred to test tubes (2.5 × 15 cm) and shaken at various temperatures. Viability of cells was determined after 72 h by the viable cell count method at 30°C using solid SMM containing 1.0% (vol/vol) methanol.

For labeling HSPs, 10 μCi of (L-[^{35}S]-methionine (Amersham) was first added to 1.0-ml aliquots of the primary cultures. The isotope-treated primary cultures were then incubated for 15 min at 30°C, immediately transferred to various temperatures, and agitated for another 15 min to label HSPs. To label other shock proteins, the 1.0-ml isotope-treated cultures were mixed with ethanol, methanol, or paraquat at a final concentration of 5.0% (vol/vol), 5.0% (vol/vol), or 200 μM, respectively, and incubated for 30 min at 30°C. The cultures were stored on ice immediately after labeling. Cells were harvested by centrifugation at 12,000 × *g* at 4°C and washed with ice-cold 50 mM Tris-HCl (pH 7.5). Protein samples for one-dimensional denaturing polyacrylamide gel electrophoresis (PAGE) were prepared by resuspending the washed cells in 60 μl denaturing sample buffer (16) followed by heating for 5 min in boiling water. The samples for two-dimensional electrophoresis were prepared by resuspending the washed pellets in 60 μl isoelectric focusing sample buffer (21) followed by incubation for 12 h at room temperature.

Electrophoresis and autoradiography

One dimensional denaturing PAGE of labeled samples in 10% acrylamide gel was carried out according to Laemmli (16) in the presence of 0.1% sodium dodecyl sulfate (SDS). Proteins were stained with Coomassie brilliant blue R-250 (CBB) by the modified method (13) of Weber and Osborn (31). Two-dimensional denaturing PAGE was performed following the method described by O'Farrell (21) with a slight modification: the first dimension of PAGE was done in a tube gel containing 4.2% acrylamide with a 2.4% final concentration of pre-blended ampholine (pH 3.5 to 9.5, Amersham Pharmacia Biotech) and the second dimension was carried out in 10% denaturing polyacrylamide gel followed by staining with silver (22). Autoradiograms were prepared by exposing Fuji medical X-ray film (Super RX) to dried gels for 6-7 days at -70°C.

Immunoblotting

Part of the shock proteins in *Methylovorus* sp. strain SS1 was identified according to the procedure described in the ECL (enhanced chemiluminescence) Western blotting protocols (Amersham) after transferring the proteins which had been subjected to denaturing PAGE to nitrocellulose membrane (Hybond-ECL; Amersham). Antiserum raised against *Escherichia coli* GroEL was purchased from Sigma Chemical Company.

Results and Discussion

Growth response to heat shock

When *Methylovorus* sp. strain SS1 growing at 30°C was transferred to 40°C, 43°C, 45°C, 47°C, and 50°C, the cells at 40°C and 43°C were found to grow faster than at 30°C (data not shown), which is in accordance with the previous observations (26), suggesting that *Methylovorus* sp. strain SS1 is a weakly thermophilic organism. The cells transferred to 45°C, 47°C, and 50°C showed no increase in cell numbers, but rather exhibited reduction in the viability of the cell population by more than 10^2-, 10^3- and

