JOURNAL OF BACTERIOLOGY, Sept. 1996, p. 5447–5451 Vol. 178, No. 18
0021-9193/96/$04.00+0

Regulation of RNA Polymerase Sigma Subunit Synthesis in *Escherichia coli*: Intracellular Levels of Four Species of Sigma Subunit under Various Growth Conditions

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By a quantitative Western immunoblot analysis, the intracellular levels of two principal σ subunits, σ^{70} (σ^{D}, the *rpoD* gene product) and σ^{38} (σ^{S}, the *rpoS* gene product), and of two minor σ subunits, σ^{54} (σ^{N}, the *rpoN* gene product) and σ^{28} (σ^{F}, the *rpoF* gene product), were determined in two *Escherichia coli* strains, W3110 and MC4100. The results indicated that the levels of σ^{54} and σ^{28} are maintained at 10 and 50%, respectively, of the level of σ^{70} in both strains growing at both exponential and stationary phases, but in agreement with the previous measurement for strain MC4100 (M. Jishage and A. Ishihama, J. Bacteriol. 177:6832–6835, 1995), the level of σ^{38} was undetectable at the exponential growth phase but increased to 30% of the level of σ^{70} at the stationary phase. Stress-coupled change in the intracellular level was observed for two σ subunits: (i) the increase in σ^{38} level and the decrease in σ^{28} level upon exposure to heat shock at the exponential phase and (ii) the increase in σ^{38} level under high-osmolality conditions at both the exponential and stationary phases.

RNA polymerase holoenzyme of *Escherichia coli* consists of the core enzyme with the subunit composition of $\alpha_2\beta\beta'$ and a σ subunit, which directs the core enzyme to initiate transcription at specific promoter sites on DNA (4). The major σ factor, σ^{70} (the *rpoD* gene product), is responsible for transcription of most genes expressed during the exponential cell growth (12, 17). Besides σ^{70}, six different molecular species of alternative σ subunits in *E. coli* have been identified. σ^{54} (the *rpoN* gene product) is concerned with expression of a wide variety of genes including those involved in nitrogen metabolism (27, 28). Temperature upshift increases transcription of the genes under the control of two heat shock σ subunits, σ^{32} (the *rpoH* gene product) (8, 41) and σ^{24} (the *rpoE* gene product) (7). Holoenzyme containing σ^{32} transcribes the heat shock genes including those encoding chaperons and proteases (42), while the regulons under the control of σ^{24} are known to be involved in extracytoplasmic functions (6, 35). σ^{38} (the *rpoS* gene product) is a key factor in the stress response during the transition from the exponential growth phase to the stationary growth phase (13, 25). σ^{28} (the *rpoF* gene product) governs transcription of the genes for flagellar formation and chemotaxis (3, 31). FecI involved in the ferric citrate transport system is now identified as a member of a new subfamily of σ subunits for extracytoplasmic functions (2).

The switch of gene expression pattern upon sudden exposure to various stresses is thought to take place by replacement of the σ subunit on RNA polymerase. The level of each form of holoenzyme is thought to be determined by the concentration of each σ subunit and its affinity to core RNA polymerase. Until recently, however, little was known of the intracellular concentrations of individual σ subunits except for the major σ subunit, σ^{70} (17). We then initiated a systematic determination of the intracellular concentrations of the σ subunits, and in a previous study (19), we reported the levels of σ^{38} in *E. coli* MC4100 growing under various conditions. As an extension of this line of research, we determined in this study the intracellular levels of four σ subunits in *E. coli* growing under steady-state conditions or various stress conditions by the same quantitative Western blot (immunoblot) method employed in the previous study (19). Since the MC4100 strain lacks σ^{28} for flagellar formation (39), we analyzed another strain, W3110, in order to understand the possible influence of the lack of one σ subunit on the levels of other σ subunits.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study were *E. coli* W3110 and MC4100. Cells were grown at 30 or 37°C under aeration in Luria broth (LB). Growth was monitored by measuring the turbidity with a Klett-Summerson photometer. The culture conditions were fixed as follows. A few colonies from a culture grown overnight on a LB agar plate were inoculated onto 5 ml of fresh LB medium. At the cell density of 30 Klett units, the culture was diluted 20-fold by adding 100 ml of fresh LB medium and incubated at 37°C with shaking at a constant rate (Taiyo Incubator M-100^{N} level 6). For osmotic stress, the culture was divided into equal halves, and to one half, a solution of prewarmed LB containing 5 M NaCl was added to make a 0.5 M solution. For heat shock stress, the culture grown at 30°C was divided into equal halves, to which an equal volume of fresh LB prewarmed at either 54°C (heat shock at 42°C) or 30°C (control) was added.

