Control of the Synthesis and Functions of RNA polymerase Sigma Subunits in *Escherichia coli*

Miki Jishage

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Department of Genetics

School of Life Science

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2 ABBREVIATIONS

bp	base pairs
CTD	carboxyl terminal domain
E. coli	*Escherichia coli*
EDTA	ethylenediamintetraacetic acid
GST	glutathion S-transferase
LB	Luria broth
N	amino
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Rsd	regulator of sigma D
SDS	sodium dodecyl sulfate

3. SUMMARY

Rapid and efficient switching of the gene expression is necessary for bacteria to suivive for a prolonged time period in the stationary phase and upon a sudden exposure to stressful environments. Replacement of the σ subunit of RNA polymerase is an efficient mechanism to change the global pattern of transcription. For understanding the regulation of σ subunit utilization and replacement in *Escherichia coli*, 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 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 *E. coli* strains, W3110 and MC4100, by a quantitative Western immnoblot analysis. The levels of σ^{54} and σ^{28} are maintained at 10 and 50% the level of σ^{70} in both strains growing at both exponential and stationary phases, while the level of σ^{38} is undetectable at the exponential growth phase but increases to 30% the level of σ^{70} at the stationary phase, supporting the concept that σ^{38} plays a key role in transcription of the stationary phase-expressed genes. Stress-coupled change in the intracellular level was observed for two σ subunits: the increase in σ^{38} level and the decrease in σ^{28} level upon exposure to heat shock at the exponential phase; and the increase in σ^{38} level under high osmolality conditions at both the exponential and the stationary phases.

In the course of this study, however, we realized that the composition of σ subunits is different between bacterial strains, reflecting the genetic backgounds. For instance, the composition of two σ subunits, $\sigma^{28}$and σ^{38}, differ between various stocks of the same W3110 strain. Five different types of W3110 were identified: A-type lineages have both σ subunits in intact forms; B-type lineages carry truncated σ^{38} subunit and intact σ^{28} subunit; C-type lineages carry intact σ^{28} subunit but lack σ^{38} subunit; D-type lineages have only σ^{38} subunit without σ^{28} subunit; and E-type stocks lack both σ subunits. All W3110 the lineages examined, however, contain the intact forms of σ^{70} and σ^{54}. As expected from the lack of σ^{28} subunit, cells of the D-type and E-type lineages are nonmotile. The truncated form of σ^{38} subunit in the B-type stocks carries two mutations near its N-terminus and lacks the C-terminal proximal region 4 due to an

amber mutation. The failure of the C- and E-type W3110 cells to express σ^{38} and that of the D- and E-type cells to express σ^{28} were found to be due to defects in transcription even though the respective σ subunit genes remain intact. These findings not only indicate that bacteria accumulate a large number of mutations during long term storage, but also raise a warning that the use of a common bacterial stock even of the same strain is quite important for construction of a whole view of the global gene regulation.

The growth transition from exponential to stationary phase is accompanied by the replacement of RNA polymerase-associated σ^{70} subunit with σ^{38}. Our estimation, however, indicated that the level of σ^{38} does not exceed that of σ^{70}. A fraction of the GST-fused σ^{70} subunit in stationary-phase cell extracts was found to exist as a complex with a novel protein, designated Rsd (Regulator of Sigma D). The intracellular level of Rsd starts to increase during the transition from growing to stationary phase. The *rsd* gene was identified at 90 min on the *E. coli* chromosome. Over-expressed and purified Rsd protein formed complexes *in vitro* with σ^{70} but not with other σ subunits, σ^{54}, σ^{38}, σ^{32}, σ^{28} and σ^{24}. Analysis of proteolytic fragments of σ^{70} indicated that Rsd binds at or downstream of the region 4, the promoter -35 recognition domain. The isolated Rsd inhibited transcription *in vitro* to various extents depending on the promoters used, implying that Rsd is an anti-sigma factor for σ^{70}.

Analysis of the transcription organization of *rsd* indicates that the *rsd* gene contains two promoters, P1 and P2, of which P2 plays a major role in *rsd* expression. The *rsd*p2 has a gearbox-like sequence with a characteristic -10 sequence, but is recognized by σ^{70}. The expression of Rsd is under the control of growth-rate inversely. Analysis of *rsd* mutant strains indicated that Rsd controls transcription of σ^{70}-dependent promoters negatively, but that of σ^{38}-dependent promoters positively.

Taken all the observations together, we propose that Rsd is a regulator that controls the relative level of various forms of the RNA polymerase, leading to the regulated switching of gene expression pattern in *E. coli*.

4. INTRODUCTION

The survival of bacterial cells in various environments depends on their abilities to sense the external conditions and adopt their internal metabolic systems by turning on and off the expression of specific genes (Gottesmann, 1984). For change of the global gene expression pattern, bacteria employs the powerful mechanism of specificity control of transcriptional apparatus. In *E. coli,* the RNA polymerase core enzyme with the molecular composition of $\alpha_2\beta\beta'$ has the ability to transcribe the genetic information on DNA into RNA. For initiation of transcription at specific promoter sites on DNA, an additional component, σ subunit, is required (Burgess and Travers, 1969). The promoter recognition specificity of RNA polymerase is conferred by one of the multiple species of σ subunit (Helmann and Chamberlin, 1988). The replacement of the σ subunit on RNA polymerase is thought to play an important role in switching of the gene expression pattern.

Up to the present time, seven different molecular species of σ subunit (Table1) have been identified in *Escherichia coli* (Ishihama, 1997). The major σ factor, σ^{70} is responsible for transcription of most genes expressed during the exponential cell growth (Osawa and Yura, 1981; Helmann and Chamberlin, 1988; Ishihama, 1997). Besides σ^{70}, six different molecular species of alternative σ subunits have been identified in *E. coli*, which are required only during certain developmental growth stages or under specific growth conditions. σ^{54} was first identified as the σ factor required for expression of the genes involved in nitrogen metabolism such as the gene for glutamine synthetase (Hunt and Magasanik, 1985) and later has been implicated in transcription of other stress-response genes such as *fdhF* (formate dehydrogenase), *hyc* and *hyp* (hydrogenase synthesis) and *psp* (phase shock protein). In the mixed-acid fermentation, as much as one-third of the carbon atoms of the carbohydrate substrate are converted to

formate, which formate is oxidized in the absence of external electron acceptors by the formate dehydrogenase (FdhF). The FdhF level is regulated in response to the oxygen and nitrate concentration and pH of the culture medium (Rossmann *et al.*, 1991). Expression of the *hyc* and *hyp* operon and of *fdhF* requires $E\sigma^{54}$ with a transcriptional activator FhlA. The phage shock protein operon is also induced in response to a variety of stressful conditions or agents such as filamentous phages infection, ethanol treatment, osmotic shock, heat shock, and prolonged incubation in the stationary phase (Brissette *et al.*, 1990). The *psp* operon is transcribed by $E\sigma^{54}$ with support of a transcription activator PspF and is stimulated by integration host factor (IHF) (Jovanovic *et al.*, 1996). In marked contrast to $E\sigma^{70}$, $E\sigma^{54}$ is unable to form the open complex in the abesence of activator protein. Moreover, σ^{54} does not contain the conserved sequences of σ family proteins (Magasanik, 1982).

When bacterial cultures is shifted to high temperature, the synthesis of a number of proteins, called the heat shock proteins (HSPs), is induced in a short period. Many HSP proteins function as molecular chaperons and facilitate protein folding, while some others function as proteases to degrade unfolded proteins (Gross *et al.*, 1996). The gene coding for HSPs are transcribed by RNA polymerase containing σ^{32} (the *rpoH* gene product). During steady-state growth at 30 °C, cells contain a low level of σ^{32}, at the order of 10-30 molecules per cell. Upon temperature upshift, the amount of σ^{32} increases (Strauss *et al.*, 1987). When *rpoH* is inactivated, cells are viable only at temperatures below 20 °C, indicating that σ^{32} is also requires for growth above 20 °C (Zhou *et al.*, 1988).

σ^{24} (the *rpoE* gene product) was initially identified as a factor required for transcription of *rpoH* (Erickson and Gross, 1989). The forth promoter of *rpoH*, called *rpoHp* 3, is activated at the lethal temperature of 50 °C and is recognized by $E\sigma^{24}$. The activity of σ^{24} responds to the rate of expression of outer membrane proteins (Mescas *et al.*, 1993). Up to date, three members of the σ^{24} regulon have been identified, *rpoHp3*,

degP (*htrA*), which encodes a periplasmic protease, and one of the two promoters driving expression of *rpoE* (Raina *et al.*, 1995; Rouviere *et al.*, 1995).

When *E. coli* stops to grow due to nutritional deprivation, the second principal σ subunit, σ^{38}or σ^{S}, begins to be synthesized. The induction of stationary phase-associated phenotypes absolutely requires σ^{38} since some genes for starvation survival are transcribed only by $E\sigma^{38}$ holoenzyme (Hengge-Aronis, 1993). Even during the exponential growth phase, the action of σ^{38} is also required under certain stress conditions such as under high osmolarity (Hengge-Aronis, 1996a) or at low temperatures (Sledjeski, 1996). A comparison of the σ^{38} amino acid sequence with other σ subunits in *E. coli* revealed that σ^{38} is the closest to σ^{70} (Lonetto *et al.*, 1992). In contrast to other alternative σ factors, vegitative or primary σ factors contain the RpoD box, highly conserved motif in region 2.3 and 2.4 (Tanaka *et al.*, 1988), and the characteristic motifs in regions 2.4 and 4.2, respectively (Lonetto *et al.*, 1992). Transcription *in vivo* of stationary-specific genes, *bolA* and *xthA*, completely depends on σ^{38} (Lange *et al.*, 1991b; Sak *et al.*, 1989). These genes are, however, transcribed *in vitro* by both $E\sigma^{38}$ and $E\sigma^{70}$.

Motitlity and chemotaxis are expedients that bacteria can adapt upon exposure to environmental stress. An ability to move away from stressful areas and into microenvironments favorable for growth is of adaptive value to bacterial cells in nature. 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 (Macnab, 1992). 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 class-3b operons which include 18 genes involving the genes for flagellar synthesis, flagellar rotation, chemotactic membrane receptors, and chemotactic signal transduction (Macnab, 1992).

E. coli possesses several iron transport systems, one of which allows for the uptake of iron complexed to citrate and consists the *fec* operon (fecIRABCDE). Expression of this operon depends not only on iron-limitation but also the presence of ferric dicitrate (Crosa, 1997). FecI is involved in the activation of this operon and is now identified as a new-subfamily member of σ subunits for extracytoplasmic functions (Angerer *et al.*, 1995).

The model of σ replacement relies on the change in the intracellular concentration of individual σ subunits, because the level of core enzyme stays almost at a constant level (Ishihama and Fukuda 1980; Kawakami *et al.*, 1979). Until recently, however little was known of the intracellular concentrations of individual σ subunits except for the major σ subunit, σ^{70} (Ishihama, 1991). We initiated a systematic determination of the intracellular concentrations of four σ subunits, σ^{70}, σ^{54} σ^{38} and σ^{28}, in two *E. coli* strains MC4100 and W3110 growing under various conditions. Since the strain MC4100 lacks σ^{28} for flagellar formation, we analyzed these two strain W3110 in order to understand possible influence of the lack of one σ subunit on the levels of other σ subunits.

In the course of these measurements, we realized that the composition of two σ subunits, σ^{38} and σ^{28}, differ between various stocks of the same W3110 strain, even though *E.coli* W3110 has been widely used for genetic and physiological studies, including the construction of the ordered library of genomic DNA the linkage map of the *E. coli* chromosome and the sequence of *E. coli* genome. We decided to carry out a systematic analysis of a number of laboratory stocks of strain W3110, collected from major laboratories of bacterial genetics in Japan, with respect to the composition of four σ subunits, σ^{70}, σ^{54}, σ^{38} and σ^{28}. Results indicate that at least five different lineages of the strain W3110 exist in this country, which differ in their content and/or the molecular structure of two stress-response σ subunits, σ^{38} and σ^{28}. The variation in σ composition and σ structure may be correlated with the fact that bacterial strains were stored as stab cultures for long periods until 20 years ago.

Upon entering into the stationary phase, sequential changes take place in cell morphology and physiology of *E. coli* (Kolter *et al.*, 1993). Cells become smaller, develop a spherical rather than a rod-shaped morphology, and their cytoplasm is condensed, whereas the volume of the periplasm increases (Reeve *et al.*, 1984). As described above, the switch of gene expression pattern under various stressful conditions is thought to take place by replacement of the σ subunit on RNA polymerase. Upon nutrient limitation, σ^{38} begins to be produced, and allows the core polymerase to transcribe more than 40 genes required for stationary phase survival (Hengge-Aronis, 1996b). The level of σ^{38} increases to as much as about 30-35% the level of σ^{70} (Jishage and Ishihama, 1995; Jishage *et al.*, 1996). Meanwhile the level of σ^{70} stays constant, even though the frequency of transcription of genes under the control of σ^{70} decreases by more than 10-fold (Ishihama, 1991).

I found in this study that in stationary phase cells, σ^{70} but not other σ subunits forms a complex with a hitherto unidentified stationary-specific protein. This protein begins to be synthesized during the transition from exponential growth to stationary phase. *In vitro* transcription studies indicated that the protein interferes with the engagement of σ^{70} in the transcription cycle. After sequencing, the protein was identified as a product of the URFs (unidentified reading frames) revealed by genome sequence analysis (Blattner *et al.*, 1993). I propose that this protein, designated Rsd (Regulator of Sigma D), with σ^{70}-binding activity plays a role in controlling the σ^{70} function, ultimately leading to the regulated switching of gene transcription pattern in *E. coli.*

5. MATERIALS AND METHODS

5.1 Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 2. A total of 11 laboratory stocks with the strain name *E. coli* W3110 were provided by a number of laboratories in Japan (Table 3). Cells were grown at 37 °C with shaking in either LB or M9 (Sambrook et al. 1989) medium supplemented with glucose (0.4%) and thiamine (1 μg/ml). In some cultures, the media were supplemented with ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), isopropyl β-D-thiogalactopyranoside (IPTG, 0.5mM) and arabinose (0.02%). Growth was monitored by measuring the turbidity with a Klett-Summerson photometer.

The culture conditions were fixed as follows: A few colonies from an overnight culture on LB agar plates were inoculated into 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 the incubation was continued 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. To one half, a solution of prewarmed LB containing 5 M NaCl was added to make 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.

For swarm assay on agar plate, 0.25% Bacto agar (Difco) was included in Tryptone broth containing 1% Bacto Tryptone (Difco) and 0.5% NaCl. Cells from LB overnight cultures were inoculated onto the center of petriplates containing 30 ml of tryptone swarm agar. The plates were incubated at 37 °C for 7 hrs.

5.2 Plasmids

The plasmids used in this study are listed in Table 2. To construct plasmids for expression of various σ subunits in GST-fusion form, the respective coding sequences were PCR-amplified using the primers described in Table 4. Each primer includes

various restriction enzyme sites (underlined in the sequences shown in Table 4). The sequences of the PCR-amplified fragments were confirmed by the dideoxynucleotide method (Sambrook *et al.*, 1989). The PCR-generated DNA fragments were treated with the appropriate restriction enzymes and cloned into the corresponding sites of the pGEX5x-1 vector (Pharmacia) to generate pGEXD, pGEXN, pGEXS, pGEXH, pGEXF, pGEXM and pGEXRD for expression of GST fusion proteins of σ^{70}, σ^{54}, σ^{38}, σ^{32}, σ^{28}, FlgM (anti-σ^{28}) and Rsd, respectively.

The *rsd* gene was also inserted into pET21a (Novagen) between the *Nde*I and *Xho*I sites to generate pET-Rsd, for expression of His_6-tagged Rsd protein. To create a Rsd expression plasmid pUCRsd33, which carries the region from the promoter to terminater containing the complete *rsd* coding sequence, a 820 bp *rsd* fragment was PCR-amplified using primers Rsd5 and Rsd6 and subcloned into the *Bam*HI and *Pst*I sites of pUC18. The *Bam*HI-*Sph*I fragment from pUCRsd33 was isolated and ligated into pACYC184 to create pACYCRsd. To create pBADRsd31-1, a *rsd* fragment was PCR-amplified with primers Rsd11 and Rsd12 (for sequences see Table4) and subcloned into the *Eco*RIand *Sph*I sites of pBAD22A. Two species of *rsd* promoter fragments were PCR-amplified using two pairs of primers, *i.e.* Rsd7 and Rsd14, and Rsd13 and Rsd14 (for sequences see Table4). The PCR products were subcloned into the *Eco*RI and *Bam*HI sites of pRS551, generating pRsdI or pRsdIII, respectively. The sequences of the insert of plamids were comfirmed by dideoxynucletide sequencing.

5.3 DNA purification

Cells in 50 mM Tris-HCl (pH 8.0) and 50 mM EDTA were treated with 10 mg/ml lysozyme for 45 min at 4 °C, and then lysed by incubation at 50 °C for 1 hr in 0.5 % SDS, 50 mM Tris-HCl (pH 8.0 at 4 °C), 0.4 M EDTA and 1 mg/ml proteinase K. After dilution with TE [10 mM Tris-HCl (pH 7.5 at 4 °C), 1 mM EDTA] buffer, the samples were extracted once with phenol-chloroform and once with chloroform. DNA was ethanol-precipitated, redissolved in TE, and treated with 10 mg/ml RNase A for 30

min at 37 °C. DNA was extracted once with phenol-chloroform and once with chloroform, and ethanol-precipitated.

5.4 DNA sequencing

DNA sequencing was performed by the dideoxy chain termination method (Sambrook *et al.*, 1989) using the Auto Read Sequencing Kit (Pharmacia) with an A.L.F DNA sequencer (Pharmacia).

5.5 PCR amplification and cloning

PCR was performed with a Perkin-Elmer DNA thermal cycler. Template genomic DNA (1 ng), 0.2 μM each of primers, and 2.5 U of ExTaq DNA polymerase (Takara Shuzo) were mixed in a volume of 100 μl containing 250 μM each of deoxynucleoside triphosphates, and ExTaq PCR Buffer (Takara Shuzo). The mixture was subjected to 30 cycles of PCR reaction, each consisting of 1 min denatration at 94 °C, 1 min reannealing at 65 °C, and 2 min DNA synthesis at 72 °C. Primers listed in Table 4 were synthesized with an Applied Biosystems Model 394 DNA synthesizer and were purified by polyacrylamide gel electrophoresis.

5.6 Protein purification

σ^{70} subunit was overexpressed using pGEMD and purified as described (Igarashi and Ishihama, 1991), while σ^{38} was expressed using pETF and purified as described (Tanaka *et al.*, 1993). σ^{54} was overexpressed using NCM668 (strain M5219 carrying pJES259) and purified as described (Popham *et al.*, 1991) and σ^{28} was expressed in BL21(DE3) using pETSF and purified as described (Kundu *et al.*, 1997).

To overproduce the His_6-tagged or GST-fused Rsd proteins, an *E. coli* BL21(λDE3) transformant containing pET-Rsd or a strain DH5 transformant containing pGEX-Rsd were grown at 37 °C in LB medium containing ampicillin, and when the culture reached 30 Klett units, isopropyl-β,D-thiogalactopyranoside (IPTG) was added at 1 mM. After 1 hr, cells were harvested and stored at -80 °C until use. For the purification

of His_6-tagged Rsd protein, the soluble fraction was applied onto a Ni^{2+}-NTA column (Qiagen) equilibrated with a binding buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl). The column was washed with 10X bed volumes of the binding buffer containing 5 mM imidazole, followed by elution with a step gradient of imidazole from 10-200 mM. The eluate containing the Rsd protein was dialyzed against TGED buffer (10 mM Tris-HCl, pH 7.6, 5% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.1 M NaCl) and loaded onto a heparin-agarose column (Hi-Trap Heparin, Pharmacia) equilibrated with TGED buffer. The adsorbed proteins were eluted with a 0.1-1.0 M linear gradient of NaCl in TGED. The Rsd protein, which eluted at 0.8 M NaCl, was dialyzed against storage buffer (10 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$ 0.2 M KCl, 50% glycerol, 0.1 mM EDTA, 1 mM DTT). The purification of GST-Rsd protein was carried out essentially as described above. IHF and Lrp were purified by Talkunder, T. A. in this laboratory. In brief, IHF was expressed in *E. coli* A6740 containing plasmid pSA5*hiphimA* and purified essentially according to the method described by Nash *et al.* (1987). IHF recovered from polyethleneimine (PEI) precipitates of the cleared cell lysate were precipitated with 50-70% saturation of ammonium sulfate precipitates and then subjected to fractionation by successive chromatography on P11-phosphocellulose (Whatman), CM cellulose (Whatman) and heparin-Sepharose (Pharmacia LKB) columns. Lrp was expressed in *E. coli* DL1582 carrying plasmid pMWD and purified essentially according to the method described by Ernsting *et al.* (1993). From the cleared cell lysate, Lrp was precipitated with 0.3% PEI, eluted from PEI precipitates with 1.0 M NaCl, and then precipitated with 50-80% saturation of ammonium sulfate. Crude Lrp was subjected to chromatography on a G-DEAE column and then to FPLC on a Mono-S column.

GST-fused σ70 proteins were overexpressed from cells growing exponentially in 100 ml LB containing ampicillin at 37 °C following induction with 1 mM IPTG (OD 600 of 0.3) for 1 hr. The purification of GST-fused σ70 proteins was performed essentially as described (Igarashi and Ishihama, 1991).

5.7 Preparation of antibodies

Antibodies against σ^{70}, σ^{38}, σ^{54}, σ^{28} subunit and Rsd were produced in rabbits by injecting the respective purified proteins.

5.8 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/ml lysozyme at 0 °C for 10 min, cells were lysed by adding 0.5% Brij-58. The Brij-lysate was supplemented with 0.01 M $MgCl_2$ and 0.2 M KCl, and digested at 37 °C for 10 min with 20 μg/ml of RNase A and 100 μg/ml of DNase I in the presence of 1 mM phenylmethylsulfonylfluoride (PMSF) followed by sonication for 1 min with a Cosmo Bio Bioruptor. The supernatant after centrifugation for 30 min at 15000 rpm was used as the cell lysate for all the experiments.