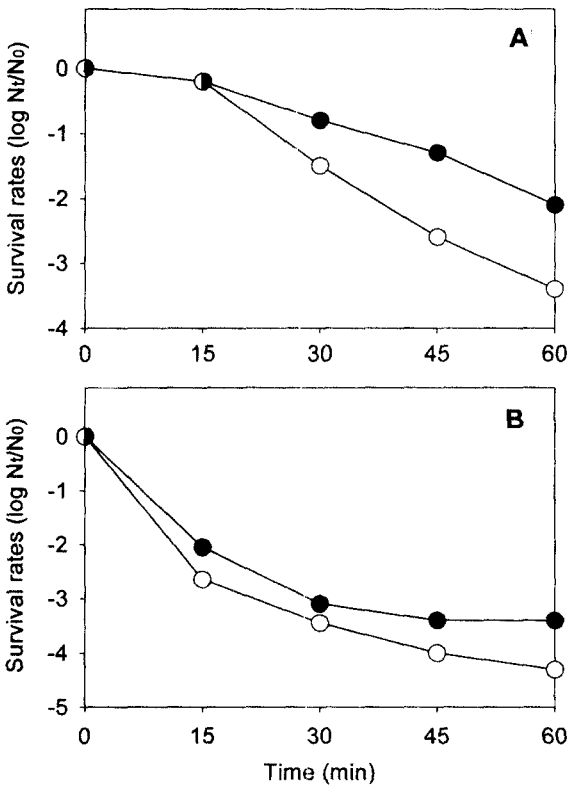

Fig. 1. Induction of thermotolerance in *Methylovorus* sp. strain SS1. Cells grown at 30°C were transferred to 47°C (A) or 50°C (B) directly (– ○ –) or after preincubation for 15 min at 43°C (– ● –). Survival rate of the cells was calculated from log (*Nt*/*No*). *Nt*: number of cells at the appropriate intervals of cultivation after transfer to shifted temperatures. *No*: number of cells at the initial stage of transfer.

10^5-fold, respectively, after 1 h cultivation. Cells transferred to 45°C, 47°C and 50°C after preincubation for 15 min at 43°C, however, revealed an approximately 10-fold increase in viability (Fig. 1), suggesting that heat resistance induced during incubation at 43°C enhanced viability of the cells at higher temperatures.

Heat shock proteins in Methylovorus sp. strain SS1

A preliminary observation revealed that no labeled protein was detectable even after over 10 days of autoradiography with cell lysates prepared from *Methylovorus* sp. strain SS1 grown for 15 min in the presence of labeled methionine, indicating that the bacterium does not absorb the methionine easily. We therefore preincubated the cells for 15 min at 30°C in the presence of labeled methionine prior to heat shock treatment.

It was found that cells incubated at 37°C did not change the protein profile of the cell (Fig. 2). A number of proteins, HSPs, were increased or newly appeared in cells growing at over 40°C (Fig. 2), indicating that *Methylovorus* sp. strain SS1 does not recognize the sudden increase in growth temperature by 7°C as a shock. In addition to the HSPs almost all proteins which were synthesized during normal incubation at 30°C were also labelled in heat shocked cells even in cells at 50°C (Fig. 2), indicating that heat shock does not result in the complete shut down of normal protein synthesis in *Methylovorus* sp strain SS1. *E. coli* which grows optimally at 37°C, however, was found to cease synthesizing most proteins except HSPs when it was exposed to 50°C (5, 17). Among the HSPs including three to four proteins with molecular weights 30,000-35,000, five proteins with molecular weights of about 90,000, 70,000, 66,000, 60,000, and 58,000 were found to be the major HSPs in this bacterium (Fig. 2), which are similar in molecular weight with HSPs found in other organisms (5, 18, 20, 32). It has been reported that one of the major functions of HSPs is to confer thermal tolerance to the cell (17-20, 28). Since *Methylovorus* sp. strain SS1 preincubated at 43°C exhibited an increase in viability at higher temperatures, it is possible to suggest that the sustained viability of *Methylovorus* sp. strain SS1 growing at over 45°C is attributable to the HSPs. These results also suggest that a sudden elevation in the growth temperature by 15°C may irreversibly damage part of the cell physiology related to cell growth, although the cells at over 45°C synthesize the same species of HSPs as those induced in cells growing at 43°C.