Preparation of cell lysates. Cells were collected by centrifugation and resuspended in 40 mM Tris-HCl (pH 8.1 at 4°C) containing 25% sucrose. After treatment with 1 mM EDTA and 500 μg of lysozyme per ml at 0°C for 10 min, cells were lysed by adding 0.5% Brij 58. The Brij 58 lysate was supplemented with 0.01 M $MgCl_2$ and 0.2 M KCl, digested at 37°C for 10 min with 20 μg of RNase A per ml and 100 μg of DNase I per ml in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF), and sonicated for 1 min with a Cosmo Bio Bioruptor. The supernatant after centrifugation for 30 min at 15,000 rpm (Tomy MRX-150) was used as the cell lysate for all the experiments. The protein concentration of cell lysates was determined with a protein assay kit (Bio-Rad).

Purification of σ subunits, preparation of antibodies, and immunological methods. Antibodies against σ^{70} and σ^{38} were produced in rabbits as described previously (19). For preparation of anti-σ^{54} and anti-σ^{28} subunit antibodies, σ^{54} was overexpressed by using NCM668 (strain M5219 carrying pJES259) and purified as described previously (33), and σ^{28} was expressed in BL21(DE3) by using pETSF and purified as will be described elsewhere (21a). Antibodies against these σ subunits were produced in rabbits by injecting the purified σ subunits. Each σ subunit was determined by the quantitative Western blot method as employed previously (19), except the enhanced chemiluminescence reagent system (Amersham) was used for detection.

RESULTS AND DISCUSSION

Levels of various σ subunits during steady-state growth. Previously we determined the intracellular concentrations of σ^{70} and σ^{38} under various growth phases of *E. coli* MC4100, which has been widely used for transcriptional analysis of stress response (15, 22). This strain, however, lacks the σ^{28} required for transcription of the genes for flagellar formation because of the lack of FlbB protein (39), a factor for transcription activation of the flagellar class 2 genes including the *rpoF* gene encoding σ^{28}. Since the intracellular concentration of RNA polymerase core enzyme stays at a constant level characteristic of the rate of cell growth (17, 18, 20), competition must take place between various σ subunits for core enzyme binding, and in this way, the lack of σ^{28} subunit may influence the levels of other subunits. To test this possibility, we analyzed in this study the intracellular levels of various σ subunits in two *E. coli* strains, MC4100 (the strain previously analyzed for σ^{38}) (19) and W3110 (the strain used for the genome project in Japan) (21), growing at various phases and under various conditions.

Cells were grown in LB medium at 37°C, and samples were taken periodically during the growth transition from the exponential growth phase (15 Klett units for W3110 and 20 Klett units for MC4100) to the stationary growth phase (220 Klett units for W3110 and 160 Klett units for MC4100). Under the culture conditions employed, the doubling time was 0.5 h for W3110 and 1 h for MC4100 (however, there are at least five different variants of W3110 in Japan, some of which showed different growth rates [19a]). Cell lysates were prepared, and the amounts of four species of σ subunit, σ^{70}, σ^{54}, σ^{38}, and σ^{28}, were determined by the same quantitative Western blot system as employed in the previous study (19).