5.9 Western blot anlysis

For the measurement of σ subunits, a quantitative Western blot analysis was employed using the mono-specific anti-σ antibodies. In brief, cell lysates were treated with a SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.025% bromophenol blue) and separated on SDS-7.5 or 10% polyacrylamide gels. Proteins in the gels were directly electroblotted onto polyvinylidene difluoride membranes (NIPPON GENETICS). Blots were blocked overnight at 4°C in 3% BSA in phosphate-buffered saline (PBS), probed with the mono-specific antibodies against each σ subunit, washed with 0.5% Tween in PBS, and incubated with goat anti-rabbit IgG conjugated with hydroxyperoxidase (CAPPEL). The blots were developed with 3,3′-diaminobenzidine, tetrahydrochloride (Dojindo) or the enhanced chemiluminescence reagent system (Amersham). Staining intensity was measured with a PDI image analyzer system equipped with a white light scanner.

5.10 Purification and analysis of σ-associated proteins

To identify σ-associated proteins, an *E. coli* W3110A strain (Jishage and Ishihama, 1997) containing pGEXD, pGEXN, pGEXS or pGEXF was grown in Luria-broth (LB) medium containing ampicillin (100 μg/ml) at 37 °C until stationary phase (3 to 4 hr after cessation of cell growth). Cell lysates were prepared as described above and applied onto a glutathione-Sepharose column (Pharmacia) previously equilibrated with phosphate-buffered saline (PBS). The column was washed with 10X bed volumes of PBS and eluted with glutathione-containing buffer (50 mM glutathione, 100 mM Tris-HCl, pH 8.0, 120 mM NaCl). Aliquots of the pooled eluate fractions were fractionated by electrophoresis on 7.5, 10 and 13.5% polyacrylamide gels in the presence of SDS. Proteins were transferred onto PVDF membranes (Nippon Genetics) and stained with Coomassie brilliant blue (CBB). Purification and analysis of σ-associated proteins were carried out by the same procedure using pGEXM and pGEXRD.

5.11 Protein sequencing

Stained protein bands were directly subjected to automated micro-sequencing (Applied Biosystems 491 Protein Sequencer).

5.12 GST pull down assay

For analysis of σ and Rsd interaction, 20 pmol each of GST or GST-Rsd were mixed with equimolar amounts of holoenzyme, core enzyme or σ subunit on ice for 30 min in a total volume of 50 μl of transcription buffer (50 mM Tris-HCl, pH 7.8 at 37 °C, 3 mM magnesium acetate, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 25 μg/ml bovine serum albumin). After adding 10 μl (bed volume) of glutathione-Sepharose beads (Pharmacia) equilibrated with phosphate-buffered saline (PBS), the mixtures were incubated on ice for 10 min. The beads carrying immobilized proteins were washed 3 times with PBS, and then the bound proteins were eluted with glutathione buffer (50 mM glutathione, 100 mM Tris-HCl, pH 8.0, 120 mM NaCl) and separated by SDS-PAGE on 5-15% gradient gels. The gels were analyzed by Western blotting using polyclonal antibodies against α, β, β' and each of the σ subunits.

5.13 RNA purification and Northern blotting

RNA was prepared from exponentially growing bacteria to 30 Klett units according to the method of Aiba *et al.* (1981). Approxymately 10 μg of RNA in 20 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 7.0) containing 5 mM sodium acetate, and 1 mM EDTA, 17.5% formaldehyde and 50% formamide was heated for 15 min at 65 °C, and immediately loaded on to a 1% agarose gel containing 18% formaldehyde and MOPS buffer. After electrophoresis at 100 V, RNA in the gel was transferred to a Hybond-N Plus membrane (Amersham) overnight in 20 X SSPE (3 M NaCl, 173 mM NaH_2PO_4 and 25 mM EDTA). After baking the filter for 120 min at 80 °C, hybridization was performed by soaking the blot for 3 hr in a prehybridization solution (5 X SSPE, 50% formamide, 5 X Denhalt' solution, 0.5% SDS, and 0.1 mg of sheared sarmon sperm DNA per ml) at 42 °C. After adding the probe, hybridization was done for 16 hr at the prehybridization temperature. The blot was washed twice in 2 X SSPE containing 0.1 % SDS at room temperature for 10 min, twice in 1 X SSPE containing 0.1 % SDS at 65 °C for 20 min, and in 0.1 X SSPE 0.1 % SDS at 65 °C for 20 min and then autoradiographed.

For analysis of *rpoS* transcript, a probe of 1,029-bp DNA corresponding to the coding region of *rpoS* was prepared by PCR using S5 and S6 primers and A-type W3110 DNA as a template. For analysis of *rpoF* RNA, a probe of 722-bp fragment covering the coding region of *rpF* was prepared by PCR using FA1 and FA2 primers. For analysis of *flhD* transcript, a probe of 1,205-bp fragment covering the coding region of *flhD* was PCR-amplified with FD1 and FD2 primers. On the other hand, a probe for *rpoD* transcript analysis was prepared by digesting plasmid pGEMD (Igarashi and Ishihama, 1991) with *Mlu*I and *Pst*I to obtain a 667-bp fragment covering the internal portion of the *rpoD* gene. All the DNA probes were gel purified and labeled by random priming using Megaprime DNA labelling systems (Amersham) and [α–^{32}P]dCTP (Amersham). Labeled probes were separated from unincorporated nucletides by using a SUPREC-02 (Takara Shuzo).

5.14 Primer extension

For primer extension analysis of *rsd* mRNA, an oligonucleotide with the sequence 5'-TGACGCGCTCCGTCAGGTTATCGAG-3' was ^{32}P-labeled by using MEGALABEL (Takara Shuzo). The reaction mixture containing 2 pmol end-labeled primer and 50 μg of total RNA in 20 μl of RT Buffer [50 mM Tris-HCl (pH 7.6), 60 mM KCl, 10 mM $MgCl_2$, 1 mM dNTP and 1 mM DTT] was heated for 5 min at 80 ℃, and then stored on ice for 5 min for annealing. After addition of AMV reverse transcriptase (12.5 U Takara Shuzo), the mixture was incubated at 42 °C for 60 min. The reaction was terminated by adding 180 μl of a stop solution (0.15 M NaOH and 5 mM EDTA), and RNA was hydrolyzed by incubation at 70 °C for 20 min. After precipitation with ethanol, the samples were resuspended in 15 μl of formamide loading buffer and analyzed by electrophoresis on a 6% polyacrylamide gel containing 8 M urea. Dideoxy sequencing reactions were carried out using pUCRsd33 as template and the same primer as that used for primer extension. Reaction products were run in parallel with the sequence ladder obtained a 7-DEAZA sequencing kit (Takara Shuzo) to determine the end point of the extension product.

5.15 Transcription *in vitro*

RNA polymerase core enzyme was purified from *E. coli* W3350 by passing the purified RNA polymerase at least three times through phosphocellulose columns (Kusano *et al.*, 1996). Holoenzymes were reconstituted by mixing the core enzyme and three-fold molar excesses of σ subunits. Single-round transcription by the holoenzyme was carried out under the standard conditions described previously (Kajitani and Ishihama, 1983) except that the concentrations of templates and RNA polymerase were varied as indicated in each experiment. In brief, mixtures of template and RNA polymerase were preincubated at 37 °C for 30 min to allow open complex formation in 35 μl of the standard transcription mixture containing 50 mM NaCl. RNA synthesis was initiated by adding a 15 μl mixture of substrate and heparin in the standard transcription buffer and

continued for 5 min at 37 °C. Transcripts were precipitated with ethanol and analyzed by electrophoresis on 6 or 8% polyacrylamide gels containing 8 M urea. Gels were exposed to imaging plates and the plates were analyzed with a BAS-2000 image analyzer (Fuji). The templates used were: a 205 bp *Eco*RI-*Eco*RI fragment for *lac*UV5 (Kajitani and Ishihama, 1983); a 233 bp *Hpa* II-*Hpa*II fragment for *gal* (Kumar *et al.*, 1994); a 205 bp *Pvu*II-XbaI fragments for wild-type *lac* (Igarashi and Ishihama, 1991); and a 287 bp *Bam*HI-*Kpn*I fragment for the *alaS* promoter (Nomura *et al.*, 1986). These truncated DNA templates produced *in vitro* transcripts 63, 45, 68 and 169 nucleotides in length, respectively.

For the anti-σ^{70} activity assay of Rsd, a fixed amount of σ^{70} (1 pmol) and increasing amounts of Rsd (1, 2, 5 and 10 pmol) were pre-incubated for 10 min at 30 °C, and then 1 pmol of core enzyme was added. The mixtures were subjected to the single-round transcription assay.

5.16 Gel shift assay

The *Eco*RI-*Bam*HI fragment from pRS551RsdI was end-labeled with [γ-^{32}P]ATP by using MEGA LABEL (Takara Shuzo). Approximately 10 fmol of the fragment was incubated for 20 min at 25 °C with various concentrations of IHF or Lrp in a total volume of 20 µl of Binding Buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 50 mM KCl, 7 mM $MgCl_2$, 3 mM $CaCl_2$, 10% glycerol, 50 µg/ml bovine serum albumin (BSA) and 2 µg of salmon sperm DNA]. For the gel shift assay of Lrp protein, L-leucine, when required, was added to a final concentration 30 mM. The samples were then loaded on a 5% nondenaturing polyacrylamide gel, prepared in 0.5 X TBE buffer. Electrophoresis was carried out at 10V/cm at room temperature. Gels were transferred to 3MM Whatman paper, dried, and autoradiographed.

5.17 DNA footprinting

Mixtures of ^{32}P-end-labeled DNA fragment (4 nM) and proteins were incubated for 20 min at 25 °C in a total volume of 50 µl of 50 mM Tris-HCl buffer (pH 8.0)

containing 0.1 mM EDTA, 0.1 mM DTT, 50 mM KCl, 7 mM $MgCl_2$, 5 mM $CaCl_2$, and 50 μg/ml bovine serum albumin (BSA). DNase I was added and the incubation was continued for 30 sec at 25 °C. Digestion was terminated by adding 50 μl of phenol. DNA was precipitated with ethanol, dissolved in 15 μl of formamide loading buffer (95% formamide, 10 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene cyanol) and analyzed by electrophoresis on a 6% polyacrylamide gel containing 8 M urea. Gels were visualized by autoradiography or with a Bioimage Analyzer BAS 2000 (Fujix).

5.18 Chemicals and Enzyms

The protein concentration of cell lysates was determined using a protein assay kit (Bio-Rad). [α-^{32}P]dCTP (3000Ci/mmol) and [α-^{32}P]UTP (3000Ci/mmol) used for radiolabelling were from Amersham Corp. X-ray film was purchased from Fuji. Limited proteolytic digestion of proteins was performed using L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma).

5.19 Construction of *rsd* disruption mutant

To construct an internal deletion of the *rsd*, a 1.4 kbp *rsd* gene fragment was PCR-amplified with primers f158-1 and f158-2 (for sequences see Table4) and subcloned into the *Sph*I and *Eco*RI sites of pUC18. The *rsd* coding region between *Bsm*I and *Sna*BI sites was replaced by a *Hinc*II fragment of pUC4K carrying the kanamycin resistance gene (Pharmacia) (pUJC-1). pUJC-1 was digested with *Bsm*I-*Sna*BI, and the digested fragment was purified by SUPREC01 (Takara Shuzo). 2 μg of this fragment was introduced into *E. coli* JC7623 by linear transformation. Kanamycin-resistant transformants harboring the mutation integrated in the chromosome were isolated. Phage P1*vir* transduction was used to transfer the mutation to strain MC4100 (MJ30).

5.20 P1 transduction

Mixture of equal volume (0.1 ml) of the P1*vir* lysate and the recipient bacteria harvested in exponential phase (OD600=0.2), in LB containing 2.5 mM $CaCl_2$, were

incubated for 15 min at 37 °C. After adding 5 ml of LB containing 1% sodium citrate, cells were incubated for 30 min at 37 °C, collected by centrifuged, and resuspended in 5 ml of LB containing 1% sodium citrate. After repeating the washing 3 times, the cells were plated on selective plates containing 1% sodium citrate.

5.21 β-galactosidase assay

β-Galactosidase activity was performed as described by Miller (1982). In brief, after recording the cell density by measuring the absorbance at 600 nm, aliquots of the cultures were immediately added to Z buffer (60 mM Na_2HPO_4 $7H_2O$, 40 mM NaH_2PO_4 H_2O, 10 mM KCl, 1 mM $MgSO_4$ $7H_2O$, and 50 mM β-mercaptoethanol), to make the final volume 1 ml. After adding 2 drops of chloroform and 1 drop of a 0.1% SDS solution, the cell suspension was mixed with vortex for 10 seconds, and stored in a 28 °C water bath for 5 min. The reaction was initiated by adding 0.2 ml of o-Nitrophenyl-β-D-galactoside (ONPG, 4 mg/ml). After sufficient levels of yellow color (0.6-0.9 at 420 nm) were developed, the reaction was terminated by adding 0.5 ml of a 1 M Na_2CO_3 solution. β-Galactosidase activity is expressed as Miller units, *i.e.* [1000x(A420nm-1.75xA550nm)]/(A600nm x reaction time x volume). The reaction was repeated at least twice, and the activity was caluculated as the average.

6. RESULTS

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

6.1.1 Measurement of the σ subunits by quantitative Western blot

For measurement of the σ subunit level in cell extracts, a quantitative Western blot system was developed using mono-specific polyclonal antibodies. In each determination, we first made a standard curve for each of the purified σ proteins to determine the range in which the linearity exists between the protein concentration and the intensity of immunostaining. For both σ^{70} and σ^{38} subunits, the linearity was detected over a 10-fold range at least between 2-20 ng (Fig. 1).

For the determination of σ subunits in test samples, we then analyzed several different volumes of the cell lysates to identify the volumes that include 2-20 ng of each σ subunit. Using the optimum volumes of cell lysates thus estimated, we finally repeated the determination of individual σ subunits at least three times, always in parallel with the determination of 6 different concentrations of the purified σ subunits as the assay standards. The maximum fluctuation between different measurements described in this report ranged ±20%.

6.1.2 Levels of various σ subunits during the steady-state growth

At first, we measured the intracellular levels of two principal σ subunits, σ^{70} and σ^{38}, at various phases of cell growth. Cells of strain MC4100 were grown in LB medium at 37 °C and samples were taken periodically during the growth transition from the exponential growth (20 Klett units) to the stationary phase (160 Klett units).
The concentration of σ^{70} is maintained at a constant level, ranging from 50 to 80 fmol per μg of total protein throughout the growth change from log to stationary phase (Fig. 2). In contrast, the level of σ^{38} increases when the cells stop to grow and reached to the maximum level of almost 30 fmol per μg of total protein in the stationary phase. Since

the level of σ^{38} is below the detection level at the early log phase, the molar ratio of σ^{38} to σ^{70} reaches from a negligible level to about 0.3 in the stationary phase.

E. coli strain MC4100 has been widely used for transcriptional analysis of the stress response (Lange and Hengge-Aronis, 1991). This strain, however, lacks σ^{28} required for transcription of the genes for flagella formation because of the lack of FlbB protein (Silverman and Simon, 1977), 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 (Ishihama, 1991; Ishihama, 1995; Kawakami *et al.*, 1979), a competition must take place between various σ subunits for core enzyme binding and thus the lack of σ^{28} subunit may influence the levels of other subunits. To test this possibility, next we analyzed the intracellular levels of various σ subunits in *E. coli* strain W3110 (the strain used for the genome project in Japan).

Cells were grown in LB medium at 37 °C and samples were taken periodically during the growth transition from the exponential growth (15 Klett) to the stationary phase (220 Klett). Under the culture conditions employed, the doubling time was 0.5 hr. Cell lysates were prepared and the amounts of four species of σ subunit, σ^{70}, σ^{54}, σ^{38}, σ^{28}, σ^{E} and FecI were determined. The maximum fluctuation between different measurements described in this report ranged 20%, and the minimum level of detection was 0.2 fmol per μg total cell lysate protein. A typical immuno-blot pattern is shown in Fig. 3 and 4, and the quantitative measurement using the standard curves for the purified individual σ subunits is summarized in Table 5.

As observed in strains W3350 (Kawakami *et al.*, 1979) and MC4100, the level of σ^{70} in W3110 is maintained at a constant level from the exponential phase to the stationary phase. The concentration of σ^{E} is maintained at a constant level, ranging from 0.7 to 2.0 fmol per μg of total protein throughout the growth change from log to stationary phase. In contrast, the level of FecI decreases when the cells stop to grow and the maximum level is almost 0.2 fmol per μg of total protein in the exponentially growing phase (Fig. 4). The levels of minor σ subunits were different between the two *E. coli*

strains. Generally, the σ levels were higher in W3110 than MC4100, *i.e,* about 3 times for σ^{70}, 6-7 times for σ^{54} (for both the exponential and the stationary phases) and about 2 times for σ^{38} (at the stationary phase).

6.1.3 Variations in the σ levels under stress conditions.

Upon exposure of bacterials cells to environmental stresses, drastic changes take place in the pattern of gene transcription. As an attempt to understand stress-coupled replacement of the σ subunits on RNA polymerase, we measured changes in the relative levels of various σ subunits in *E. coli* upon exposure to various stresses: (i) exposure of both exponential and stationary phase cells to temperature upshift from 30 to 42 °C; and (ii) exposure of both exponential and stationary phase cells to osmotic shock by increasing NaCl concentration to 0.5 M.

At first we measured the levels of σ^{70} and σ^{38} in MC4100. Among all the stress conditions tested, a significant increase in σ^{38} level was observed for heat shock treatment at the exponentially growing phase and osmotic shock at the stationary phase. Following the temperature upshift, for instance, the concentration of σ^{38} increased to 13 fmol per μg of total protein by 20 min at 42 °C; and at 60 min, it dropped to the steady state level at 30 °C (Fig. 5A). The results support the notion that, besides σ^{32}, σ^{38} plays a role in heat-shock protection although the time reached to the maximum level of σ^{38} was later than that of σ^{32}.

High osmolality stress at the stationary phase also induced the production of σ^{38} from 10 fmol per μg of total protein to 50 fmol by 30 min, *i.e.*, about 5-fold increase compared to the control culture without osmotic stress (Fig. 5B). In the both cases, the level of σ^{70} remained constant at least within the time ranges examined. Temperature upshift at the stationary phase and osmotic upshift at the exponential phase gave no effect on the levels of both σ subunits (data not shown).

Next we measured the σ subunit levels in *E. coli* W3110 under stress conditions. As observed in MC4100, significant changes were also observed in σ^{38} and σ^{28} levels , but not in σ^{70} and σ^{54} levels in W3110. Under steady-state culture

conditons, the level of σ^{38} subunit is higher for 30 °C than 37 °C (compare Fig. 6A and 6C). Our observation is consistent with the recent finding that at 20 °C, the expression of *rpoS* is high even in the exponentially growing phase (Sledjeski *et al.*, 1996). 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 protein by 10 min or 4-fold by over the control culture without heat shock (Fig. 6A). 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 the same as in the case of MC4100. The pattern of heat-shock response is, however, markedly different from that of σ^{32} or heat-shock σ factor, which inreases 10-15 fold within the first few minutes after heat-shock and decreases again to the steady-state level by 10-20 min (Strauss *et al.*, 1987; Yura *et al.*, 1993). 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 (Fig. 6C) and the stationary phase (Fig. 6E). 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 protein (Fig. 6C), while in the stationary phase, about 2-fold increase was observed at 20 min after osmotic shock (Fig. 6E). High osmolarity gave no effect on σ^{28} level at the both growth phases (Fig. 6D and F), except for a slight decrease observed after 20 min of the exponential phase culture (Fig. 6D). Upon exposure to 42 °C, however, the level of σ^{28} started to decrease, and at 20 min dropped to 28 fmol per μg of total protein or less than half the level of control without heat shock (Fig. 6B).

6.2 Variation in RNA Polymerase Sigma Subunit Composition within Different Stocks of *Escherichia coli* W3110

6.2.1 Variation in the σ subunit composition between different stocks of strain W3110

Some W3110 strains are known to have truncated forms of the σ^{38} subunit (by K. Tanaka, personal communication). We then collected a total of 11 laboratory stocks

with the strain name of *E. coli* W3110 from a number of laboratories in Japan (Table 3) and examined the molecular forms and intracellular concentrations of σ subunits in these strains.

Cells were grown at 37 °C under aeration in Luria broth (LB). Cell lysates were prepared at both exponential growth and stationary phases. The levels of σ^{70}, σ^{54}, σ^{38} and σ^{28} in the cell lysates were determined by the quantitative Western blot method. All the W3110 stocks examined contained σ^{70} and σ^{54} subunits of apparently intact sizes, as fractionated by SDS-polyacrylamide gel electrophoresis (Fig. 7). To our surprise, however, marked differences were found in their contents of two other σ subunits, σ^{28} and σ^{38}. From the difference in the immuno-blot pattern of these two σ subunits, we classified the bacterial stocks into five types, A, B, C, D and E (Table 6).

A typical pattern of σ subunits from each type strain is shown in Figure 7, which indicates that: A-type lineage carries both σ^{28} and σ^{38} subunits of intact sizes; B-type lineage contains a truncated form of σ^{38} subunit with the apparent size of 42 kDa on SDS-PAGE (intact σ^{38} gave 48 kDa under the same running conditions) and intact σ^{28} subunit; C-type lineage contains only σ^{28} subunit and lacks detectable level of σ^{38} subunit; D-type lineage carries only σ^{38} subunit without σ^{28} subunit: and E-type bacteria lack both $\sigma^{28}$and σ^{38} subunits. The levels of σ^{70}, σ^{54} and σ^{28} subunits stayed constant throughout the growth transition from exponential to stationary phases, but σ^{38} was detected only in the stationary phase cells as in the case of W3350 and MC4100 (Jishage *et al.*, 1996).