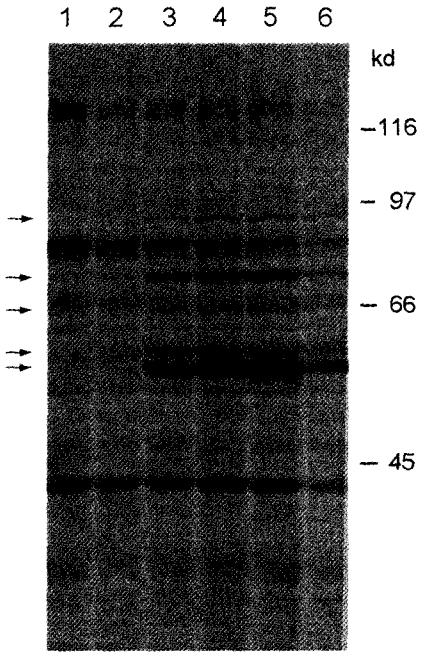

Fig. 2. HSPs in *Methylovorus* sp. strain SS1. Cells were preincubated in the presence of [^{35}S]-methionine at 30°C for 15 min and incubated for another 15 min at 30°C (lane 1), 37°C (lane 2), 40°C (lane 3), 45°C (lane 4), 47°C (lane 5), and 50°C (lane 6). Arrows indicate major HSPs. Molecular size markers are indicated on the right.

Shock proteins induced by other stresses

Methylovorus sp strain SS1 was also found to produce a specific subset of proteins when subjected to oxidative

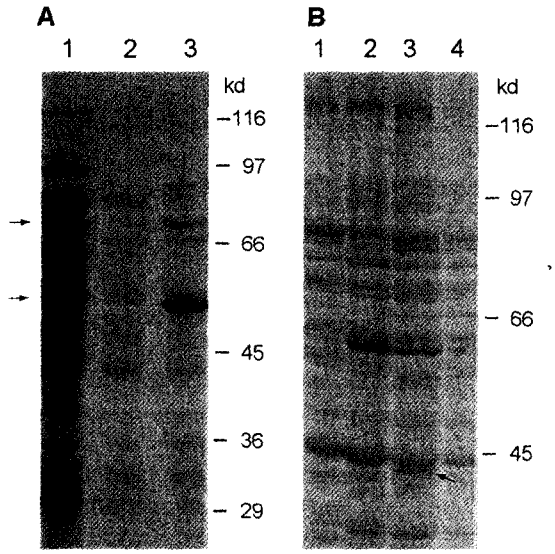

Fig. 3. Oxidative, ethanol, and methanol stress responses. (A) Oxidative stress response. To label oxidative shock proteins, cells grown at 30°C were mixed with [^{35}S]-methionine (10 μCi/ml) and paraquat at a final concentration of 200 μM and incubated for 30 min at 30°C (lane 1). Cells incubated at 30°C (lane 2) and 43°C (lane 3) for 30 min in the presence of [^{35}S]-methionine were used as controls. The major shock proteins over-expressed in both heat shocked and oxidative stressed cells are indicated with arrows. (B) Ethanol and methanol stress proteins. Cells grown at 30°C were mixed with [^{35}S]-methionine (10 μCi/ml) together with ethanol (lane 3) or methanol (lane 4) at a final concentration of 5 % (v/v) and incubated for 30 min at 30°C. Cells incubated at 30°C (lane 1) and 43°C (lane 2) for 30 min in the presence of [^{35}S]-methionine were used as controls. An arrow indicates ethanol-specific protein. Size markers are indicated on the right.

stress, most of which were different from HSPs except for the 70,000 and 60,000 proteins (Fig. 3A), indicating that the bacterium responds to oxidative stress in a way different from that against heat shock. It has also been reported for *E. coli* that only one protein, GroEL with a molecular weight of 56,500, among several oxidative shock proteins induced by paraquat was found to overlap with HSPs (29).

Methanol-treated cells synthesized new proteins identical in size to the five major HSPs (Fig. 3B). Ethanol stress, on the other hand, was found to induce a unique protein with a molecular weight of 40,000 in addition to the major HSPs (Fig. 3B). It has been reported that *Bacillus subtilis* under ethanol stress synthesized eleven shock proteins identical to the twelve major HSPs besides fifteen unique ethanol stress proteins (3). *M. methylotrophus* under methanol stress has also been reported to produce six proteins, in addition to three unique methanol stress ones, identical to eight HSPs (30). These observations indicate that the similarity in cellular response of *Methylovorus* sp. strain SS1 to alcohol and heat shocks is greater than that of *B. subtilis* and *M. methylotrophus* and that the HPSs of *Methylovorus* sp. strain SS1 may act as general stress proteins in this bacterium.