For measurement of σ subunits, we first made a standard curve for each of the purified σ subunits and determined the range in which the linearity exists between the protein concentration and the intensity of immunostaining. Since this linearity was observed between 2 and 20 ng for all of the four σ subunits examined, we then analyzed several different volumes of the cell lysates to identify the volume that includes this level of σ protein for each of the four σ subunits examined. Using the optimum volumes of cell lysates thus estimated, we finally repeated the measurement of individual σ subunits at least three times, always in parallel with the determination of six different concentrations of the respective purified σ subunit as the assay standard. The maximum fluctuation between different measurements described in this report was 20%, and the minimum level of detection was 0.5 fmol per μg of total cell lysate proteins. A typical immunoblot pattern is shown in Fig. 1, and the quantitative measurements using the standard curves for the purified individual σ subunits are summarized in Table 1.

As observed in strains W3350 (20) and MC4100 (19), the level of σ^{70} in W3110 is maintained at a constant level from the exponential phase to the stationary phase. However, a significant difference was observed in σ^{70} level between two *E. coli* strains, ranging from 50 to 80 fmol per μg of total proteins in strain MC4100 or from 150 to 170 fmol per μg of total proteins in strain W3110. The levels of minor σ subunits were different in the two *E. coli* strains. Generally, the σ levels were higher in W3110 than in MC4100, i.e., about 3 times for σ^{70}, 6 to 7 times for σ^{54} (for both the exponential and stationary phases), and about 2 times for σ^{38} (at the stationary phase). The difference in growth rate between two strains, i.e., two doublings per h for W3110 and one doubling per h for MC4100, may lead to the observed difference in σ^{70} level, probably because the fast-growing W3110 cells express σ^{70}-dependent essential genes constitutively at higher levels. In spite of the differences in the absolute levels of various σ subunits, the relative levels of σ^{70} to minor σ subunits were almost the same between the two strains. For instance, σ^{38} is undetectable at exponential phase and reaches about 30% in stationary phase in strain W3110, as in the case of MC4100 (19), suggesting that the balance between two principal σ subunits is important for the growth phase-coupled switching in transcription pattern.

σ^{54} was identified as a positive regulatory factor needed for expression of the gene encoding glutamine synthetase in enteric bacteria (9, 33). Later, σ^{54} was found to be required for expression of a wide variety of genes including *fdhF* (formate dehydrogenase), *hyc* and *hyp* (hydrogenase synthesis), *psp* (phage shock protein), and *glnA* (glutamine synthetase) (27, 28). Our measurements indicated that the level of σ^{54} in both MC4100 and W3110 strains remains constant throughout the growth transition from exponential phase to stationary phase in LB medium at 37°C. The result is consistent with the observation that the expression of *rpoN* encoding σ^{54}, as measured by β-galactosidase synthesis in a *rpoN-lacZ* fusion strain, is constitutive under different conditions of nitrogen availability (5). In comparison with the number of genes under the control of σ^{54}, however, the molar ratio of σ^{54} to σ^{70} is high, i.e., about 16% of the level of σ^{70} in strain W3110 and about 6% in strain MC4100. Holoenzyme containing σ^{54} is, however, completely inactive in the absence of a functional activator (28). It remains unknown how the unused σ^{54} protein is stored without being degraded. One possibility is that σ^{54} forms a complex with an as yet unidentified protein, as in the case of σ^{28}–anti-σ^{28} complex (see below).