6.2.2 Sequence analysis of the truncated σ^{38} subunit in B-type W3110

The B-type W3110 contained a truncated form of σ^{38} subunit which crossreacted against mono-specific anti-σ^{38} antibodies. In order to determine the structural change(s) in the truncated form of σ^{38} subunit, we cloned the *rpoS* gene from a B-type stock and determined its DNA sequence. As shown in Fig. 8, three single base changes were detected, which resulted in Leu-to-Phe substitution at position 25, Gly-to-Tyr substitution at position 45, and generation of an amber codon at position 270. Thus,

the σ^{38} protein in the B-type *E. coli* W3110 is 269 amino acid residues in length (intact σ^{38} is composed of 330 residues) with Mr of 30,854, almost entirely devoid of the region 4, which is highly conserved among the σ family proteins and thought to be involved in promoter -35 recognition (Helmann and Chamberlin,1988; Lonetto *et al.*, 1992).

The amino acid sequence of σ^{38} displays a high homology to σ^{70} (Lonetto *et al.* 1992), especially in regions 2.3, 2.4 and 4.2, and it has been suggested that it may be a second vegetative σ factor rather than an alternative σ factor. In fact, a number of promoters are recognized by both $E\sigma^{70}$ and $E\sigma^{38}$ (Klob *et al.*, 1995; Tanaka *et al.*, 1995; Tanaka *et al.* 1993). Analysis of chimeric promoters formed from the σ^{70}- and σ^{38}-dependent promoters indicates that the specificity of σ^{38} recognition is determined mainly by the -10 sequence which is recognized by the region 2.4 of σ subunits (Tanaka *et al.*, 1995). It is possible that the truncated form of σ^{38} lacking the region 4 is functional in B-type variant cells.

6.2.3 Expression of the *rpoS* gene in C- and E-type W3110

Type-C and type-E lineages lacked detectable amounts of σ^{38} subunit. Transcripts of the *rpoS* gene were than analyzed by Northern blot hybridization. As shown in Fig. 9, one clear band was observed when RNA isolated from A-type cells was hybridized with the *rpoS* probe. The size of *rpoS* mRNA detected is in good agreement with the previous observation that transcription is driven by a single promoter located within the *nlpD* gene upstream of *rpoS*, giving a product of about 1,600 bases (Lange *et al.*, 1995; Takayanagi *et al.*, 1994). The *rpoS* transcript in the A-type cells was detected albeit different levels for both the exponential and stationary phases, supporting the concept that in the exponential phase, translational repression operates in the synthesis of σ^{38} (Yamashino *et al.*, 1995). On the contrary, *rpoS* transcripts from C- and E-type cells were very faint or even absent (Fig. 9A). As an internal control, we checked the same RNA blots with a probe of the *rpoD* gene. As shown in Fig. 9B, one transcript of

similar intensity was observed for all three stocks of the strain. These results indicated that the defect in the expression of *rpoS* in the C- and E-type cells arises at the transcriptional level.

We then determined the DNA sequence from the *Cla*I site located 1,397 bp upstream of the initiation codon of the *rpoS* gene to the stop codon. No difference was, however, detected in the DNA sequence at least within the range from 286 bp upstream from the initiation codon to the stop codon of *rpoS* (data not shown). Several candidates have been suggested as signals for activation of *rpoS* transcription. For example, a gradual reduction in growth rate stimulates *rpoS* transcription (Lowen and Hengge-Aronis, 1994); cAMP-CRP represses it (Lange and Hengge-Aronis 1994); but ppGpp activates (Gentry *et al.*, 1993, Lange *et al.*, 1995). It has not yet been determined how the transcription of *rpoS* is blocked in these cell lines.

The expression of *rpoS* is, however, under exceedingly complex regulation, involving a number of regulatory signal molecules and operating at the levels of transcriptional and post-transcriptional regulation as well as stability control of the protein (Lange and Hengge-Aronis, 1994, Yamashino *et al.*, 1995). Thus, it is not excluded yet that *rpoS* transcript in the type-C and type-E lineages is extremely unstable.

6.2.4 Expression of the *fliA* gene in D- and E-type W3110

Both D- and E-type bacteria were found to lack the σ^{28} subunit, which is required for the expression of flagellar genes. In agreement with this finding, both stocks are non-motile as analyzed by the plate assay for motility (Fig. 10).

In order to investigate whether the D- and E-type lineages carry the gene encoding intact σ^{28} protein, we cloned the *rpoF* gene from these cells and sequenced DNA from the *Stu*I site located 253 bp upstream of the initiation codon of the *rpoF* gene to the stop codon. The sequences were completely identical with that reported (Mytelka and Chamberlin, 1996) except for two single-base changes at positions -130 and -188 bp upstream from the initiation codon for σ^{28} translation. For determination of the

defective step of *rpoF* expression in these bacteria, we then analyzed transcripts of not only *rpoF* but also *flhDC*, which encode a class-I transcription factor for expression of stage I genes of the flagellar regulon, including *rpoF* (Liu *et al.*, 1994). Northern blot analysis with a *rpoF* probe, shown in Fig. 11A, indicates two transcripts for RNA isolated from the A type cells. The *rpoF* gene maps in the *fliAZY* operon (*fliA* has been renamed *rpoF*), which gives a transcript of approximately 2,270 bases (Mytelka and Chamberlin, 1996). The upper band, migrating faster than the 2.9 kbp marker, might be a complete transcript of this operon while the lower, stronger and more smeared band detected below the 1.5 kbp marker might be a product terminated after the first one or two genes. In contrast to the A-type pattern, virtually no transcript of the *rpoF* gene was detected in RNA from D- and E-type bacteria. Moreover, no detectable transcript of *flhD* was observed in D- and E-type samples (Fig. 11B), even though one clear band of similar intensity was observed for all 3 samples when the same RNA blot was hybridized with a probe of the *rpoD* gene (Fig. 11C). These results together indicate that the lack of *rpoF* mRNA in the D- and E-type bacteria is due to a defect in FlhDC production, but it remains unsolved why *flhD* mRNA is not synthesized in these W3110 variants.

Over 3% of the *E. coli* K-12 genome is concerned with the synthesis, assembly, and function of its flagellar (Macnab, 1992). In addition, the biosynthesis of a flagellum is costly, mainly because of the large number of flagellin subunits needed for assembly of the filament (Macnab, 1992). Under the environmental conditions favorable for bacterial growth, it is reasonable that costly processes such as flagellar biosynthesis, motility and chemotaxis would be shut off at the level of the master operon. Thus, the lack of σ^{28} may not be disadvantageous under laboratory culture conditions, and might even have selective advantage.

6.3 A Stationary Phase Protein in *Escherichia coli* with Binding Activity to the Major Sigma Subunit of RNA Polymerase

6.3.1 Analysis of σ-associated proteins

We determined the intracellular concentrations of various σ subunits in *E. coli* strain MC4100 and W3110 at various phases of cell growth using a quantitative Western blot assay (Jishage and Ishihama, 1995; Jishage *et al.*, 1996). The level of σ^{70}, the major σ subunit, was found to stay constant at about 600-700 molecules per cell, in both growing and stationary phases. The results were in good agreement with those obtained by a combination of immuno-precipitation and gel electrophoresis of radio-labeled cell extracts of the strains W3350 and B/r (Saitoh and Ishihama, 1977). These observations raised a question as to why σ^{70} is largely inactive in the stationary phase. In growing cells, most of the σ^{70} subunit is associated with the core enzyme and is involved, through the σ cycle, in transcription (Ishihama, 1991). We therefore tried to identify any proteins which might be associated with σ^{70} or other σ subunits in the stationary phase. For this purpose, four species of σ subunits, σ^{70}, σ^{54}, σ^{38} and σ^{28}, were expressed as fusions with GST in *E. coli* W3110 grown into the stationary phase in LB medium at 37 °C. The expression levels of GST-σ fusion proteins were analyzed by Western blotting of induced and uninduced cell lysates. Due to the leaky expression, the level of GST-σ^{70} subunit in the absence of IPTG was as high as that of the chromosome-coded σ^{70} (Fig. 12). Thus, in order to minimize possible artifacts due to over-production of σ subunits, σ^{70}-associated proteins were analyzed using uninduced cell extracts.

Cell lysates were passed directly through glutathione-Sepharose columns. The column-bound proteins were eluted with glutathione and separated by SDS-PAGE on 5-15% gradient gels (Fig. 13). All four GST-σ fusion proteins were bound to the columns and eluted with glutathione (the major band in each lane). By immuno-staining, the core enzyme subunits, α, β and β', were all detected in the column-bound fractions for each of the GST fusion-σ subunits (GSTσ^{70}, GSTσ^{54} and GSTσ^{28} lanes) except GST-σ^{38} (GSTσ^{38} lane), indicating that the holoenzymes containing GST-fused σ^{70}, σ^{54} and σ^{28} can be retained on the columns, but the GST-σ^{38} does not form a stable holoenzyme or the holoenzyme containing GST-σ^{38} has a low affinity to the glutathione-Sepharose column.

For identification of σ-associated proteins, most of the minor bands detected by protein staining were subjected to protein micro-sequencing. DnaK was found to be associated with all GST fusion-σ subunits, but its content was the highest for σ^{54}. Several minor bands associated with all GST-σ fusions were identified as GST without σ and some other degradation products of GST-σ fusion proteins, because all these proteins contained the N-terminal sequences of GST. In addition, one or two unique proteins were identified for each σ subunit. For instance, the 14 kDa band observed in the bound protein fraction using GST-σ^{28} was found to have the protein sequence of FlgM (Fig. 13, GST-σ^{28} lane), which is the anti-σ factor for σ^{28} (Ohnishi *et al.*, 1992). To verify this finding, we also expressed GST-FlgM fusion and found that σ^{28} can be recovered as a complex with the GST-FlgM fusion protein (Fig. 13, GST-FlgM lane). These results confirmed the possibility that as yet unidentified anti-σ factors against other σ subunits could be detected using this experimental system.

6.3.2 Identification of an σ^{70}-associated proteins

In the GST-σ^{70} lane, at least five additional bands were observed besides the three core subunits, α, β and β' (Fig. 13, lane GSTσ^{70}). After protein micro-sequencing, four of these were identified as breakdown products of the GST-σ^{70} fusion protein, and these bands were also observed for other GST-σ fusions. However, one specific band with a molecular mass of around 21 kDa (indicated by an arrow) was identified only in the GSTσ^{70} lane but not in the other GSTσ lanes. The N-terminal sequence of this 21 kDa protein was found in the *E. coli* genome data base as an unidentified reading frame (URF) f158 (Blattner *et al.*, 1993). The f158 URF gene is located near 90 min on the *E. coli* chromosome, probably forming a single gene operon (Fig. 14A).

We next cloned the f158 gene and confirmed that its nucleotide sequence is identical with that in the *E. coli* genome data base. The predicted gene product shows a 31% identity with the alginate regulatory protein AlgR2 of *Pseudomonas aeruginosa* (Kato *et al.*, 1989) (Fig. 14B). More strikingly, there is a 45% identity in its N-terminal

region of 74 amino acid residues with the first 69 residues of AlgR2, which regulates the production of alginate, a virulence factor for *P. aerruginosa*, by controlling some enzymes in the pathway of alginate production such as nucleoside diphosphokinase (Schlictman *et al.*, 1995). Since the isolated 21 kDa protein formed binary complexes with σ^{70} and interfered with its function (see below), we tentatively designated this 21 kDa protein as Rsd (Regulator of Sigma D).

6.3.3 Direct interaction of Rsd with σ^{70} subunit

In order to test whether this σ^{70}-associated Rsd interacts directly with σ^{70}, we constructed an *E. coli* plasmid expressing a GST-Rsd fusion protein. Overexpressed and purified GST-Rsd was mixed with either σ^{70} or $E\sigma^{70}$ holoenzyme, and then with glutathione-Sepharose beads. Proteins tightly bound to the beads were eluted with glutathione and fractionated by SDS-PAGE. For detection of the proteins at a high sensitivity, the gels were subjected to Western blotting using a mixture of anti-α, anti-β, anti-β' and anti-σ^{70} antibodies (Fig. 15A). Although there were low level backgrounds of non-specific binding to GST without Rsd under the washing conditions employed, it is clear that: free σ^{70} binds to GST-Rsd (lane 6) but not to GST (lane 5); a fraction of σ^{70} in the holoenzyme preparation also binds to GST-Rsd (lane 2); but neither α nor $\beta\beta$' in both core and holoenzyme associate with GST-Rsd (lanes 4 and 6). These results suggest that Rsd associates preferentially with free σ^{70}.

In order to identify the intracellular state of Rsd-σ^{70} complexes, we expressed GST-σ^{70} in *E. coli* and analyzed σ^{70}-bound proteins after isolation of complexes using glutathione-beads. As shown in Fig. 15B (*log* lane), the major proteins associated with σ^{70} in extracts of exponentially growing cells were core enzyme subunits. In addition, several minor bands were detected, which were identified by N-terminal sequencing to be degradation products of GST-σ^{70}. When the stationary-phase cell extract was analyzed (Fig. 15B, *stationary* lane), two additional bands, Rsd and ω, were identified, suggesting that Rsd is produced or becomes active only in the stationary phase cells. The level of RNA polymerase-associated ω protein also increases in stationary phase.

The glutathione bead eluate of stationary phase extracts was then fractionated by heparin-agarose column chromatography. As shown in Fig. 15C, two major peaks were identified, core-associated GST-σ^{70} (holoenzyme fractions) and free form of GST-σ^{70}, to which Rsd was associated (Rsd-GSTσ^{70} fractions). The result indicates that the Rsd-bound σ^{70} is unable to associate with the core enzyme.

6.3.4 Rsd-binding site on σ^{70} subunit

The specificity of σ^{70} recognition by Rsd was analyzed using six *E. coli* σ subunits, *i.e.*, σ^{70}, σ^{54}, σ^{38}, σ^{32}, σ^{28} and σ^{24}, which were all over-expressed in *E. coli* and purified to apparent homogeneity. A mixture of six different σ subunits was subjected to the GST-Rsd pull-down assay and the Rsd-bound σ species was identified by immuno-staining with mono-specific polyclonal antibodies against each of the six σ subunits. Among the six σ subunits examined, only one species, σ^{70}, was found to bind Rsd (Fig. 16A). Since the antibodies against six different σ factors are not equally efficient in binding with their respective σ factors, we repeated the assay using increasing amounts of the GST-Rsd fusion and increasing amounts of the antibodies. Under all the conditions employed, σ^{70} was the only species tightly bound with Rsd. This suggests that Rsd recognizes a unique structural signal of σ^{70}, which is not present in other σ subunits.

We next determined the contact site on σ^{70} for Rsd. For this purpose, σ^{70} was partially digested with trypsin and mixtures of the digestion products were incubated with either GST or GST-Rsd. Proteins tightly bound to the glutathione-Sepharose beads were eluted with glutathione and analyzed by Western blotting using anti-σ^{70} antibodies. As shown in Fig. 16B, only specific tryptic fragments were retained bound with the GST-Rsd (compare GST-Rsd and σ^{70}-trypsin lanes). Fragments which were recovered in the Rsd-bound fractions were then examined for their amino-terminal sequences. Results indicated that the GST-Rsd bound all the C terminus-proximal fragments containing the region 4 of σ^{70}. Two small proteins, R3-4 and R4, shown in Fig. 16B correspond to C-terminal fragments downstream from amino acid residues 449 and 500, respectively.

The initial tryptic cleavage sites of σ^{70} were identical with those determined by Severinova *et al.* (1996). Since the 18.5 kDa R4 fragment contains only region 4, we conclude that Rsd interacts at or downstream of the region 4 of σ^{70}. One possibility raised by these observations is that Rsd interferes with the σ^{70} activity of either promoter -35 recognition or interaction with class-II (σ-contact) transcription factors.

To determine the contact site on region 4 of σ^{70} precisely, the nineteen GST-fused σ70 alanine substitution mutants (Table 8) were purified and GST pull down assay was performed. The expression vectors for these mutants were constructed by C. A. Gross *et al.* As shown in Fig. 17A, GST-fused σ^{70} mutants which are substituted with alanine at position amino acid residue 595 or 598 were not bound with Rsd. While under the same condition, β and β' were found to associated with these two GST-fused σ^{70} mutants. To test the transcriptional activity of these mutants, *in vitro* transcription was carried out using *lac*UV5 or extended -10 promoter. As shown in Fig. 17B, the transcriptional activity by 595 or 598 σ^{70} mutants with *lac*UV5 promoter was found to decrease compared to the activity using extended -10 promoter. Results indicated that 595 and 598 amino acid residue in σ^{70} were important for Rsd to associate with σ^{70} and these two mutants could form the complex with core, however these two mutations had an effect on transcription related -35 promoter recognition.

6.3.5 Inhibition of transcription *in vitro* by the Rsd protein

To test directly whether the Rsd protein acts as an anti-σ factor, we purified it and tested its influence on *in vitro* run-off transcription assays, using linear DNA templates containing σ^{70}-regulated promoters. When *lac*UV5 was used as the promoter, the addition of Rsd did not produce any detectable effect on σ^{70} activity. On the other hand, *alaS* promoter-directed transcription was almost completely inhibited in the presence of excess Rsd (Fig. 18). Transcription inhibition was also measured using other promoters. As summarized in Table 7, various extents of inhibition were observed depending on the promoter used. The effect of Rsd was also examined at increasing concentrations of templates but all produced essentially the same effects, suggesting that

the target of Rsd is RNA polymerase but not template. When further σ^{70} were added, the activity was restored in a dose-dependent manner, indicating that the target of Rsd action is σ^{70}. The variations in sensitivity to Rsd between promoters may be related to the fact that the holoenzyme concentration required to express the maximum activity is different between the promoters used.

6.3.6 Intracellular level of the Rsd protein

The intracellular level of Rsd was measured using a quantitative Western blot method. Whole cell extracts were prepared from *E. coli* W3110 A-type strain (Jishage and Ishihama, 1997) growing at various growth phases. As shown in Fig. 19, Rsd started to increase when the rate of cell growth began to decrease. The level became maximum, reaching about 20% of the level of σ^{70} subunit, when the cell growth stopped completely and thereafter stayed constant. This observation is in good agreement with the interpretation that Rsd binds to unused σ^{70} and store it in an inactive form.

6.4 Regulation of *rsd* expression and physiological roles of Rsd.

6.4.1 Identification of transcriptional start sites of *rsd*.

In order to clarify the regulation mechanism underlying growth-dependent expression of Rsd protein, we first determined the transcriptional start site of the *rsd* gene. Total RNA was isolated from both log- and stationary- phase cells of strain W3110A, and was subjected to primer extension analysis. Only one major product (P2) was observed for RNA from the log-phase cells (Fig. 20A). Besides the P2 transcript, another product (P1) was identified for RNA from the stationary-phase cells (Fig. 20A). The transcriptional start point of P1 transcript is located at 148 bp upstream of the translational initiation codon (Fig. 20A), while the start site of P2 transcript is located at 54 bp upstream of the initiation codon, the two promoters being separated by 94 bp.

The P1 start site at -148 is preceded by a -10 promoter-like sequence (CATAAT) with reasonable similarity to the σ^{70} consensus sequence. Separated from the -10 signal

by a 17 bp spacer, a promoter -35-like sequence (ATGGCA) exists, which shows four of six bases match to the σ^{70} consensus sequence (Fig. 20C and D). On the other hand, the transcription of P2 is initiated at -54 G and leads to the synthesis of RNA having an untranslated sequence of 54 nucleotides. Although the -10 hexamer of P2 (CATGTT) is not in good agreement with the σ^{70} consensus sequence, the presence of upstream TG characterizes it as an extended -10 promoter (TGGCATGT). Such a sequence alignment is similar to several gearbox promoters (Fig. 20D). The -35 region (TTGCAC) is separated from -10 region by a 17 bp spacer and in agreement with the -35 hexamer associated with the gearbox promoters (Fig. 20D). Two gearbox promoters, *bolA*p1 and *ftsQ*p1, are known to be recognized by σ^{38} subunit (Bohannon *et al.*, 1991; Lange and Hengge-Aronis, 1991). To identify whether σ^{38} is responsible for transcription from P1 and P2 promoters, total RNA was isolated from strain ZK1000 which lacks *rpoS* and analyzed by primer extension. As shown in Fig. 20E, P1 product was not detected in the absence of *rpoS*, whereas the P2 product was observed. These results suggest that the P1 promoter is dependent on σ^{38} whereas P2 promoter is transcribed by $E\sigma^{70}$. This conclusion was confirmed by an *in vitro* transcription assay. As shown in Fig. 20E, $E\sigma^{38}$ can transcribe only from the P1 promoter, whereas $E\sigma^{70}$ can transcribe from both promoters.

6.4.2 Influence of growth-rate on and growth phase the expression of *rsd*.

The level of transcription from the gearbox promoters, *bolA*p1 and *ftsQ*p1, have been demonstrated to be inversely related to growth rate (Aldea *et al.*, 1990). To examine whether the expression of *rsd* is dependent on growth phase or growth rate, we constructed the *rsd-lacZ* transcriptional fusion on a phage lambda and inserted the phage at its normal attachment site on the *E. coli* gene Using *rsd-lacZ* transductant, the β-galactosidase activity was measured at various time points during the growth transition from exponential growth to stationary phase grown in LB at 37 °C. The β-galactosidase activity increased 7- to 8- fold in the stationary phase compared to the exponential phase (Fig. 21A).

To confirm that this activation of *rsd* promoter in stationary phase is dependent on σ^{38}, the β-galactosidase activity was measured at different growth phases with the cells carrying the *rpoS* null mutation. Results shown in Fig. 21A indicated that the β-galactosidase activity in the *rpoS* null mutant also increased during the growth transition from exponential to stationary phase, but the maximam level of expression in the *rpoS* mutant (1698 Miller Units) is lower than that in the wild-type (2364 Miller Units) in the early stationary-phase (3 to 4 hrs after the stop of cell growth). After prolonged culture in the stationary phase, however, the β-galactosidase activity of the *rpoS* mutant reached to the same level as that observed for the wild-type, suggesting that the *rsd*p1 promoter is transcribed by σ^{38} only in the early stationary phase. To measure the β-galactosidase activity from the P2 promoter, another *rsd-lacZ* transcriptional fusion without the P1 promoter was constructed. As shown in Fig. 21B, the maximum activity was observed during the transition from exponential phase to stationary phase and the basal activity is two fold higher than the activity from both P1 and P2. One possible explanation of this observation is that there a regulatory signal with the silencing activity of *rsd* transciption is located upstream of the P2 promoter. We next investigated the effect of growth rate on *rsd* expression. The expression level of *rsd -lacZ* fusion was compared between exponentially growing cultures in media supplemented with various carbon sources. As shown in Fig. 21C, the β-galactosidase activity was low in cells growing at high rates, but substantially increased in cells growing at lower rates (spots 4 and 5). This result indicates that *rsd* expression is inversely related to the growth rate.