Persistence of HSP production and stability of HSPs

It has been reported that the induction of HSPs in *E. coli* occurs almost immediately after heat shock treatment, reaches a maximum at about 5 min, and declines to a new steady-state level in 20-30 min (32). The HSPs in *Methylovorus* sp. strain SS1, however, were synthesized in large amounts for 1 h after the temperature shift (Fig. 4). The amounts of HSPs induced 2 h after heat shock were also found to be significantly more than that produced before heat shock treatment although much less than that induced 1 h after the treatment. These results suggest that the expression pattern of HSPs in *Methylovorus* sp. strain SS1 is different from that in *E. coli*. *Streptomyces albus* (11) and *Caulobacter crecentus* (23) were also reported to synthesize GroEL, not all major HSPs, in increased amounts for a certain extended period.

The pulse-chase experiment showed that the major HSPs produced at 43°C were present in a stable form for at least 90 min in the cell (Fig. 5), indicating that the HSPs in *Methylovorus* sp. strain SS1 are stable enough to act for a long time after induction.

HSPs after two-dimensional gel electrophoresis

Two-dimensional SDS-PAGE of HSPs produced at 43°C revealed that the HSP with a molecular weight of 58,000 was increased most prominently among the five HSPs induced in *Methylovorus* sp. strain SS1 (Fig. 6), which is in good agreement with the result obtained from one-dimensional SDS-PAGE. The autoradiograms also showed

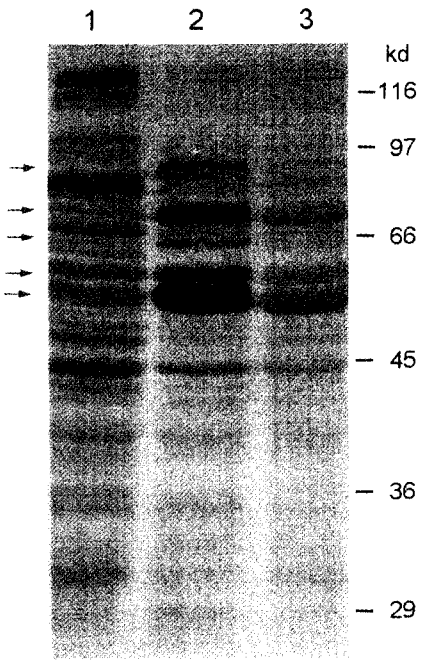

Fig. 4. Persistent induction of HSPs. Cells grown at 30°C were preincubated at 43°C for 1 h (lane 2) or 2 h (lane 3), mixed with [^{35}S]-methionine (10 μCi/ml), and then incubated for 15 min at the same temperature. Cells incubated in the presence of [^{35}S]-methionine for 1 h at 30°C were used as a control (lane 1). Arrows indicate HSPs. Size markers are indicated on the right.

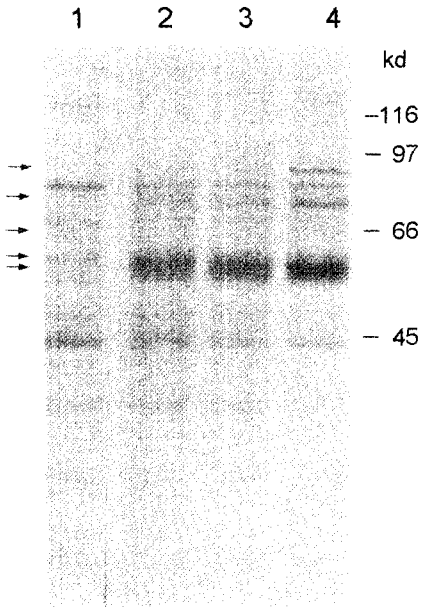

Fig. 5. Pulse-chase studies for the stability of HSPs. Cells grown at 30°C were preincubated at 30°C for 15 min in the presence of [^{35}S]-methionine, then treated with an excessive amount of unlabeled methionine to inhibit further incorporation of labeled methionine followed by immediate transfer to 43°C. Aliquots were removed after 15 (lane 2), 45 (lane 3), and 90 min (lane 4). Cells incubated for 30 min at 30°C were used as a control (lane 1). Arrows indicate the major HSPs in *Methylovorus* sp. strain SS1. Size markers are indicated on the right.