The flagellar chemotaxis regulon in *E. coli* contains over 40 genes that are arranged in a hierarchy, in which the expression of an operon in a given class is necessary for the expression of operons which are organized downstream in the hierarchy (26). Four classes, classes 1, 2, 3a, and 3b, have been defined in this complex regulon. The *rpoF* gene encoding σ^{28} belongs to a class 2 operon, and σ^{28} is needed for expression of the class 3a and 3b operons which include 18 genes (11, 26). As can be seen in Fig. 1, the level of σ^{28} stays constant from the middle of the exponential phase to the stationary phase. In early log phase, however, its level is significantly lower than this log-phase level, presumably because early log-phase cells degrade serine and synthesize acetylphosphate, which can function as a phosphate donor to phosphorylate OmpR that leads to repression of the expression of *flhDC*, the positive regulatory genes for σ^{28} synthesis (34, 38). To our surprise, the level of σ^{28} in *E. coli* W3110 was found to be as much as 50% of the level of σ^{70}. If all these σ^{28} subunits were active and the binding affinity to core enzyme were the same between σ^{70} and σ^{28}, the level of RNA polymerase holoenzyme containing σ^{28} should be half the level of the regular holoenzyme containing σ^{70}. It is possible that the high level of σ^{28} is related to the high-level expression of flagellar proteins, even though there are only 18 genes under the control of σ^{28} (10, 26). Alternatively, σ^{28} is used for transcription of as yet unidentified genes except for the flagellar genes. In the case of σ^{28}, however, it has been established that the majority of σ^{28} subunit forms a complex with an anti-sigma factor and stays in a stored form for rapid reuse (10, 16, 32).

On the other hand, σ^{38} was virtually undetectable during the exponential growth phases of the two test strains and started to appear when the cells stopped growing. The maximum level of σ^{38} at the stationary phase reaches 40 to 60 or 20 to 30 fmol per μg of total proteins for W3110 and MC4100, respectively (Table 1). The σ^{38} level in MC4100 agreed well with the previous determination (19).

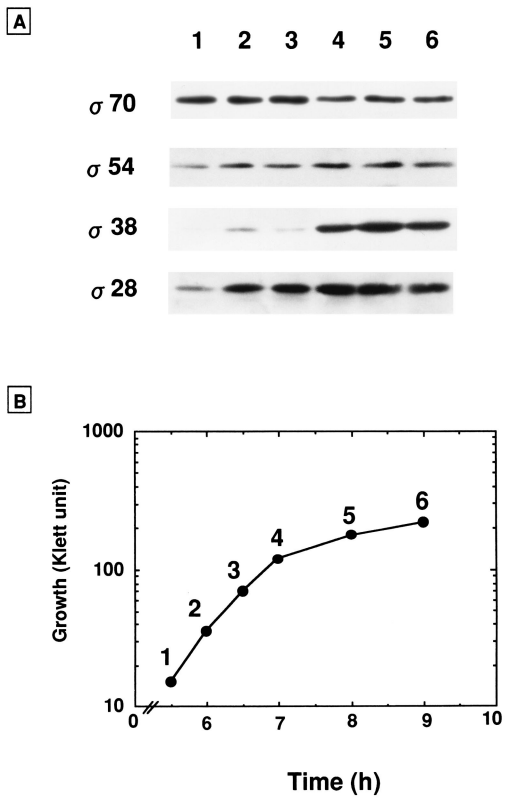

FIG. 1. Growth phase-dependent variation in the intracellular levels of σ^{70}, σ^{54}, σ^{38}, and σ^{28} subunits in *E. coli* W3110. (A) Aliquots containing 1 μg (for σ^{70}) or 10 μg (for σ^{54}, σ^{38}, and σ^{28}) of total proteins from cell lysates of *E. coli* W3110 prepared at various times of the cell culture (see panel B for the growth curve) were analyzed by the quantitative Western blot system. (B) *E. coli* W3100 was grown in LB medium at 37°C, and the growth was monitored by measuring the turbidity with a Klett-Summerson photometer. The numbers on the growth curve represent the time points at which samples were taken.