Some growth rate dependent genes are known to be under the control of ppGpp. To determine whether ppGpp also affects *rsd* expression, we measured the β-galactosidase activity in a strain which does not produce ppGpp. As shown in Fig. 21D, the β-galactosidase activity in the ppGpp mutant strain was reduced to 38% the level of wild type at the maximum expression and then the expression increased to 70% in the late stationary phase. This suggests that ppGpp is partly involved in stimulation of the transcription of *rsd* , but may not be responsible for the full expression of *rsd*.

6.4.3 Effects of IHF and Lrp on *rsd* expression.

Upon deletion of a region upstream from the *rsd*p2 promoter, the basal activity of *rsd* transcription increased compared to that of promoter fusion containing p1 and p2 promoters (see above). Assuming that there is a sequence signal affection on *rsd* expression, we searched for the consensus sequences for transciption factors, and found the binding sequence for IHF and Lrp. To test possible effect of IHF and Lrp on *rsd* expression, we examined the β-galactosidase activity using various mutant strains defective in these DNA-binding proteins. As shown in Fig. 22A, the activity was reduced approximately three fold in strains deficient in IHF or Lrp.

To test the binding of IHF and Lrp to this region, gel shift assays *in vitro* were carried out using a linear DNA fragment from pRS551 digested with *Bam*HI and *Eco*RI, corresponding to the region from -207 to +20, and purified IHF and Lrp proteins. The shifted-band was observed with IHF at concentrations higher than 100 nM (Fig. 22B). By DNase I footprinting analysis, however, no clear protecting region could be found. Lrp also bound to the *rsd* promoter fragment, and the addition of leucine reduced the level of Lrp binding to *rsd* DNA (Fig. 22C). To identify the Lrp binding site(s), on *rsd* DNA, DNase I footprinting analysis was also performed using the same DNA fragment. As observed for other promoters with Lrp-binding sites (Nou *et al.*, 1993; Marschall *et al.*, 1998), Lrp protected DNA at multiple sites, extending at least positions between -160 and -40 (Fig. 22D). The Lrp-binding was found to be in a highly cooperative manner. Lrp is known to bend DNA (Wang and Calvo, 1993), thereby inducing looping as to be wrapped around a core of Lrp (Calvo and Matthews, 1994). In consistent with this model, the periodical protection pattern on *rsd* DNA fragment was observed.

6.4.4 Effect of *rsd* mutation on the transcription of σ^{38}-dependent *bolA*p1 promoter.

Upon entry into the stationary phase, σ^{38} begins to be produced and allows the core polymerase to recognize and transcribe the genes required for stationary phase survival (Hennge-Aronis, R., 1996). Previously we showed that Rsd can interact

preferentially *in vitro* with free σ^{70} but not $E\sigma^{70}$ (Jishage and Ishihama, 1998). If Rsd interacts with free σ^{70} *in vivo* leading to switching of the transcription from σ^{70}-dependent genes to σ^{38}-dependent genes, the transcription of σ^{38}-dependent promoters may be affected by *rsd* mutation.

To test the above possibility, we constructed a mutant *E. coli* lacking the *rsd* gene, and measured the expression of σ^{38}-dependent *bol*Ap1-*lacZ* transcription fusion under the exogenous supply of various levels of Rsd. As shown in Fig. 23A, the expression of *bol*Ap1-*lacZ* in the *rsd* mutant strain was markedly reduced to about 30% the level of wild-type strain. Western blotting analysis indicated that this reduction in β-galactosidase synthesis was not caused by decrease or increase in the levels of σ^{38} and σ^{70} proteins, respectively (see the Western blot pattern shown above Fig. 23A). Thus, the decrease in Rsd level led to reduction in the utilization of σ^{38} for expression of the σ^{38}-dependent *bol*Ap1-*lacZ* fusion gene. We then tested the opposite case, *i.e.* effect of increased expression of Rsd, on σ^{38}-dependent gene expression. Expression of Rsd in wild-type cells using a *rsd* expression vector, constructed using plasmid pACYC184, resulted in significant (about 1.5-fold) increase in the expression level of *bol*Ap1-*lacZ* (Fig. 23B). Western blot analysis indicated that the maximum level of Rsd expression was higher than that of σ^{70}. To confirm that the observed decrease or increase in *bol*Ap1-*lacZ* expression was due to the direct effect of decrease or increase, respectively, in Rsd level, we introduced the Rsd-expression vector into the *rsd* mutant. As expected, the β-galactosidase activity increased by about 2 fold after induction of Rsd (Fig. 23C).

The increase of Rsd should lead to decrease in the concentration of functional σ^{70} subunit and as a result, the relative amount of $E\sigma^{38}$ holoenzyme may increase because the the intracellular level of core enzyme stays constant at a level characteristic of the rate of cell growth (Ishihama et al., 1976). Likewise the decrease in Rsd level may result in the increase in $E\sigma^{70}$ holoenzyme, ultimately leading to the reduction in $E\sigma^{38}$ level. In order to further confirm this hypothesis, we next examined possible effect of the exogenous supply of σ^{38} on the expression of *bol*Ap1-*lacZ* fusion. For this purpose, σ^{38} was overexpressed using the σ^{38} expression vecter under the control of an arabinose-

inducible promoter (see the Western blot pattern shown above Fig. 23D). The activity of *bolA*p1-*lacZ* indeed increased more than 2 fold (Fig. 23D), reaching the level as high as that observed when Rsd was expressed in the *rsd* mutant strain (Fig. 23C).

These phenomena altogether support the prediction that the decrease in intracellular level of functional σ^{70} by forming complexes with Rsd leads to the increase in $E\sigma^{38}$ level and in activation of transcription from σ^{38}-dependent promoters.

6.4.5 Effect of *rsd* mutation on transcription of σ^{70}-dependent *ompF* promoter.

The intracellular level of RNA polymerase stays at a constant level characteristic of the rate of cell growth (Ishihama, 1981). Thus, the relative level of various forms of holoenzyme should reflect the amounts of σ subunits. If this is the case, the increase or decrease of one σ subunit should affect the levels of holoenzymes containing other σ subunits. The *ompF* gene encoding an outer membrane porin protein is transcibed by E σ^{70} and regulated by OmpR. The level of *ompF* transcription seems to be directly related to the level of $E\sigma^{70}$ (Pratt and Silhavy 1996). For instance, *rpoS* mutation results in overproduction of OmpF (Nystrom, 1994) and high level expression of σ^{38} leads to repress the *ompF* expression (Pratt and Silhavy 1996; Pratt *et al.*, 1996). If *ompF* transcription is repressed by the increased level of σ^{38}, *rsd* mutation may lead to the increase in functional σ^{70} (and the decrease in $E\sigma^{38}$ level) and ultimately to induction of *ompF* transcription.

To test this possibility, we next measured the β-galactosidase activity of *ompF-lacZ* fusion. As shown in Fig. 24A, the expression of *ompF-lacZ* fusion increased about two-fold in the *rpoS* mutant or *rsd* mutant compared to that in wild type cells. The high level expression of *ompF-lacZ* was also observed in the *rsd* mutant (Fig. 24A), but this induction of *ompF-lacZ* expression was surpressed by the supply of Rsd protein by an expression plasmid (Fig. 24B). The high level expression of Rsd protein in the wild-type *E. coli* results in the decrease in β-galactosidase activity (Fig. 24C). Likewise the repression was observed when σ^{38} was overexpressed using the σ^{38} expression vecter

under the control of arabinose-inducible promoter (Fig. 24D). These results altogether support our prediction that the Rsd protein interacts with σ^{70} and thereby decreases the intracellular level of $E\sigma^{70}$.

6.4.6 Influence of Rsd expression on σ^{70}-dependent transcription

To confirm our prediction, we next exmined possible influence of the high-level expression of Rsd on σ^{70}-dependent *ompF* transcription at various times of cell growth. In order to achieve high-level induction of the Rsd protein, the *rsd* gene was inserted into an expression vector under the control of arabinose-inducible promoter and the resulting Rsd expression plasmid was transformed into *E. coli* strain. When Rsd was induced in the early exponential phase, the expression level of *ompF-lacZ* fusion, as measured by β-galactosidase activity, was essentially the same with that in the abesence of Rsd induction (Fig. 25A). On the other hand, the expression of Rsd in the late exponential phase or the early stationary phase markedly inhibited the induction of *ompF-lacZ* fusion, down to 60% the level in the abesence of Rsd expression (Fig. 25B). In the late stationary phase, the inhibitory effect of Rsd on the *ompF-lacZ* induction again became weaker, giving 80% activity of the level without Rsd expression (Fig. 25C).

The fluctuation of the inhibitory effect of Rsd on the *ompF-lacZ* expression can be observed if the level of Rsd expression alters depending on the cell growth phase. After checking the expression level of Rsd using quantitative Western blot analysis, however, we found that the level of Rsd expression stays almost constant at least during the growth transition from log to stationary phase, suggesting that the inhibitory activity of Rsd varies depending on the growth conditions. Since the pattern of growth phase-dependent variation in the inhibition of *ompF-lacZ* expression by over-expressed Rsd correlates with the change in σ^{38} production level, we assumed that the effect of Rsd is observed only under the situation where σ^{38} is present and competes with σ^{70} in binding to the core enzyme. This is consistent with the proposal that the presence of functional σ^{38} represses the expression of σ^{70}-dependent genes such as *ompF*.

7. DISCUSSION

7.1 Regulation of RNA Polymerase Sigma Subunit Level

Previously, Kawakami *et al.* (1979) showed that the intracellular concentration of σ^{70} subunit is maintained at a constant level throughout the growth transition from the exponentially growing to stationary phase, although the levels of core enzyme subunits decrease concomitantly with the arrest of cell growth. We confirmed this conclusion using a quantitative Western blot assay. The concentration of σ^{70} in strain MC4100 and W3110 grown in LB is maintained at a constant level throughout the growth change from log to stationary phase, although a significant difference was observed in the absolute level of σ^{70} between two *E. coli* strains, ranging from 50 to 80 fmol per μg of total protein in the strain MC4100 or from 150 to 170 fmol per μg of total protein in the strain W3110. The difference in growth rate between two strains, *i.e.*, two doublings per hr for W3110 and one doubling per hr for MC4100, may be corelated with the observed difference in σ^{70} level, probably because the fast growing W3110 cells express σ^{70}-dependent essential genes constitutively at higher levels. The level of σ^{38} increases when the cell growth enters into the stationary phase, and reaches the maximum level of almost 30 fmol (MC4100) or 60 fmol (W3110) per μg of total protein. These results indicate that the molar ratio of σ^{38} to σ^{70} increases to about 30% in the stationary phase, suggesting that the balance between two principal σ subunits is important for the growth phase-coupled switching in transcription pattern. In spite of the difference in the absolute levels of various σ subunits, the relative levels of σ^{70} to minor σ subunits are also nearly the same between the two strains.

The level of σ^{54} in both MC4100 and W3110 strains stays constant throughout the growth transition from the 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 (Castano and Bastarachea, 1984). 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% 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 (Merrick, 1993). It remains unsolved 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 inhibitory protein as in the case of σ^{28}-anti-σ^{28} complex (see below).

The level of σ^{28} stays constant from the middle of exponental phase to the stationary phase. In the early exponential-phase, however, its level is signficantly lower than this steady state level, presumably because early log-phase cells degrade serine and synthesize acetylphosphate, which functions as a phosphate donor to phosphorylate OmpR, and the phosphorylated OmpR represses transcription of *flhDC*, the positive regulatory genes for σ^{28} synthesis (Pruess and Matsumura, 1996; Shin and Park, 1995). To our surprise, the level of σ^{28} in *E. coli* W3110 was found to be as much as 50% the level of σ^{70}. If all these σ^{28} subunits are active and the binding affinity to core enzyme is 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 the number of genes under the control of σ^{28} is only 18 (Gillen and Hughes; 1991; Macnab, 1992). 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 is established that the majority of σ^{28} subunit forms a complex with an anti-sigma factor FlgM and stays in an inactive stored form for rapid reuse (Gillen and Hughes, 1991; Hughes *et al.*, 1993; Ohnishi *et al.*, 1992).

On the basis of these measurements, we can estimate the intracellular state of RNA polymerase in *E. coli*. The steady-state cells at the exponentially growing phase contain 1500-2000 molecules of RNA polymerase core enzyme and 500-700 molecules of σ^{70} per cell (Ishihama, 1991). Hence, we can now estimate the approximate numbers of

σ^{54}, σ^{38} and σ^{28} to be 110, 0 and 350 at the exponential phase, and 110, 230, and 350 molecules per cell at the stationary phase, respectively. Thus, the total number of alternative σ proteins in *E. coli* cell 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 RNA synthesized in exponentially growing cells are transcripts of the constitutive and essential genes under the control of σ^{70} (Ishihama, 1991). The total number of genes on the *E. coli* genome is estimated to be about 4000, among which about 1000 genes are expressed in exponentially growing cells. When cells stop growing at the stationary phase, most of these genes are shut-off and instead approximately 100 stationary-specific genes begin to be expressed (Hengge-Aronis, 1993; Ishihama, 1995). The numbers of σ^{54}- or σ^{28}-dependent operons so far identified are only 4 (Magasanik, 1982) or 18 (Gillen and Hughes, 1991; Macnab, 1992), respectively, while in the stationary phase the σ^{38} subunit of RNA polymerase controls the expression of more than 40 genes or operons (Hengge-Aronis, 1996b). If the number of genes under the control of a particular σ subunit correlates with its abundance, the levels of alternative σ factor, σ^{54} or σ^{28}, are very high. The high concentrations of these two alternative σ factors may be related to the capability of *E. coli* cells to rapid adaptation to changes in the environment.

For transcription of the heat shock response genes, σ^{32}, the alternative σ factor is involved (Straus *et al.*, 1987). σ^{32} is a highly unstable protein *in vivo*, but following temperature shift to 42 °C, the level of σ^{32} increases transiently. Upon temperature up-shift, we demonstrated in this study the increase of σ^{38} level even during the exponential phase in both strain MC4100 and W3110. In the case of σ^{32} induction, two possibilities have been proposed: the increase in translation efficiency of *rpoH* mRNA at 42 °C; and the transient stabilization of σ^{32} protein (Yura *et al.*, 1993). Likewise, the level of σ^{38} is controlled by a complex mechanism involving not only transcriptional and translational regulation but also control of the protein stability (Lange and Hengge-Aronis, 1994). The σ^{38} protein is also highly unstable in exponential growing cells, and the secondary structure of σ^{38} mRNA is similar to that of σ^{32} mRNA (Lange and Hengge-Aronis,

1994). From these considerations, the life-time of σ^{38} protein may be under the control of chaperone or protease. Otherwise, the translation of *rpoS* mRNA may increase at 42 °C. Even though we have not determined whether σ^{38} induced under heat shock conditions is functional or not, it may play a role even in the exponentially growing cells under certain stress conditions.

Osmotic upshift at the stationary phase also induces the production of σ^{38}. The concentration reaches the same level as that of σ^{70} at 30 min after the osmotic shock. This is consistent with the observations that the σ^{38} subunit of RNA polymerase controls the expression of some osmotically regulated genes (Hengge-Aronis *et al.*, 1993; Muffler *et al.*, 1996). Even in the stationary-phase cells of both strain MC4100 and W3110 expressing high levels of σ^{38}, the increase in medium osmolarity induces further increase in the σ^{38} level (Fig. 6E), suggesting that *E. coli* cells control the level of σ^{38} by monitoring the demand for expression of the stress-response genes even in the stationary phase.

Flagellar formation in *E. coli* is sensitive to changes in the environmental condition. For instance, the flagellar synthesis is under the regulation of catabolite repression (Alder and Templeton, 1967) and upon exposure to high temperature or high salt concentrations (Alder and Templeton 1967; Morrison and McCapra, 1961). In agreement with these observations, *E. coli* cells become non-motile at high temperature or under high salt concentrations (Li *et al.*, 1993; Shi *et al.*, 1993). This decrease may be due to 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 (Shi *et al.*, 1992). The reduction of FlhDC, a class-I transcription factor which contacts RNA polymerase α subunit C-terminal domain (CTD) (Liu *et al.*, 1995), results in 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} increases to recover the steady state level (Fig. 6B) even though the cell motility is not yet regained. The apparent but transient 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 flagella-specific σ factor, FliA (Gillen and Hughes, 1991; Ohnishi *et al.*, 1992), can be exported to sense the structural state of the flagellar organelle (Hughes *et al.*, 1993). 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.

7.2 Variation in RNA Polymerase Sigma Subunit Composition within *Escherichia coli* W3110

Until 20 years ago, bacterial strains were stored as stab cultures on agar for long periods. However, stab cultures do not guarantee the genetic stability, because under the steady state of stationary phase, transposition of mobile genetic elements, which does not depend on DNA replication, contributes to a high degree of genetic instability (Rodriguez *et al.*, 1992). Recent quantitative analyses based on restriction fragment length polymorphism (RFLP) indicate that a high degree of genetic polymorphism accumulates between subclones isolated from an old stab culture of the strain W3110 and that mobile genetic elements contributes to a major portion of the spontaneous mutagenesis during bacterial storage (Naas *et al.*, 1994; 1995).

Even though our knowledge of the bacterial physiology in stab cultures is poor, it is considered that life in stabs represents particular conditions of stress. In fact, the stationary-phase *E. coli* cells develop a marked resistance against various stresses such as exposure to heat shock (>50 °C), hydrogen peroxide (Jenkins *et al.*, 1988) or high salt concentrations (Jenkins *et al.*, 1990). Thus, the lack of σ^{38} and/or σ^{28} may be quite serious for survival in stab cultures. Accordingly, it is likely that these stock strains have acquired additional mutations which can suppress the σ mutations, even though a majority of those mutantions may be indistinguishable by their phenotypes when grown under laboratory culture conditions. It is, however, not excluded yet that some minor σ subunits are not needed for survival on stab cultures. Supporting this prediction is that the motility is not expressed in the stationary phase on stab cultures, and therefore the lack of σ^{28} may not be so disadvantageous. Likewise, the lack of σ^{38} might have some

unexpected advantageous properties on stab cultures. It is also possible that mutations in the σ proteins may some times result in aquitition of recognition properties of the genes which contribute for long-term survival.

Most of the genetic, biochemical and physiological studies of *E. coli* have so far been carried out using exponentially growing cells under laboratory culture conditions. Non-sporulating bacteria such as *E. coli* in the resting state have long been neglected in genetic, biochemical and physiological studies, even though starvation is the most frequent state in nature. Therefore, the lack of stress-response σ factors have been left unidentified. Recently, however, studies of the regulation of global gene expression in *E. coli* in the stationary phase of cell growth and under stress conditions have become a developing trend in the field of bacterial molecular genetics. Furthermore, the strain W3110 was used as the standard material for construction of the *E. coli* ordered clones (Kohara *et al.*, 1987) and was used for the sequencing project in Japan. Taking the previous (Naas *et al.*, 1994; 1995) and our findings (Jishage and Ishihama, 1997) into account, we propose that for the experimental research on stress response in *E. coli*, special care should be taken to use a common bacterial stock such as the strain W3110A maintained in the Bachmann's collection, which contains all four σ subunits in intact form.

7.3 Regulatory Proteins of the Function of RNA polymerase σ Subunits

We discovered a novel stationary-phase *E. coli* protein, Rsd, which specifically associates with σ^{70}. Several lines of evidence support the prediction that Rsd is involved in control of the activity of the σ^{70} subunit: i) Rsd is formed during the growth transition from exponential to stationary phase in parallel with the shut-off of transcription of σ^{70}-dependent genes (Fig. 19); ii) some of the σ^{70} subunit in stationary-phase cell extracts exists as a complex with Rsd, which can be separated from the remaining σ^{70}-core complexes (Fig. 15C); iii) the purified Rsd forms a specific complex *in vitro* with σ^{70} but not with other σ subunits (Fig. 16A); iv) the binding site on σ^{70} with Rsd is located at or downstream from the promoter -35 binding region 4 (Fig. 16B), where

some class-II transcription factors also interact (Ishihama, 1997; Kumar *et al.*, 1994); and v) Rsd interferes with σ^{70}-dependent transcription *in vitro* directed by at least some promoters (Fig. 18 and Table 7). However, the level of transcription inhibition *in vitro* by Rsd was not high, presumably because: i) the fraction of active Rsd in the over-expressed and purified Rsd fraction was low (the purified Rsd tends to form aggregations) ; ii) an as yet unidentified factor(s) is involved in the formation *in vivo* of stable Rsd-σ^{70} complexes because the Rsd-σ^{70} complex isolated from cells was stable and was not dissociated even after isolation; or iii) Rsd-bound σ^{70} loses the recognition activity of only a set of σ^{70}-dependent promoters.

The role of anti-σ factors in the control of σ subunit activity is being increasingly recognized as a global regulatory system for transcription in prokaryotes. Regulation of the σ subunit activity by anti-σ factors is well-established in *Bacillus subtilis* (Brown and Hughes, 1995; Straiger and Losick, 1996). The existence of anti-σ factors in *S. typhimurium* and *E. coli* was first identified in a regulatory system for inhibition of σ^{28} activity after completion of flagella formation (Ohnishi *et al.*, 1992). Synthesis of σ^{28} and FlgM (anti-σ^{28}) is induced in an early stage of the flagella cascade. The genes involved in flagella formation and chemotaxis are then transcribed by the RNA polymerase holoenzyme Eσ^{28} only as long as FlgM is being excreted from the cell through immature flagella tubes. This provides a unique feed-back regulation system for gene transcription, depending on the level of formation of a cellular structure. The FlgM protein is unusual because it is mostly unfolded even in the native state (and thus can be secreted through narrow flagella tubes), but it becomes structured when it binds to σ^{28} (Daughdrill *et al.*,1997). The FlgM protein binds to the region 4 of σ^{28} subunit and inhibits the σ function (Kutsukake *et al.*, 1994).