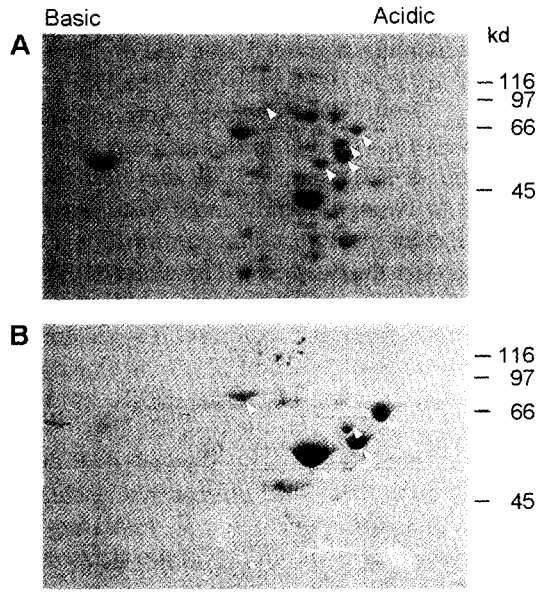

Fig. 6. Two-dimensional analysis of HSPs. Autoradiography was performed after two-dimensional gel electrophoresis of cell lysates prepared from cells incubated for 30 min at 30°C (A) and 43°C (B). White arrow heads indicate the major HSPs. Size markers are indicated on the right.

that the 70,000 protein has the lowest pI among the five HSPs. Comparison of the autoradiograph with that of other organisms indicates that the 70,000 and 58,000 proteins are DnaK- and GroEL-like proteins, respectively (15, 24). The comparison also indicates that one of the two proteins induced in *Methylovorus* sp. strain SS1 under both heat shock and oxidative stress (Fig. 3B) is a DnaK-like protein.

Presence of two GroEL-like proteins

Immunoblot after denaturing PAGE of cell lysates prepared cells incubated at shifted temperatures revealed that the 60,000 and 58,000 proteins cross-reacted with the anti-GroEL antiserum (Fig. 7), indicating that the two GroEL-like HSPs are present in *Methylovorus* sp. strain SS1. The presence of two kinds of GroEL-like protein has also been reported for *S. albus* (11, 27), *Pseudomonas aeruginosa* (1), *Mycobacterium tuberculosis* (14), and *Rhizobium meliloti* (25). The result also supports the indications deduced from comparative analysis of the autoradiographic data of two-dimensional gels that the 58,000 protein is a GroEL-like protein which is induced in larger amounts than the 60,000 protein. This result, together with the protein profiles of cell lysates prepared from cells incubated under oxidative and heat stresses (Fig. 2, 3A, and 6), clearly show that the inductions of the two GroEL-like proteins in *Methylovorus* sp. strain SS1 are under different control; i.e., oxidative stress induces the gene for 60,000 protein more strongly than the gene for 58,000 protein, while heat shock results in an opposite effect on the expression of the two genes.

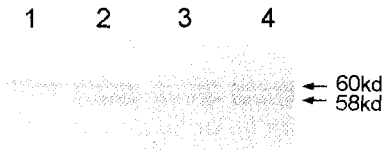

Fig. 7. Western blot analysis of HSPs. Immunoblot of HSPs was performed with antiserum raised against *E. coli* GroEL after denaturing PAGE of cells lysates prepared from cells incubated for 30 min at 30°C (lane 1), 40°C (lane 2), 43°C (lane 3), and 45°C (lane 4). Arrows indicate the 60,000 and 58,000 HSP proteins cross-reacted with the anti-GroEL antiserum. Size markers are indicated on the right.

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