Previously we estimated that the intracellular level of σ^{70} subunit is about 700 molecules per *E. coli* W3350 cell throughout the change in growth phase, even though the level of core enzyme increases concomitantly with the increase in cell growth rate (17, 18, 20). Taking the previous determination and the present measurement together, we can now estimate the approximate numbers of σ^{54}, σ^{38}, and σ^{28} to be 110, 0, and 350 molecules per W3110 cell at the exponential phase, respectively, and 110, 230, and 350 molecules per W3110 cell at the stationary phase, respectively. Thus, the total number of alternative σ proteins in *E. coli* cells is roughly 80% at the exponential phase and almost equal to that of primary σ subunit at the stationary phase. The result was rather unexpected because most RNAs synthesized in exponentially growing cells are transcripts of the constitutive and essential genes under the control of σ^{70} (17). The total number of genes of the *E. coli* genome is estimated to be about 4,000 genes; about 1,000 genes are expressed in exponentially growing cells, and when cells stop growing at the stationary phase, most of these genes are shut off and approximately 100 genes begin to be expressed (13, 18). The numbers of σ^{54}- or σ^{28}-dependent genes so far identified are only 4 (27) or 18 (10, 26), respectively, while in the stationary phase, the σ^{38} subunit of RNA polymerase controls the expression of at least 20 to 30 genes or operons (25). If the number of genes under the control of a particular σ subunit correlates with its abundance, the level of alternative σ factor, σ^{54} or σ^{28}, seems to be very high. The high concentrations of these two alternative σ factors may be related to the capability of *E. coli* cells to rapidly adapt to changes in the environment.

TABLE 1. Intracellular levels of four species of σ subunit in *E. coli* W3110 and MC4100[a]

σ subunit	Level (fmol/μg) of σ subunit in strain:			
	W3110		MC4100	
	Exponential phase	Stationary phase	Exponential phase	Stationary phase
σ^{70}	150–170	150–170	50–80	50–80
σ^{54}	20–30	20–30	3–5	3–5
σ^{38}	0	40–60	0	20–30
σ^{28}	70–100	70–100	0	0

[a] Cells were grown in LB medium at 37°C with shaking. At the middle of exponential growth phase, the levels of σ subunits were determined by the quantitative Western blot system. The determination was repeated at least three times, and the values are the averages.

Variations in the σ levels under stress conditions. Upon exposure of bacterial cells to environmental stresses, drastic changes take place in the pattern of gene transcription. As an attempt to understand stress-coupled replacement of σ subunits on RNA polymerase, we measured changes in the relative levels of various σ subunits when strain W3110 was exposed to various stresses: (i) exposure of both exponential and stationary-phase cells to temperature upshift from 30 to 42°C; and (ii) exposure to osmotic shock by increasing NaCl concentration to 0.5 M. Of all four σ subunits examined, significant changes were observed in σ^{38} and σ^{28} levels, but not in σ^{70} and σ^{54} levels.

Under steady-state culture conditions, the level of σ^{38} subunit is higher at 30°C than 37°C (compare Fig. 2A and C). Our observation is consistent with the recent finding that at 20°C, the expression of *rpoS* is high even in the exponentially growing phase (40). Upon exposure to temperature upshift at the exponential phase from 30 to 37°C, the σ^{38} level increased to 23 fmol per μg of total proteins by 10 min or fourfold over the control culture without heat shock (Fig. 2A). The increased level was maintained up to 20 min, but at 60 min, σ^{28} again decreased to the steady-state level at 30°C. The response in σ^{38} level is essentially as in the case of MC4100 (19). The pattern of heat shock response is, however, markedly different from that of σ^{32} or heat shock σ factor, which increases 10- to 15-fold within the first few minutes after heat shock and decreases again to the steady-state level by 10 to 20 min (41, 42). It is therefore possible that σ^{38} plays a role in the recovery process from the transient response to heat shock.