Recently a similar control of the σ activity has been identified for *E. coli* σ^{24}, which is involved in transcription of the extreme heat-shock genes required to deal with damage to extracytoplasmic proteins (Missiakas *et al.*, 1997; De Las Penas *et al.*, 1997). A protein, designated RseA (Regulator of Sigma E), is associated with the cell membrane and forms a complex with σ^{24} under normal growth conditions, but upon exposure to

certain stress conditions σ^{24} is released from the RseA complex. σ^{24} can then be used for transcription activation of the relevant genes. Thus, RseA is considered to function as an anti-sigma factor for σ^{24}.

The repression of host cell gene transcription by phage T4 involves the inhibition of σ^{70} activity by the phage-coded, 10 kDa AsiA protein (Brody *et al.*, 1995; Orshini *et al.*, 1993). The AsiA protein modulates initial DNA binding by the RNA polymerase containing σ^{70} subunit (Adelman *et al.*, 1997). The contact site for AsiA protein on σ^{70}, like that identified for Rsd in the present work, appears to lie near the regions 3 and 4 (Hinton *et al.*, 1996). However, there is no significant similarity in overall sequence between AsiA and Rsd.

Sequence analysis of the *rsd* gene indicates that Rsd has a high similarity in primary structure with *Pseudomonas* AlgR2, a regulatory protein for exopolysaccharide alginate production (Schlictman *et al.*, 1995). The AlgR2 protein is considered to regulate some enzymes involved in the pathway leading to alginate formation such as nucleoside diphosphate kinase (Sevelinova *et al.*, 1996), but its action mechanism remains unsolved. The identification of Rsd as an important anti-σ^{70} factor needs more studies *in vivo*, including characterization of *rsd* mutants.

7.4 Switching the gene expression from exponential to stationary phase by Rsd

The *rsd* gene was found to have two transcriptional start sites, *rsd*p1 and *rsd*p2. Transcription from the upstream *rsd*p1 is dependent on σ^{38}, while the downstream *rsd*p2 is driven by σ^{70}. The sequence of *rsd*p2 shows a strong similarity with the gearbox promoters such as *bolA*p1, which are induced in the stationary phase (Aldea *et al.*, 1989; 1990; Mao and Siegele, 1998). Like other gearbox promoters, the *rsd*p2 activity is inversely propotional to the growth rate. The sequence of -10 promoter region, however, shows a slight difference from the gearbox consensus sequence. Transcription from the *bolA*p1 completely depends on $E\sigma^{38}$ (Bohannon *et al.*, 1991; Lange and Hengge-Aronis, 1991), but *rsd*p2 is not transcribed by $E\sigma^{38}$ *in vivo* (Fig. 18E) and *in vitro* (Fig. 18F).

The gearbox promoter for *mcb* also does not require σ^{38} for its stationary phase induction (Bohannon *et al.*, 1991; Lange and Hengge-Aronis, 1991), but instead the expression of *mcb*p is controlled by some transcription factors including OmpR and EmrR (Mao and Siegele, 1998).

Even though the *rsd* gene carries the σ^{38}-dependent P1 and σ^{70}-dependent P2 promoters, the contribution of σ^{38} in the expression of *rsd* seems to be not so much, because the overall expression level in wild type-cells is higher rather than that in the *rpoS* mutant. Moreover, we found that the deletion of *rsd*p upstream region including the P1 promoter resulted in the increase in the activity from *rsd*p2.

In exponentially growing cells in a mimimal medium, the activity of *rsd-lacZ* fusion stays nearly constant but the expression level of *rsd*p decreases with the increase in growth rate. The expression of several genes including *cspD*, *lrp* (Landgraf *et al.*, 1996), *rmf* (Yamagishi *et al.*, 1993), and *sspA* (Williams *et al.*, 1994) shows inverse relation with the growth rate even though the promoters of these genes do not share common sequences. The *cspD* gene encodes a protein of high sequence similarity with the cold shock protein CspA, but, *cspD* expression is not induced by cold shock and drivened by σ^{70} with a growth phase-dependent manner (Yamanaka *et al.*, 1997). The function of CspD is suggested as an RNA and /or DNA chaperone during the stationary phase (Yamanaka *et al.*, 1997). The *rmf* gene encoding a protein associated with 100S ribosome dimers is expressed in stationary phase, but does not require σ^{38} (Yamagishi *et al.*, 1993). Disruption of *rmf* resulted in loss of ribosome dimers and reduction of cell viability in the stationary phase (Yamagishi *et al.*, 1993). The expression of stringent starvation protein, SspA, is induced by glucose, nitrogen, phosphate or amino acid starvation (Williams *et al.*, 1994). SspA can be isolated as a complex with RNA polymrase (Ishihama and Saitoh, 1979) and the isolated SspA rebinds to either core-RNA polymerase or holoenzyme (Williams, 1992). These gene products transcribed by σ^{38}-independent pathway may be play important roles for survival in the stationary phase. Detailed analysis is needed to define the promoter elements that are important for inverse relationship with the growth rate.

After entry into the stationary phase or under carbon source starvation, the cellular level of ppGpp is known to increase (Cashel *et al.*, 1996). Direct interaction of ppGpp with RNA polymerase has been demonstrated in both *in vitro* (Reddy *et al.*, 1995; Chatterji *et al.*, 1998) and *in vivo* (Cashel *et al.*, 1996; Hernandez *et al.*, 1995). The ppGpp-associated RNA polymerase looses transcription activity of the growth-related genes such as those for rRNA, r-proteins and t-RNA. The promoter activity of the genes, *rsd* (this study), *cspD* (Yamanaka *et al.*, 1997) and *lrp* (Landgraf *et al.*, 1996), *sspA* (Williams *et al.*, 1994), showing inverse relationship with the growth rate is positively regulated by ppGpp. At present, it is not known whether ppGpp acts ,directly or indirectly, on *rsd* expression.

Lrp is known as a major regulatory protein that controls the expression of many operons, positively or negatively, in *Escherichia coli* depending on the availability of nutrients (Calvo and Matthews, 1994). The *in vivo* data indicates that Lrp plays a positive modulatory role in expression of *rsd*. As observed in other Lrp-controlled promoters (Nou *et al.*, 1993; Wang and Calvo, 1993; Marschall *et al.*, 1998), Lrp binds cooperatively to several sites of the *rsd* promoter region. It has been proposed that Lrp recognizes some structural features of the DNA rather than a specific sequence and participates in the maintenance of chromosomal structure (D' Ari *et al.*, 1993). On the *rsd* promoter region, the binding of multiple Lrp molecules may affect DNA structure and support the basal level transcription of *rsd*.

IHF, one of the nucleoid-associated core proteins, was discovered originally as an accessory factor for the integration of bacteriophage λ but is known to contribute to a wide variety of macromolecular processes in bacteria (Friedman, 1988). For instance, this heterodimeric protein is involved in regulation of transcription of some σ^{54}-dependent promoters and the initiation of DNA replication. In these cases, IHF binds tightly to the target DNA and produces distinct footprints. In our study, IHF mutation reduced the β-galactosidase activity of *rsd-lacZ* fusion, but, IHF did not give a clear protected region on the *rsd* promoter DNA by DNaseI footprinting. In addition, the dissociation constant (Kd) estimated from the results of gel-shift assay is far from the Kd (<25 nM) measured using

DNA with the IHF consensus (Yang and Nash, 1995). According to the study by Yang and Nash (1995), at least 50% of the *E. coli* chromosome is accessible to IHF. Therefore the observed effect of IHF mutation on the *rsd* expression is probably indirect and possibly some factor(s) under the control of IHF may be involved in the regulation of *rsd* expression.

To get insight into the function of Rsd *in vivo*, we constructed the *rsd* null mutant strain. The mutant, however, showed apparently no distinct phenotype from the wild-type parental strain as analyzed by measuring growth-curve and viability in various media. Several lines of evidence, however, indicated that the variation in Rsd level influenced the level of σ^{70}- and σ^{38}-dependent transcription of at least some specific genes: (i) the level of σ^{70}-dependent *ompF-lacZ* and σ^{38}-dependent *bolA-lacZ* fusions increases and decreases, respectively, in the absence of Rsd but (ii) the expression of *ompF-lacZ* and *bolA-lacZ* decreases and increases, respectively, in the presence of over-expression of Rsd.

The intracellular concentration of RNA polymerase core enzyme stays constant (Ishihama, 1997; Ishihama *et al.*, 1976) while the levels of seven species of the σ subunit vary depending on the rate and phase of cell growth (Ishihama, 1997; Ishihama, 1998). In exponentially growing *E. coli* cells, only three species of the σ subunit, σ^{70}, σ^{32} and σ^{28}, are present at detectable levels (Jishage *et al.*, 1996), but upon entry into the stationary phase, the levels of both σ^{38} and σ^{32} increases markedly while the levels of other σ do not change significantly (Jishage *et al.*, 1996; Jenkins *et al.*, 1991). Under the steady state of cell growth, the vast majority of core enzyme is associated with the nucleoid and involved in dynamic cycle of transcription (Ishihama *et al.*, 1976; Pettijohn et al., 1970; Stonington, *et al.*, 1971). The level of free core enzyme, not involved in transcription, is considered to be 10-30% of the total number of RNA polymerase (Ishihama *et al.*, 1976). Thus, the competition must take place between seven species of the σ subunit for binding to a fixed number of the core enzyme. The observations herewith described support the prediction that Rsd binds to free σ^{70} subunit and thereby affects the relative level of $E\sigma^{70}$ and $E\sigma^{38}$ holoenzymes. The prediction is supported by

the observations that: (i) the reduction of σ^{38}-dependent gene expression by the *rsd* null mutation is suppressed by the expression of σ^{38} (see Fig. 23D); (ii) the over-expression of σ^{70} results in reduction of σ^{38}-dependent transcription (Farewell *et al.*, 1998); and (iii) an *rpoS* mutant strain exhibits an increased expression of the σ^{70}-dependent genes (Farewell *et al.*, 1998; Pratt and Shilhavy, 1996). Similar situations have been observed between σ^{70} and σ^{32}. For instance, the decrease in the intracellular level of σ^{70} results in super induction of σ^{32}-dependent genes (Osawa and Yura, 1981). On the other hand, over-expression of σ^{70} leads to a reduction in the expression of some σ^{32}-dependent genes (Zhou *et al.*, 1992).

The inhibitory effect of σ^{70}-dependent transcription by the over-expressed Rsd was observed only at certain stages of the cell growth although the level of Rsd expression is not so much different between the early and the late exponential phases (see the Western blot pattern above Fig. 25A). Possible mechanisms to explain the apparent lack of inhibitory effect of Rsd on the σ^{70} function in the early exponential growth phase include that: (i) The reduction in the concentration of functional σ^{70} by Rsd might not affect the concentration of $E\sigma^{70}$ holoenzyme if the level of σ^{70} subunit exceeds that of free core enzyme available for binding of the σ subunit. (ii) The affinity of various σ subunits to the core enzyme may vary depending on the cytoplasmic conditions. Under the conditions favorable for transcription *in vitro* by the $E\sigma^{70}$ holoenzyme, the core enzyme-binding affinity of σ^{70} is stronger than the σ^{38} subunit (Maeda *et al.*, in preparation). However, transcription *in vitro* by the $E\sigma^{38}$ holoenzyme is markedly enhanced in the presence of high concentrations of glutamate (Ding *et al.*, 1995), trehalose (Kusano and Ishihama, 1997) and polyphosphate (Kusano and Ishihama, 1997). (iii) Under the steady-state growth conditions, Rsd is not synthesized but the artificially expressed Rsd might be inactivated by an as yet unidentified mechanism, or it is also not excluded yet that an additional factor present only in the stationary phase cells is involved for Rsd binding to σ^{70}.

A number of anti-σ factors have been discovered in both *Bacillus subtilis* and *E. coli* (Hughes and Mathee, 1998). The T4 AsiA protein binds to the *E. coli* σ^{70} subunit and

inhibits σ^{70}-dependent transcription, but AsiA plays a positive factor for transcription of the middle class of T4 genes (Ouhammouch *et al.*, 1994). *E. coli* FlgM can interact with $E\sigma^{28}$ and induces the dissociation of σ^{28} from the core enzyme (Chadsey *et al.*, 1998). The ECF anti-σ factors of *E. coli* are inner membrane proteins which have the sensor domains in the periplasm and the σ subunit-binding domains in the cytoplasm. Extracytoplasmic signals are likely involved in the interaction between the ECF σ subunits and their cognate anti-σ factors (Hughes and Mathee, 1998). By definition, the anti-σ factors have the activity of not only binding to the cognate σ subunits but also inducing the dissociation of target σ subunits from the core enzyme (Hughes and Mathee, 1998). Rsd has the binding activity with σ^{70}, but appears to lack the activity of dissociating σ^{70} from $E\sigma^{70}$. The putative accessory factor or some specific reaction conditions may be required for the enhancement of Rsd activity or the modulation of its specificity. Along this line, the possibility remains that Rsd is a different type of the regulatory protein which promotes σ switching from σ^{70} to other minor σ subunits under stress conditions.

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9. TABLES

Table 1.

σ subunits of *E. coli* RNA polymerase

Species	Gene	Min	Size(aa)	pI/Mw	Target genes
σ^{70}	*rpoD*	69.2	613	4.69/70263.28	Gene expressed during exponential phase
σ^{54}	*rpoN*	72.0	477	4.63/53989.78	Genes induced under nutrient imblance
σ^{38}	*rpoS*	61.7	330	4.89/37971.86	Stationary-phase and osomotic-shock genes
σ^{32}	*rpoH*	77.5	284	5.64/32468.80	Heat-shock genes
σ^{28}	*rpoF*	43.1	239	5.20/27521.11	Flagellar-chemotaxis genes
σ^{24}	*rpoE*	58.2	191	5.38/21695.74	Extracytoplasmic and periplasmic genes
σ^{19}	*fecI*	97.3	173	5.82/19480.41	Iron-transport genes

Table 2. Strains and plasmids used in this work

Strain/Plasmid	Relevant genotype	Source/reference
Strain		
W3110	F-IN[*rrnD-rrnE*]	Backmann, (1972)
MC4100	F-*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	
JC7623	*argE3 his4 leu6 proA2 thr1 thi1 rpsL31 galK2 lacY1 ara14 xyl15 mtl1 supE44 kdgK51 recB15 recC22 sbcB15*	Oishi and Cosloy (1972)
BL21(DE3)	F⁻ *ompT* $hsdS_B$ (r_B-m_B-) *gal* (λ*cI*857 ind1 Sam7nin5 *lacUV5*-T7gene1) *dcm* (DE3)	Novagen
M5219	M72 $lacZ_{am}trp_{am}Sm^R$ (λ *bio*252 *cI*857 H1)	Popham *et al.*, 1991
NCM668	M5219+pJES259	Popham *et al.*, 1991
DH5	*supE*44 *hsdR*17 *recA*1 *endA*1*gyrA*96 *thi-*1*relA*1	TOYOBO
ZK126	W3110 Δ*lacU169 tna2*	Bohannon *et al.*, (1991)
ZK1000	ZK126 *rpoS*::Km	Bohannon *et al.*, (1991)
HP4110	MC4100 *himA*::Tn10	Pratt *et al.*, (1997)
DL1784	MC4100 Δ*lrp*	Woude *et al.*, (1996)
CF1946	CF1943 Δ*relA*251::Km Δ*spoT*207::Cm	
MH20	F⁻Δ*lacU169 rpsL relA thiA flbB*	Hall and Shilhavy (1981)
MH513	MH20 ϕ(*ompF-lacZ*) 16-13	Hall and Shilhavy (1981)
MJ30	MC4100 *rsd*::Km	This work
MJ23	MC4100 *rpoS*::Km	This work
MJ6	MC4100 λϕ(*rsdI-lacZ*)	This work
MJ27	MJ23 λϕ(*rsdI-lacZ*)	This work

MJ39	MJ6 *spoT*::Cm	This work
MJ19	MC4100 λφ(*rsdIII-lacZ*)	This work
MJ34	MJ30 λφ(*rsdI-lacZ*)	This work
MJ8	HP4110 λφ(*rsdI-lacZ*)	This work
MJ15	DL1784 λφ(*rsdI-lacZ*)	This work
MJ31	MC4100 λφ(*bolA-lacZ*)	This work
MJ35	MJ30 λφ(*bolA-lacZ*)	This work
MJ46	MJ35+pACYC184	This work
MJ47	MJ35+pACYCRsd	This work
MJ53	MJ31+pACYC184	This work
MJ54	MJ31+pACYCRsd	This work
MJ57	MH513 *rsd*::Km	This work
MJ59	MJ35+pBAD22A	This work
MJ60	MJ35+pBF1	This work
MJ67	MH513+pACYC184	This work
MJ68	MH513+pACYCRsd	This work
MJ69	MJ57+pACYC184	This work
MJ70	MJ57+pACYCRsd	This work
MJ71	MH513+pBAD22A	This work
MJ72	MH513+pBF1	This work
MJ83	MH513 *rpoS*::Km	This work
MJ130	MH513+pBADRsd31-1	This work

Plasmids

pGEX5X-1	Expression vector for GST fusion carrying tac promoter	Pharmacia
pGEXD	pGEX5X-1 carrying *rpoD*	This work
pGEXN	pGEX5X-1 carrying *rpoN*	This work
pGEXS	pGEX5X-1 carrying *rpoS*	This work
pGEXH	pGEX5X-1 carrying *rpoH*	This work
pGEXF	pGEX5X-1 carrying *rpoF*	This work
pGEXM	pGEX5X-1 carrying *flgM*	This work
pGEXRSD	pGEX5X-1 carrying *rsd*	This work
pET-Rsd	pET21a carrying *rsd*	This work
pETF	pET3b carrying *rpoS*	Tanaka *et al.*,1993
pETSF	pET21 carrying *rpoF*	Tapas *et al.*, 1997
pGEMD	pGEMX1 carrying *rpoD*	Igarashi *et al.*, 1991
pUJC-1	Δ*rsd* cloned into pUC18	This work
pUCRsd33	pUC18-derived vector carring *rsd*	This work
pACYCRsd	pACYC184-derived vector carring *rsd*	This work
pBAD22A	Expression vector carrying arabinose inducible promoter	Guzman *et al.*, 1995
pBF1	pBAD22A carrying *rpoS*	K.Tanaka unpublished
pBADRsd31-1	pBAD22A carrying *rsd*	This work
pRsdI	pRS551 carrying *rsd*p1 and p2 promoter	This work
pRsdIII	pRS551 carrying *rsd*p2 promoter	This work

Table 3.

Laboratory stocks of *E. coli* W3110 analyzed in this study [a]

Sources (location)	Stock no.	Sigma type[b]
B. Backmann → Y. Kohara (NIG, Mishima)	1	A
J. Tomizawa (NIH, Tokyo) → H. Ozeki (Kyoto) → C. Wada (Kyoto)	2	A
A. Matsushiro (Osaka) → T. Yura (Kyoto) → C. Wada (Kyoto)	3	A
A. Matsushiro (Osaka) → T. Yura (Kyoto) → C. Wada (Kyoto)	4	A
A. Matsushiro (Osaka) → T. Yura (Kyoto) → K. Ito (Kyoto) → A. Ishihama (NIG, Mishima)	5	C
A. Matsushiro (Osaka) → T. Yura (Kyoto) → R. Okazaki (Nagoya) → Y. Kohara (Nagoya) → A. Nishimura (NIG, Mishima [Kohara Library])	6	B
B. Backmann → K. Kutsukake (Hiroshima)	7	C
T. Iino (Tokyo) → K. Kutsukake (Hiroshima)	8	C
Y. Hirota (NIG, Mishima) → A. Nishimura (NIG, Mishima)	9	C
M. Cashel → K. Tanaka (Tokyo)	10	D
C. Yanofsky → F. Imamoto (Osaka/Kyoto) → Y. Kano (Kyoto)	11	E

a Strain W3110 was a K-12 derivative of *E. coli* constructed in the J. Lederberg laboratory. This strain was distributed directly to Japanese scientists shown in the first column or via American scientists, and thereafter transferred to other Japanese scientists from whom the stocks were obtained. Details of the storage conditions and of the inoculation history of stab cultures were not documented, but all these stocks were transferred from stab cultures at room temperature into glycerol stocks at -80 ℃ until the mid 1980's. Stock 3 and 4 are the permanent and working stock, respectively, in C. Wada's laboratory. The Kohara library was constructed using a type-B stock of strain W3110. NIH, National Institute of Health; NIG, National Institute of Genetics.

b For details see Table 6.

Table 4.

Primers used for gene amplification

Primer	Sequence
D1	5'-CCCAAGCTTGAATTCATGGAGCAAAACCCGCAGTCAC-3'
D4	5'-CCCAAGCTTGTCGACTTAATCGTCCAGGAAGCTACG-3'
N1	5'-GGAATTCCATATGAAGCAAGGTTTGCAACTCAGG-3'
N2	5'-CATGCATGCCTCGAGTCAAACGAGTTGTTTACGCTG-3'
S5	5'-GCGAATTCCATATGTTCCGTCAAGGGATCACG-3'
S6	5'-GCGGATCCCTCGAGTTACTCGCGGAACAGCGCTTC-3'
F1	5'-CGAGCTCGGATCCCCATGAATTCACTCTATACCGCT-3'
F2	5'-GGGGTACCCTCGAGTTATAACTTACCCAGTTTAGT-3'
FA1	5'-GCCGGATCCCAGGCCTACAAGTTGAATTGC-3'
FA2	5'-CCCAAGCTTCTGACGTTATAACTTACCCG-3'
FD1	5'-GCCGGATCCGATCTGTCATCACGAATTATTG-3'
FD2	5'-CCCAAGCTTAAACAGCCTGTACTCTCTGTTC-3'
M1	5'-GCGAATTCCATATGAGTATTGATCGCACTTCGCC-3'
M2	5'-GCGGATCCCTCGAGGTTACTCTGCAAGTCTTGCTG-3'
RsdU	5'-GCGAATTCCATATGCTTAACCAGCTCGATAACC-3'
RsdR	5'-AACTGCAGCTCGAGAGCAGGATGTTTGACGCGGGC-3'
f158-1	5'-CATGCATGCCACAAGATCGAAATTTGCCCGTTC-3'
f158-2	5'-CCGGAATTCCATTTCCGGCGTGATGATGCCCTG-3'
Rsd5	5'-CGCGGATCCAACCAAACAGGTTCCCCCTGCCAT-3'
Rsd6	5'-AACTGCAGCTCGAGCTCAGCCAGTTAAGGCACTCC-3'
Rsd11	5'-CCGGAATTCACCATGCTTAACCAGCTCGATAAC-3'
Rsd12	5'-CATGCATGCTCAAGCAGGATGTTTGACGCGGGC-3'
Rsd7	5'-CCGGAATTCAACCAAACAGGTTCCCCCTGCCAT-3'
Rsd13	5'- CCGGAATTCACGACCCACCAGCCGTGATCTAAT-3'
Rsd14	5'-CGCGGATCCCAGTGAGAAATGTAAAAACCATGT-3'

Table 5.

Intracellular levels of 6 species of σ subunit in *E. coli* W3110 and MC4100

σ subunit	Concetration (fmol/μg)			
	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}	-	40-60	-	20-30
σ^{28}	70-100	70-100	-	-
σ^{E}	0.7-2.0	0.7-2.0	Not tested	
FecI	0.2	-	Not tested	

Cells were grown in LB medium at 37 °C with shaking. At both middle of exponential phase and the stationary phase, the level of σ subunits were determined by the quantitative Western blot system. The determination was repeated at least three times and the values represent the averages.

Table 6.

σ subunit composition and swarming ability of different stocks of *E. coli* W3110

σ subunit	Lineage of laboratory stocks				
	A	B	C	D	E
σ^{70}	+	+	+	+	+
σ^{54}	+	+	+	+	+
σ^{38}	+	+ (truncated)	-	+	-
σ^{28}	+	+	+	-	-
Swarming ability	+	+	+	-	-

Table 7

Effect of Rsd on *in vitro* transcription

Promoter	Transcription level (%)
*lac*UV5	100
*gal*P1	83
nusA	83
*dnaQ*P2	72
rnh	72
*dnaQ*P1	66
leuX	62
trp	61
*lac*P1	48
alaS	24

Single-round transcription *in vitro* was carried out as described in Fig. 16, using the indicated promoters (1 pmol each for both core enzyme and promoters). σ^{70} was pre-incubated for 5 min with 10-fold molar excess of Rsd before the addition of core enzyme (core/σ^{70} = 1).

Table 8. GST-σ^{70} alanine substitution mutants

No.	Strain	Plasmid	Position of alanine substitution
1	CAG 20410	pML152	IA590
2	CAG 20412	pML153	KA593
3	CAG 20413	pML148	LA595
4	CAG 20414	pML149	RA596
5	CAG 20415	pML150	KA597
6	CAG 20416	pML158	LA598
7	CAG 20417	pML151	RA599
8	CAG 20420	pML156	RA603
9	CAG 20421	pML159	MA1
10	CAG 20411	pML160	EA591
11	CAG 20418	pML154	HA600
12	CAG 20419	pML155	SA602
13	CAG 20422	pML157	MA2
14	CAG 20715	pML165	SA604
15	CAG 20716	pML166	RA605
16	CAG 20717	pML167	RA608
17	CAG 20718	pML168	DA612
18	CAG 20719	pML169	DA613
19	CAG 20720	pML164	EA574

10. FIGURES

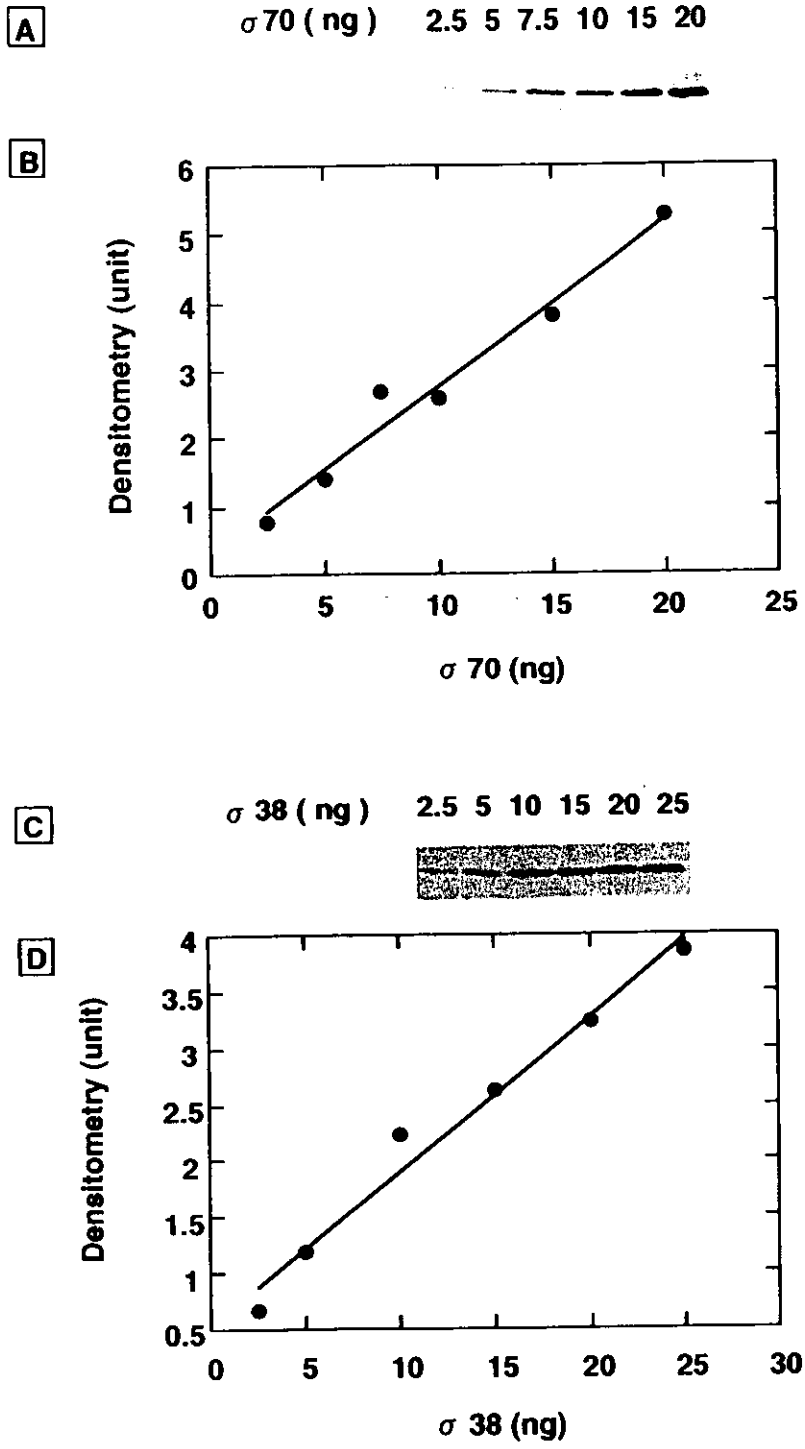


Figure 1. Standard curves for the determination of σ^{70} and σ^{38} subunits. [A and C] Various concentrations of purified σ^{70} (A) or σ^{38} (C) subunit were analyzed by the quantitative Western blot system. [B and D] The intensity of σ^{70} (B) and σ^{38} (C) bands after immunostaining was quantitated with a PDI image analyzer system equipped with a white light scanner.

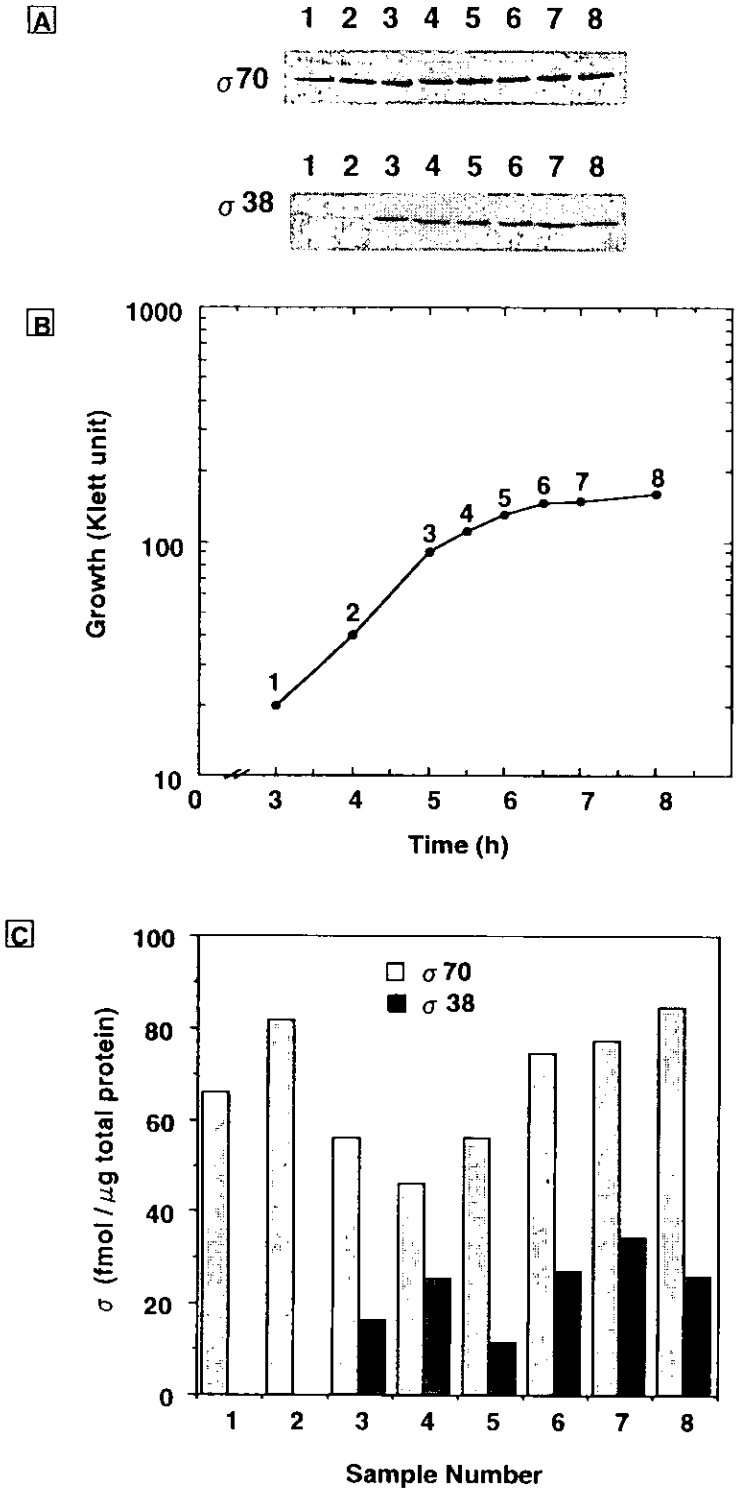


Figure 2. Growth phase-dependent variation in the intracellular levels of of σ^{70} and σ^{38} proteins. [A] Either 5 μg (for σ^{70}) or 10 μg (for σ^{38}) of total protein in cell lysates prepared at various times of the cell culture (see B for the growth curve) was subjected to analyze by the quantitative western blot system. [B] Cells of MC4100 were grown in LB medium at 37°C and the growth was monitored by measuring the turbidity with a Klett-Summerson photometer. [C] The immunostained blot was analyzed with a PDI image analyzer system equipped with a white light scanner to determine the intensity of σ^{70} and σ^{38} proteins.

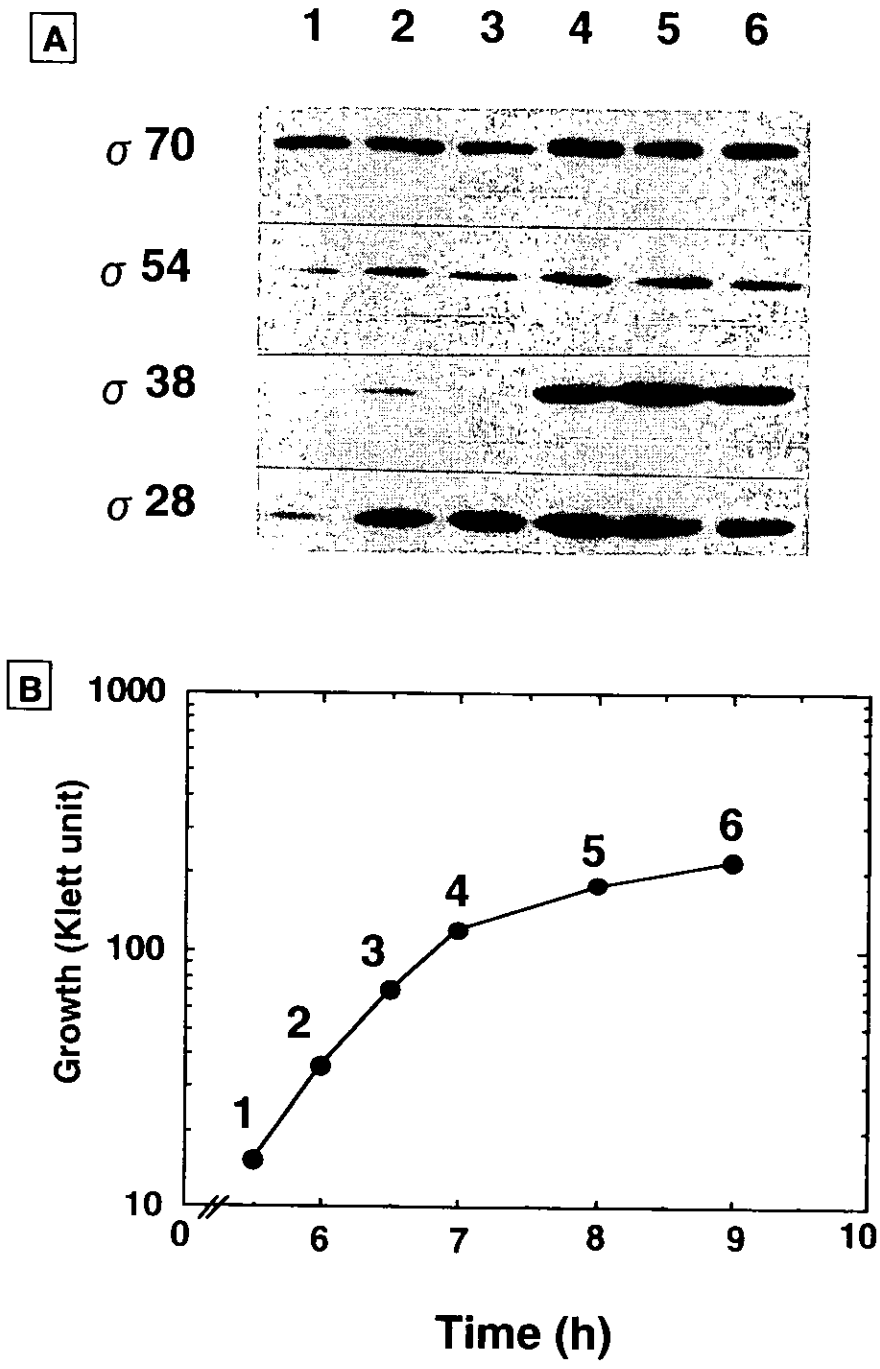


Figure 3. Growth phase-dependent variation in the intracellular levels of σ70, σ54, σ38 and σ28 subunits in W3110. [A] Aliquots containing 1 μg (for σ70) or 10 μg (for σ54, σ38 and σ28) of total proteins from cell lysates of *E. coli* W3110 prepared at various times of the cell culture (see B for the growth curve) were analyzed by the quantitative Western blot system. [B] *E. coli* W3110 was grown in LB medium at 37 °C and the growth was monitored by measuring the turbidity with a Klett-Summerson photometer.

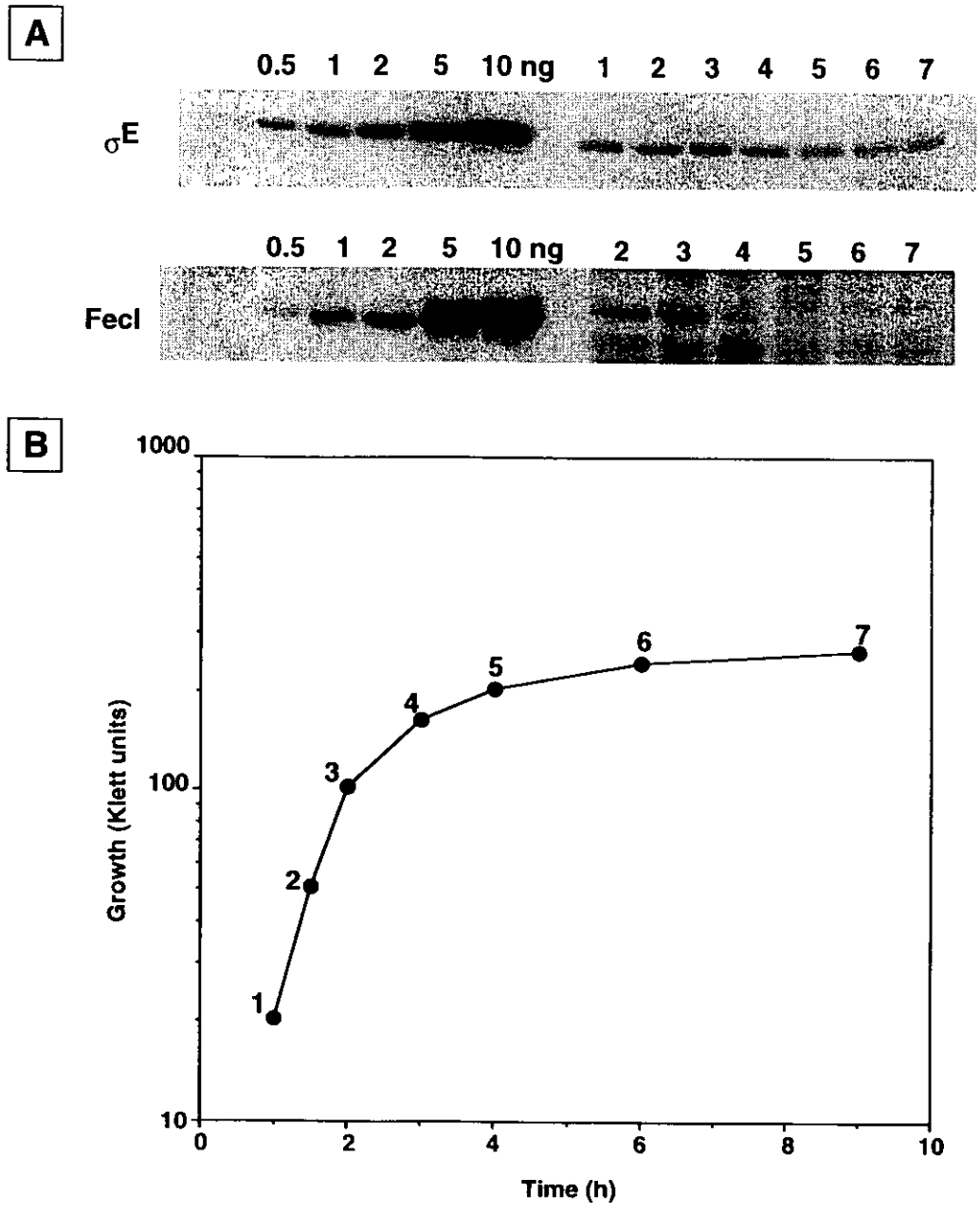


Figure 4. Growth phase-dependent variation in the intracellular levels of of σ^E and FecI proteins. [A] Either 20 μg (for σ^E) or 60 μg (for FecI) of total protein in cell lysates prepared at various times of the cell culture (see B for the growth curve) was subjected to analyze by the quantitative western blot system. [B] Cells of W3110A were grown in LB medium at 37°C and the growth was monitored by measuring the turbidity with a Klett-Summerson photometer.

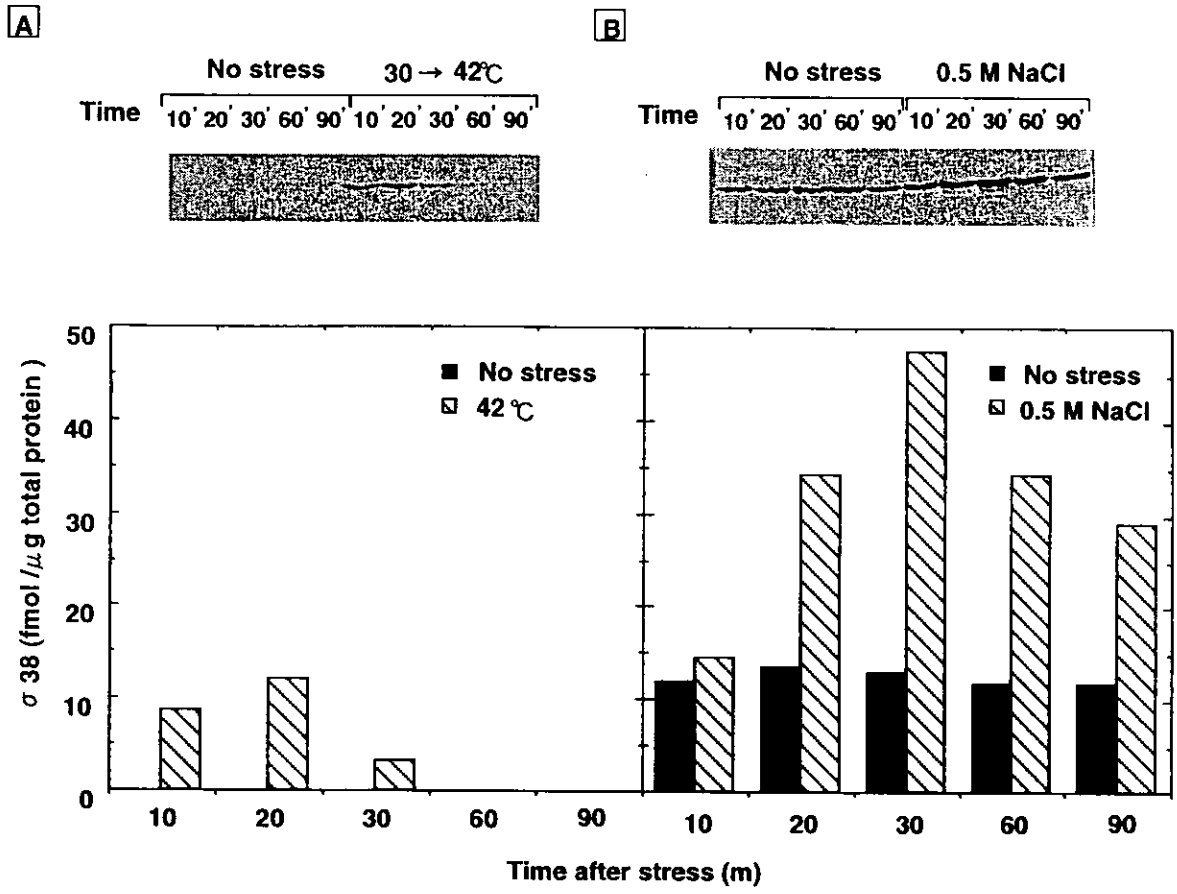


Figure 5. Variation in the σ^{38} protein level under stress conditions. [A] *E. coli* Cells of MC4100 were grown to the exponential phase in LB medium at 30 °C. At the cell density of 15 Klett units, the culture temperature was raised to 42 °C adding an equal volume of LB warmed at 54 °C. min. [B] Cells were grown in LB medium at 37°C and at 5 h after the culture dilution (about 2 h after the growth arrest), NaCl was added to make final 0.5 M solution. Samples were taken at 10, 20, 30, 60 and 90 min after the addition of NaCl. Aliquots of the cell lysates containing 20 μg of total protein were analyzed by quntitative Western blot system.