High-osmolality stress resulted in the increase in σ^{38} level at both the exponential phase (Fig. 2C) and the stationary phase (Fig. 2E). At the exponential phase, the level of σ^{38} increased dramatically at 30 min from an undetectable level to 77 fmol per μg of total proteins (Fig. 2C), while in the stationary phase, about twofold increase was observed at 20 min after osmotic shock (Fig. 2E). This is consistent with the observations that the σ^{38} subunit of RNA polymerase controls the expression of some osmotically regulated genes (13–15, 30). Even in the stationary-phase cells of both strains MC4100 (19) and W3110 (this study) expressing high levels of σ^{38}, the increase in medium osmolarity induced further increase in the σ^{38} level (19) (Fig. 2E), suggesting that *E. coli* cells control the level of σ^{38} by monitoring the demand for expression of the stress response genes.

Flagellar formation in *E. coli* is sensitive to environmental conditions. For instance, flagellar synthesis is inhibited by ca-

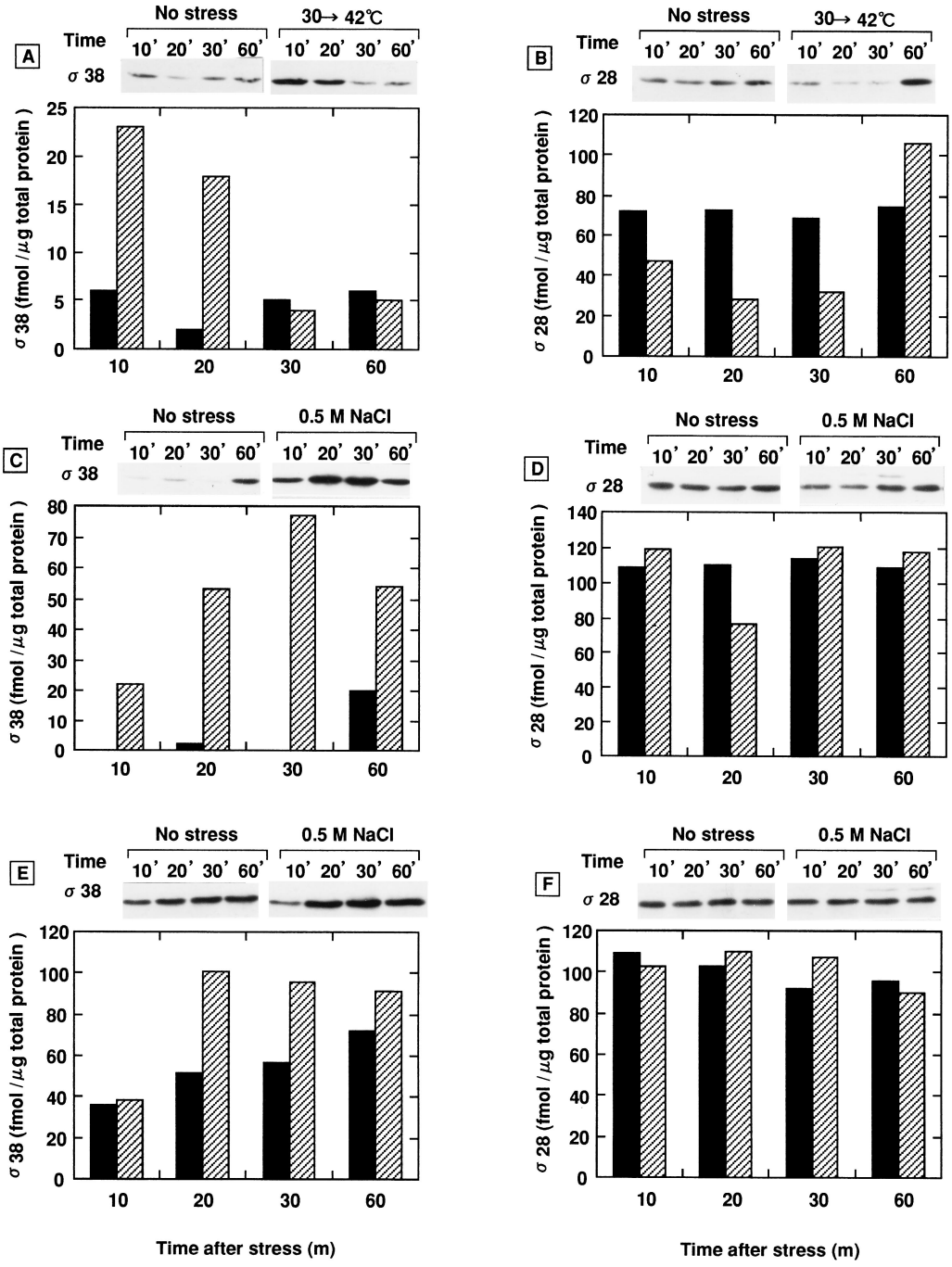

FIG. 2. Variation in the σ^{38} and σ^{28} levels under stress conditions. (A to D) Cells of *E. coli* W3100 were grown to the exponential phase in LB medium at 30°C (A and B) or 37°C (C and D). At the cell density of 15 Klett units, the culture temperature was raised to 42°C by adding an equal volume of LB prewarmed at 54°C (A and B) or the NaCl concentration was increased by adding 5 M NaCl to make the final solution 0.5 M (C and D). Samples were taken at 0, 10, 20, 30, and 60 min after the stress treatment. Aliquots of the cell lysates containing 10-μg amounts of total proteins were analyzed by the quantitative Western blot system. Black bars represent the control values determined using untreated cell extracts. (E and F) Cells of *E. coli* W3100 were grown to the stationary phase in LB medium at 37°C. At 5 h after the culture dilution (about 2 h after the growth arrest), 5.0 M NaCl was added to make final 0.5 M solution. Samples were taken at 0, 10, 20, 30, and 60 min after the addition of NaCl. Aliquots of the cell lysates containing 10 mg of total proteins were analyzed as above. Black bars represent the control values determined using untreated cell extracts. The increase in the σ^{38} control levels in Fig. 2C is due to the induction of σ^{38} synthesis upon the transition into stationary growth phase.