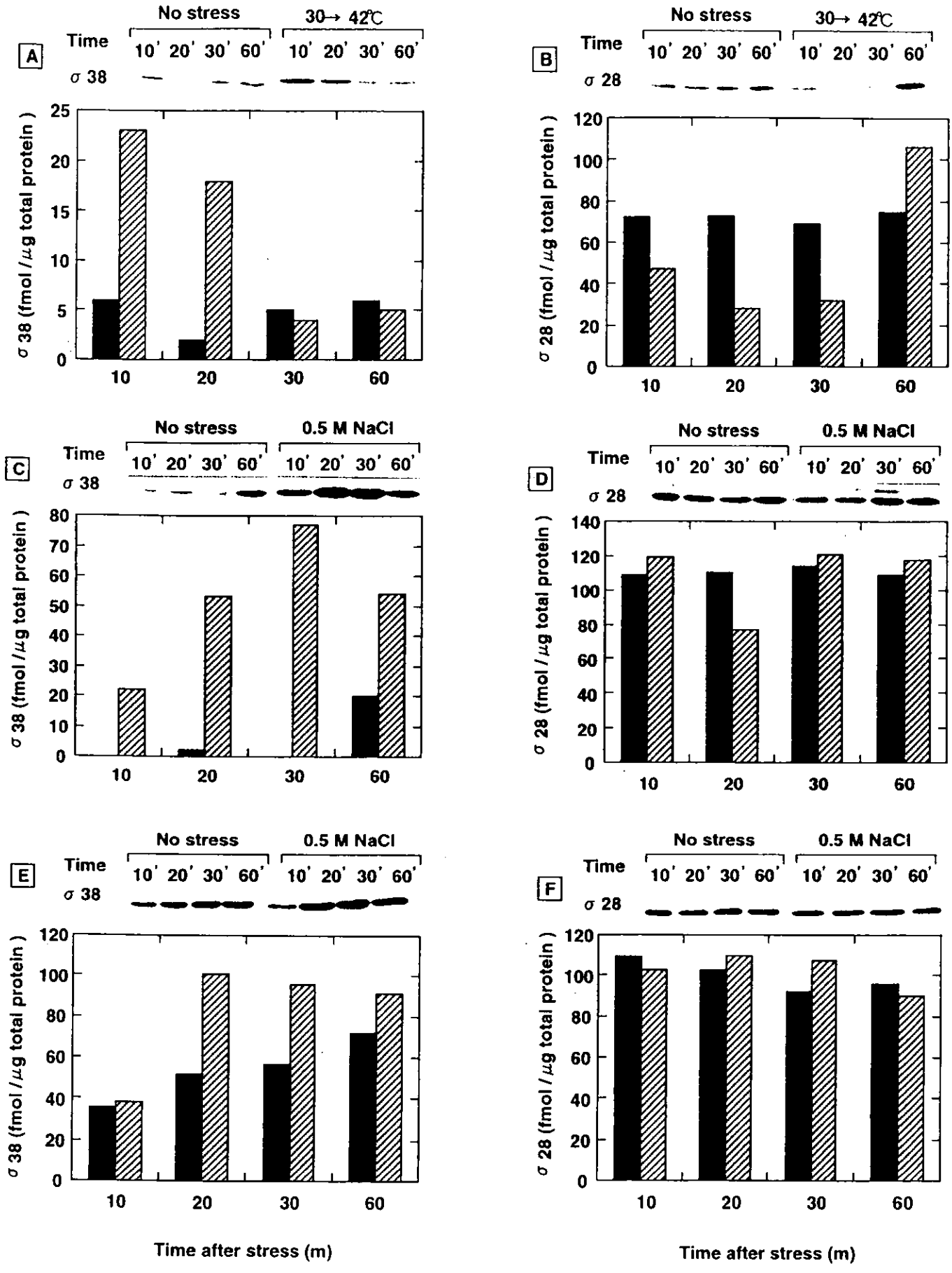

A
No stress
30→ 42℃
Time
10' 20' 30' 60'
10' 20' 30' 60'
σ 38
σ 38 (fmol / μg total protein)
B
σ 28
σ 28 (fmol / μg total protein)
C
0.5 M NaCl
D
E
F
10 20 30 60
Time after stress (m)

Figure 6. Variation in the σ^{38} and σ^{28} levels under stress conditions. [A, B, C and D] Cells of *E. coli* W3110 were grown to the exponential phase in LB medium at 30 (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 final 0.5 M solution (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 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* W3110 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 μg 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. 5E is due to the induction of σ^{38} synthesis upon the transition into stationary growth phase.

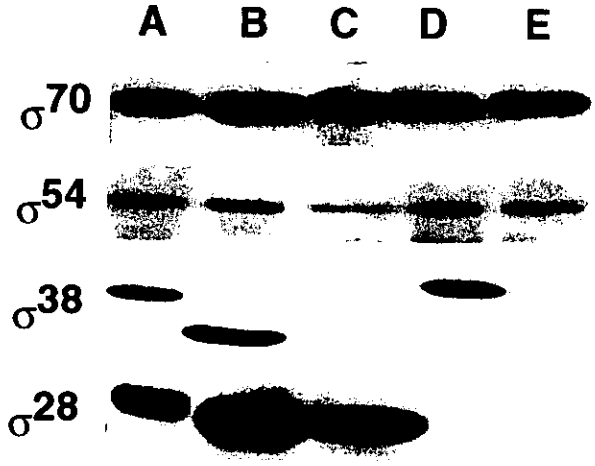


Figure 7. Content of RNA polymerase σ subunits. *E. coli* A-, B-, C-, D- and E-type W3110 were grown at 37 °C in LB until the early stationary phase. The protein concentration of cell lysates was determined using a protein assay kit (Bio-Rad). The total proteins used were: 1 μg (for σ^{70}) or 10 μg (for σ^{54}, σ^{38} and σ^{28}).

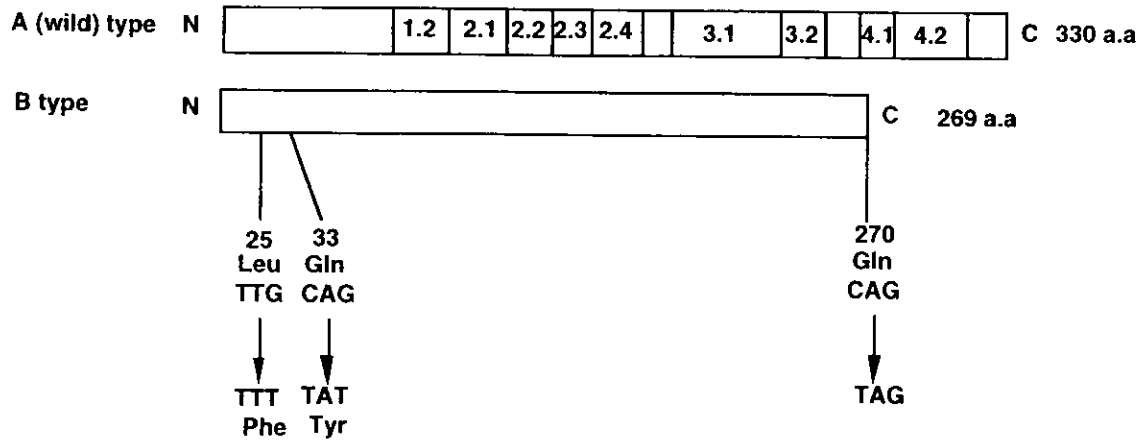


Figure 8. Nucletide and amino acid substitutions of *rpoS* gene in B type W3110. Nucleotide sequence of the *rpoS* gene was determined in B-type W3110. Wild-type σ^{38} protein consists of 330 amino acids while the truncated σ^{38} protein in the B-type lineage consists of 269 amino acids. N and C indicate the N- and C-termini of the σ^{38} protein.

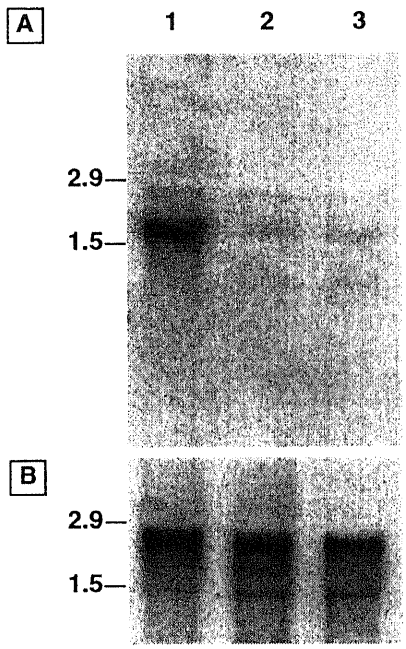


Figure 9. Northern blot analysis of *rpoS* and *rpoD* gene transcripts. Total RNA from A-, C-, and E-type W3110 were analyzed by Northern blotting using specific probes for the*rpoS* (A) and *rpoD* (B) genes. Lane 1, A type; lane 2, C type; and lane 3, E type. Migration positions of ribosomal RNAs (1.5 and 2.9 kbp) are shown on left.

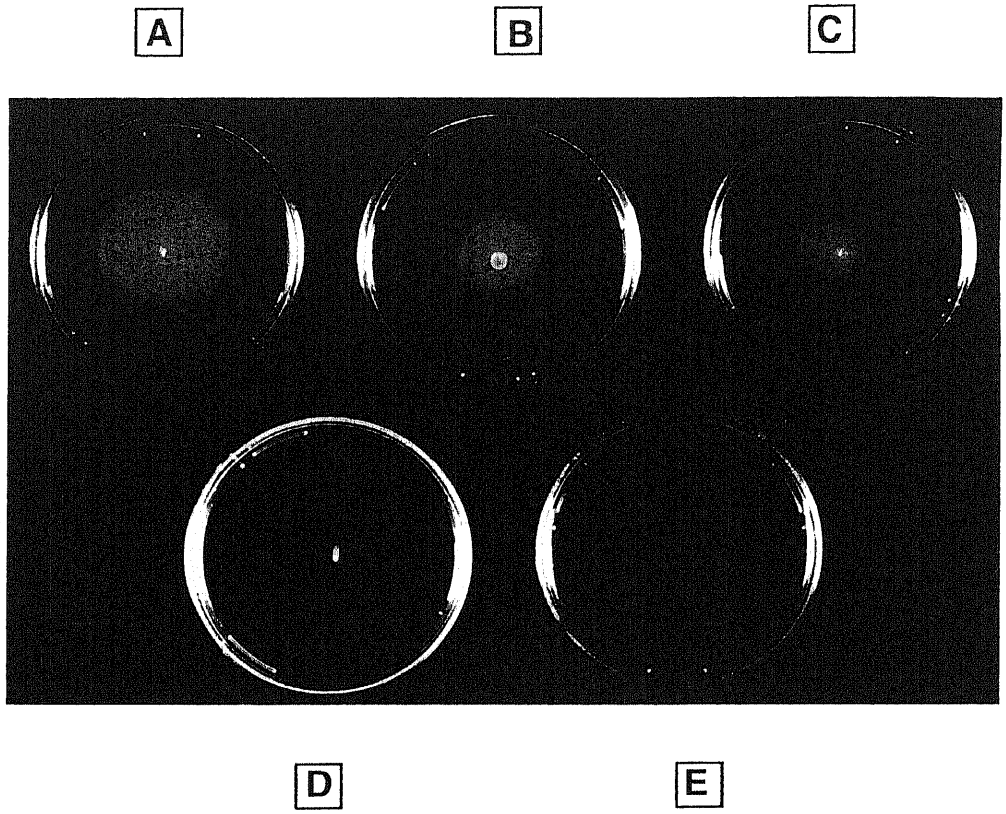


Figure 10. Swarming ability of W3110 stocks. Cells from LB overnight cultures were inoculated into the centers of petriplates containing 30ml of tryptone swarm agar. The plates were incubated at 37 °C for 7 hours, and photographed when the diameter of swarms reached larger than 4 cm.

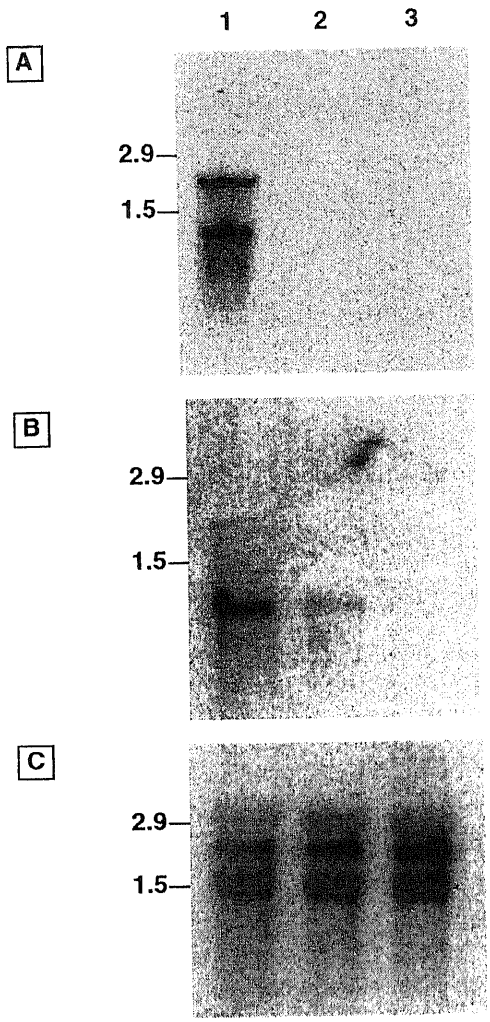


Figure 11. Northern blot analysis of *fliA*, *flhDC* and *rpoD* transcripts. Total RNA from A-, D-, and E-type W3110 were analyzed by Northern blotting using probes for the *fliA* gene (A), *flhDC* (B) and *rpoD* (C) genes. Lane 1, A type; lane 2, D type; and lane 3, E type. Migration positions of ribosomal RNAs (1.5 and 2.9 kbp) are shown on left.

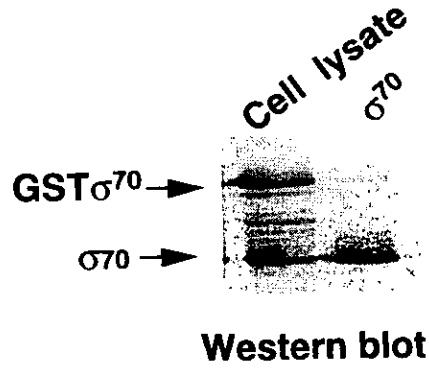


Figure 12. Expression level of GST-σ. Cell lysate from pGEX-σ^{70} transformant was analyzed by Western blotting using σ^{70} antibody. The level of leaky expression of GST-σ^{70} was as high as that of chromosome-coded σ^{70}.

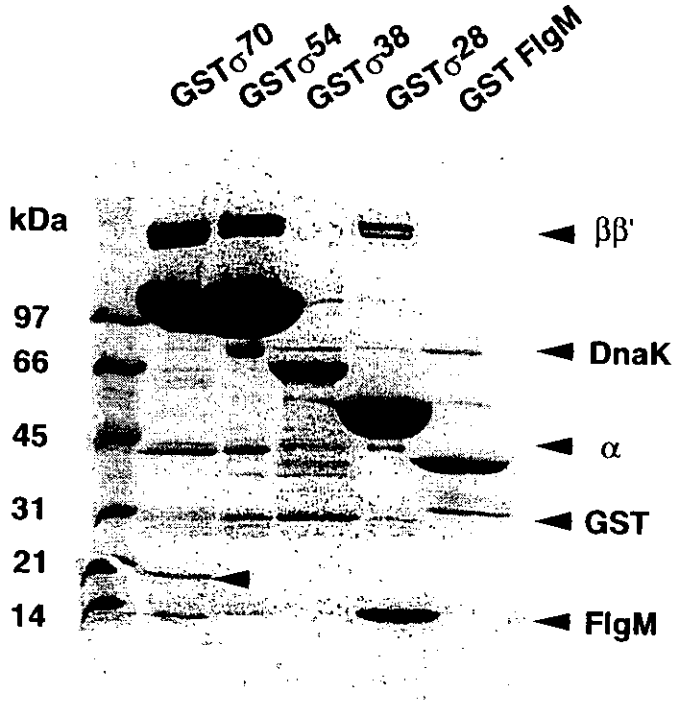


Figure 13. Identification of σ-associated proteins. Cells of *E. coli* W3110 containing pGEXD (lane GSTσ^{70}), pGEXN (lane GSTσ^{54}), pGEXS (lane GSTσ^{38}), pGEXF (lane GSTσ^{28}) or pGEXM (lane GST FlgM) were grown in LB medium at 37 °C into stationary phase (3-4 hr after the cessation of cell growth). GST-fusion protein complexes were isolated and separated by SDS-PAGE on a 5-15% gradient gel. The gel was stained with Coomassie brilliant blue. The migration positions of core enzyme subunits, DnaK, GST (without Rsd) and FlgM are indicated on right. The arrow in the GSTσ^{70} lane indicates Rsd (Regulator of Sigma D).

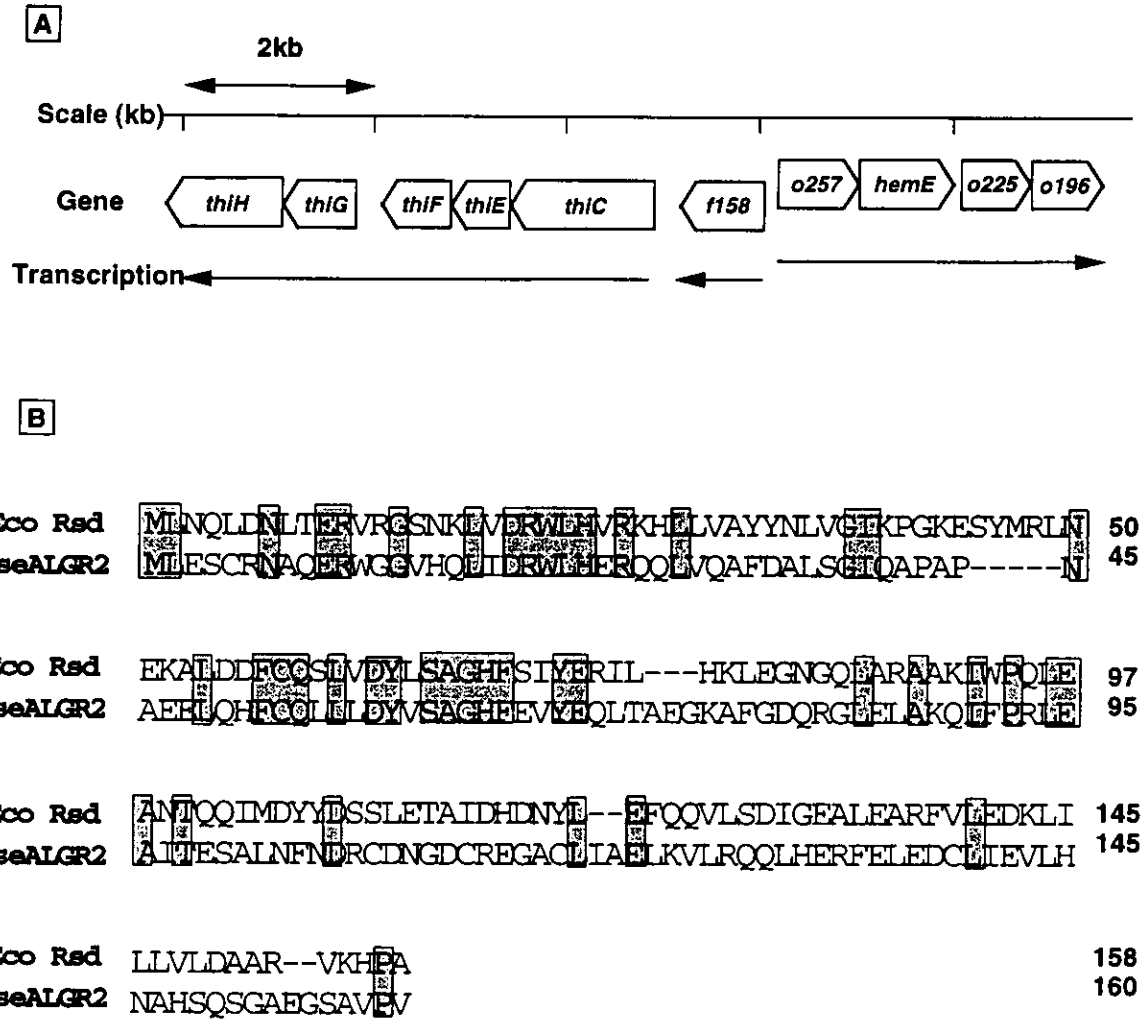


Figure 13. Map position of the *rsd* gene and the amino acid sequence of Rsd protein. [A] Map position of the *rsd* gene. From the amino acid sequence analysis of Rsd, the *rsd* gene was found to be identical with the f158 gene in the genome sequence. [B] The amino acid sequences of the Rsd protein and the *Pseudomonas aeruginosa* AlgR2 protein are compared in optimized alignment. Identical residues are shaded.