tabolite repression caused by growth on D-glucose as the carbon source (1) and upon exposure to high temperature or high salt concentrations (1, 29). In agreement with these observations, *E. coli* cells become nonmotile at high temperature or under high salt concentrations (23, 36). High osmolarity gave no effect on σ^{28} level at the both growth phases (Fig. 2D and F), except for a slight decrease observed after 20 min of the exponential-phase culture (Fig. 2D). Upon exposure to 42°C, however, the level of σ^{28} started to decrease, and at 20 min, it dropped to 28 fmol per μg of total proteins or less than half the

level of the control without heat shock (Fig. 2B). This decrease may be due to the lack of available chaperons because the heat shock proteins DnaK, DnaJ, and GrpE are required for transcription of both the *flhD* master operon and the *fliA* operon (37). The reduction of FlhDC, a class I transcription factor which contacts RNA polymerase α subunit carboxy-terminal domain (24), causes the decrease in transcription of all the flagellar regulon genes including the *rpoF* gene encoding σ^{28}. At 60 min after heat shock, the level of σ^{28} recovered to the steady-state level (Fig. 2B), even though cell motility was not yet regained. The apparent conflict between the loss of motility and the increase in σ^{28} level may be due to accumulation of anti-σ factor. In *S. typhimurium*, FlgM, a negative regulator of the flagellum-specific σ factor, FliA (10, 32), can be exported to sense the structural state of the flagellar organelle (16). The exposure of cells to adverse conditions such as high salt concentrations or high temperature would inhibit the flagellar assembly by inhibiting the export of anti-σ^{28} factor.

ACKNOWLEDGMENTS

We thank Shuichi Kusano and Nobuyuki Fujita (NIG) for discussions and Susan Garges (NIH) for critical reading of the manuscript.

This work was supported by grants-in-aid from the Ministry of Education, Science and Culture of Japan and Proposal-Based Avanced Industrial Technology R&D Program.

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