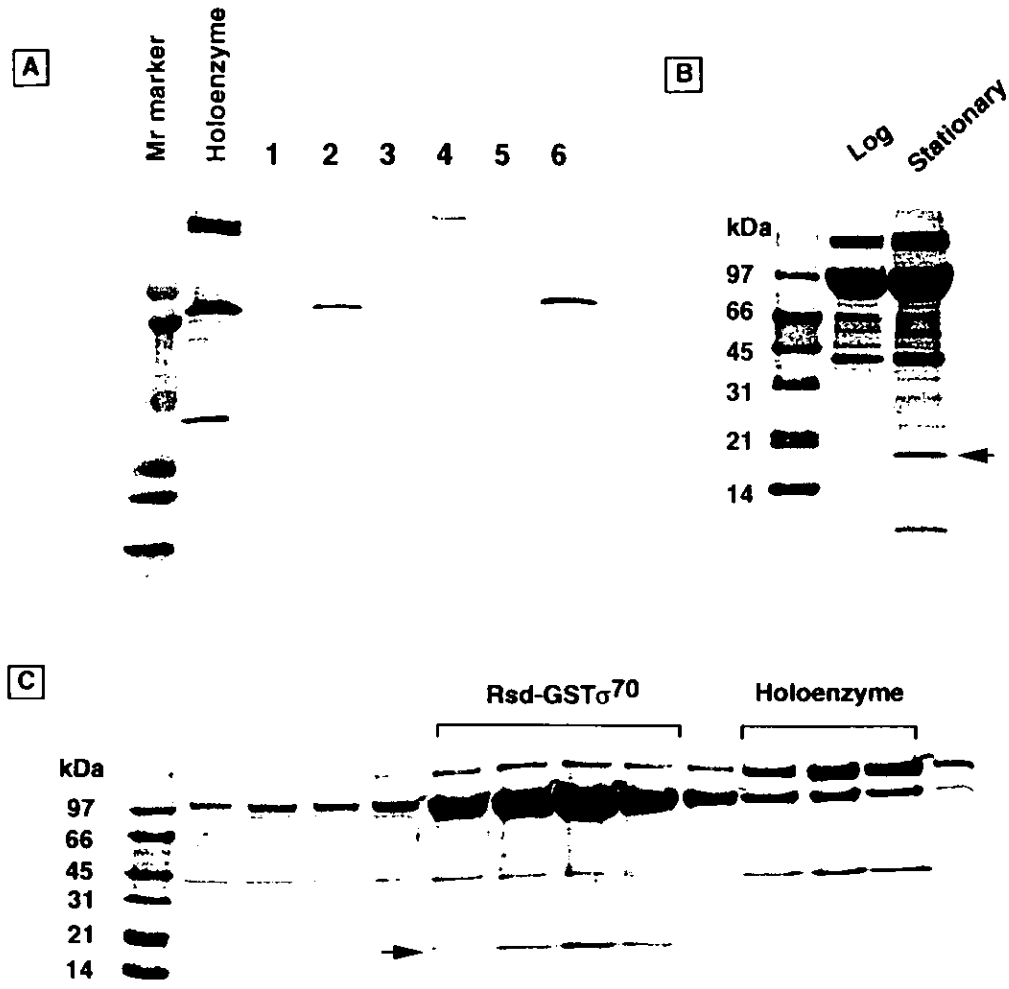


Figure 15. Identification of complex formation between σ^{70} and Rsd. [A] Complex formation *in vitro* between Rsd and σ^{70} was analyzed by the GST pull-down assay. GST (lanes 1, 3 and 5) or GST-Rsd (lanes 2, 4 and 6) was mixed with equimolar amounts of E σ^{70} holoenzyme (lanes 1 and 2), core enzyme (lanes 3 and 4) or σ^{70} subunit (lanes 5 and 6). Complexes formed were bound to glutathione-Sepharose beads. The bead-bound proteins were eluted with 50 mM glutathione and separated by SDS-PAGE on a 5-15% gradient gel. The gel was subjected to Western blot analysis using a mixture of mono-specific antibodies against RNA polymerase α-, β-, β'- and σ^{70}-subunits. The migration positions of RNA polymerase subunits can be identified in the holoenzyme lane. [B] Isolation of GST σ^{70}-associated proteins in cell extracts. Cell lysates of a pGEXD transformant of *E. coli* W3110 were prepared at both exponential (lane, *log*) and stationary (lane, *stationary*) phases. GST σ^{70}-bound proteins were isolated by the GST pull-down assay and the σ^{70}-bound proteins were separated by SDS-13.5% PAGE. The migration positions of core enzyme subunits, GST σ^{70} and proteins are indicated on right. [C] Proteins isolated from stationary phase cells (see *stationary* lane in B) were subjected to heparin-Sepharose column chromatography. Proteins were eluted by a linear gradient of NaCl, and aliquots were analyzed by SDS-13.5% PAGE.

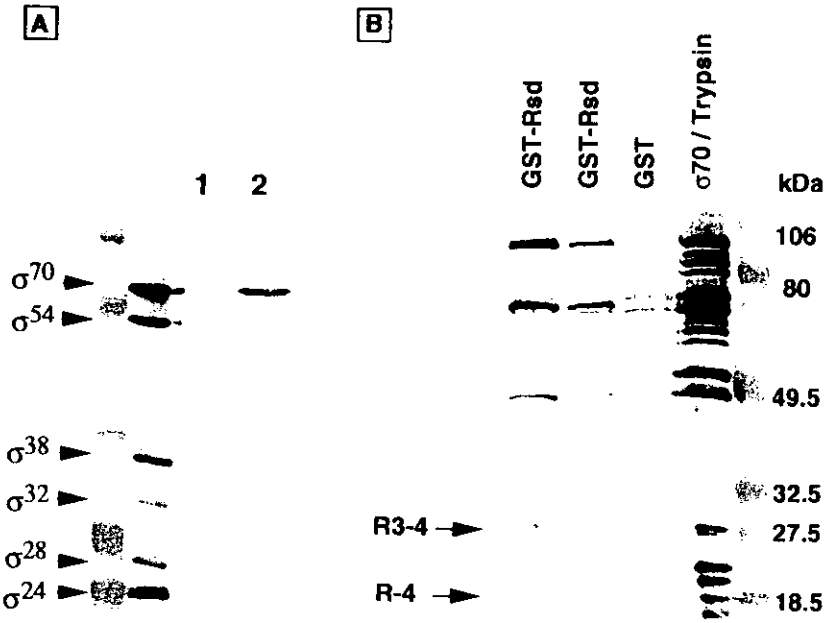


Figure 16. Identification of the Rsd-binding subunit and Rsd-contact site. [A] GST (lane 1) or GST-Rsd (lane 2) was mixed with an equimolar mixture of σ^{70}, σ^{54}, σ^{38}, σ^{32}, σ^{28} and σ^{24}. Complexes were isolated by the GST pull-down method using glutathione-Sepharose beads. The bead-bound proteins were eluted with 50 mM glutathione and analyzed by SDS-13.5% PAGE. The gel was subjected to Western blot analysis using a mixture of antibodies against all six σ subunits. The control lane contained all six σ subunits which all reacted against the antibody mixture. [B] Trypsin-treated σ^{70} (starting material, 1 nmol), shown in lane σ^{70}/trypsin, was mixed with two different concentrations of GST-Rsd (lane 1, 40 pmol; lane 2, 20 pmol), and the complexes formed were isolated by the GST pull-down assay with glutathione-Sepharose beads. The bead-bound proteins were eluted with 50 mM glutathione and separated by 5-15% SDS-PAGE. The gel was analyzed by Western blotting using monospecific polyclonal antibodies against σ^{70}. After amino-terminal sequence analysis, R3-4 and R4 peptides were identified as C-terminal fragments downstream from 449 and 500, respectively.

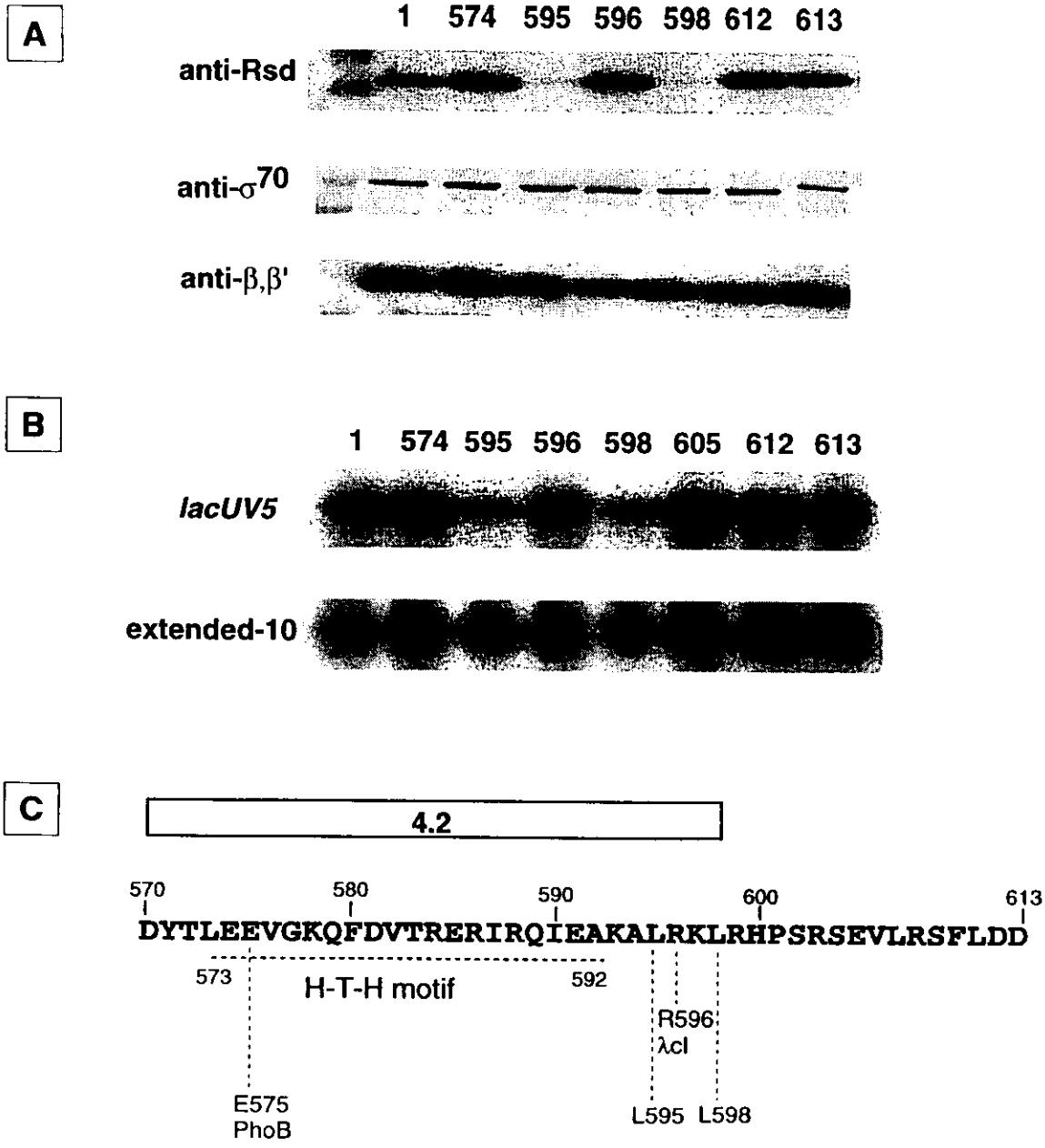


Figure 17. Identification of the Rsd-contact site. [A] GST-σ70 alanine substitution mutants were mixed with an equimolar mixture of Rsd. Complexes were isolated by the GST pull-down method using glutathione-Sepharose beads. The bead-bound proteins were eluted with 50 mM glutathione and analyzed by SDS-PAGE. The gel was subjected to Western blot analysis using antibodies against Rsd, σ70, β and β'. [B] Transcriptional activity of GST-σ70 alanine substitution mutants. In vitro transcription was performed using GST-σ70 mutants (1 pmol), core (1 pmol) and lacUV5 or extended-10 promoter (1 pmol). [C] Position of the Rsd contact sites in region 4.2.

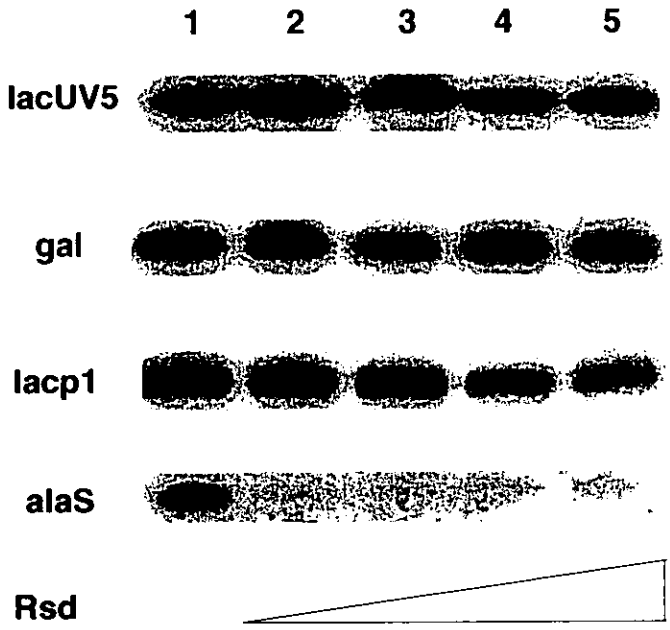


Figure 18. Effect of Rsd on *in vitro* transcription. A fixed amount of σ^{70} (1 pmol) and increasing amounts of Rsd (lanes 2-5, 1, 2, 5 and 10 pmol) were preincubated for 10 min at 30 °C in our standard transcription mixture containing 50 mM NaCl and then 1 pmol of core enzyme was added. After 10 min at 37 °C, a DNA fragment (1 pmol) carrying the indicated promoter was added and the mixture was incubated at 37 °C for 30 min to allow the open complex formation. RNA synthesis was initiated by adding a substrate-heparin mixture and continued for 5 min at 37 °C. Transcripts were analyzed by electrophoresis on 6% or 8% polyacrylamide gels containing 8 M urea.

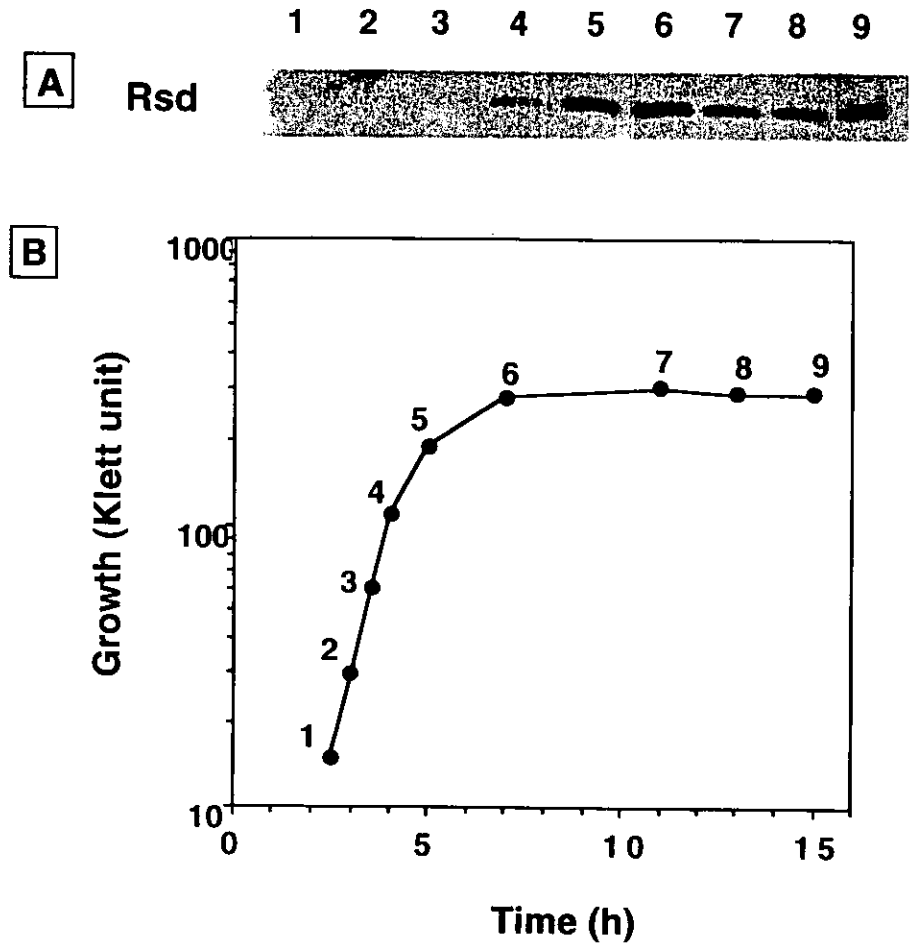
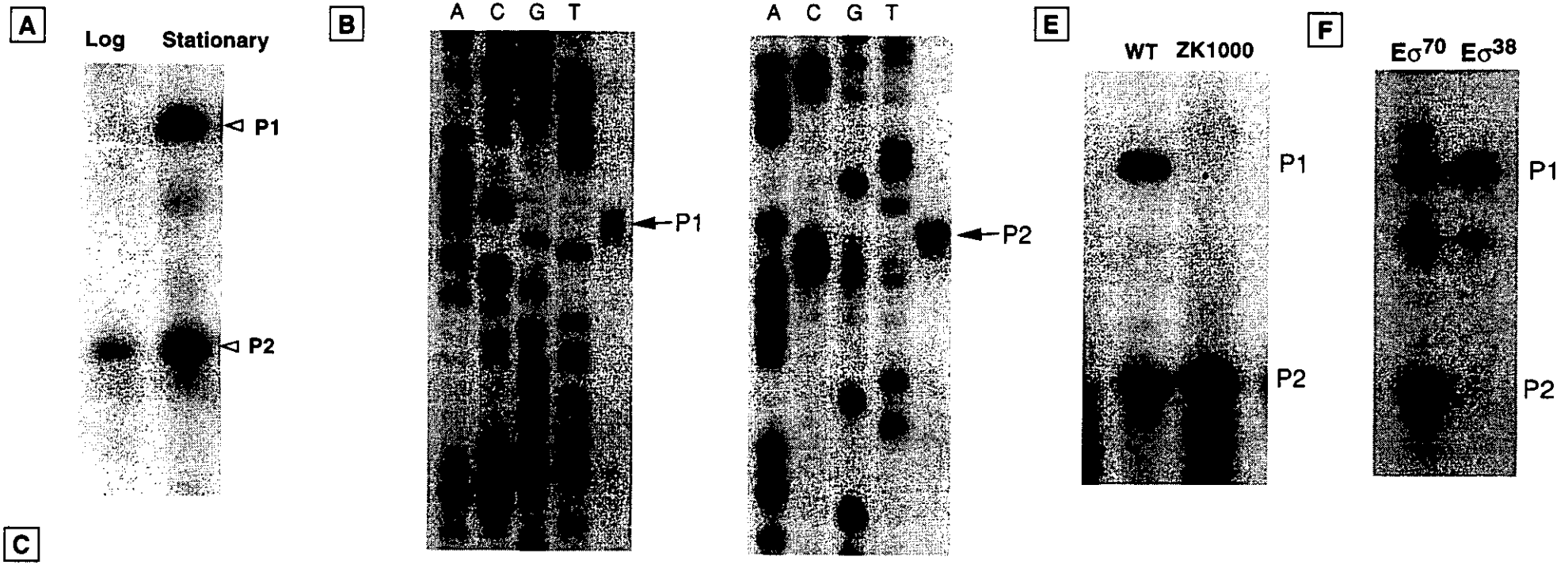


Figure 19. Measurement of the intracellular level of Rsd. *E. coli* W3110 type-A strain was grown in LB medium at 37 °C. Growth was monitored with a Klett-Summerson photometer. At the times indicated, cell lysates were prepared. The protein concentration was determined using a protein assay kit (Bio-Rad). Aliquots containing 10 μg of total proteins were subjected to the quantitative Western blot assay using the ECL reagent system (Amersham) for detection of the membrane-bound anti-Rsd antibodies.



C

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P1

AT**ATGGCA**AT TCTCCCTTCG GCAAC**CATAA T**TTTTGTTCA TGGCTGACGA CCCACCAGCC GTGATCTAAT TTTTCAATTA

-35 -10

P2

TACGATCCAT AGCTC**TTGCA C**TACCTTTGC ATCACTGG**CA TGTT**TAACAT GGTTTTTACATTTCTCACTGAGCAGTTTTTGA

-35 -10

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D

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Gear box consensus	CTGCAA	(14-16)	CGGCAAGT
rsdp2	TTGCAC	(14)	TGGCATGT
mcbp	TTGCAA	(14)	CGGCAAGT
bolAp1(rpoS)	CTGCAA	(15)	CGGCTAGT
ftsQp1(rpoS)	CGTCAA	(16)	CGGAACCT

Figure 20. Identification of the *rsd* promoters. [A] Primer extension analysis was carried out for RNA extracted from exponentially growing and stationary-phase cells. [B] Primer extension products were analyzed on a denatured polyacrylamide gel together with a sequence ladder. [C] Nucleotide sequence of the upstream region of *rsd*. [D] Comparison of *rsd*p2 with the gearbox promoter sequences. [E] Primer extension analysis was carried out for RNA extracted from stationary phase cells of strains ZK126 and ZK1000. [F] In vitro transcription by $E\sigma^{70}$ or $E\ \sigma^{38}$ was performed using the *rsd* promoter.

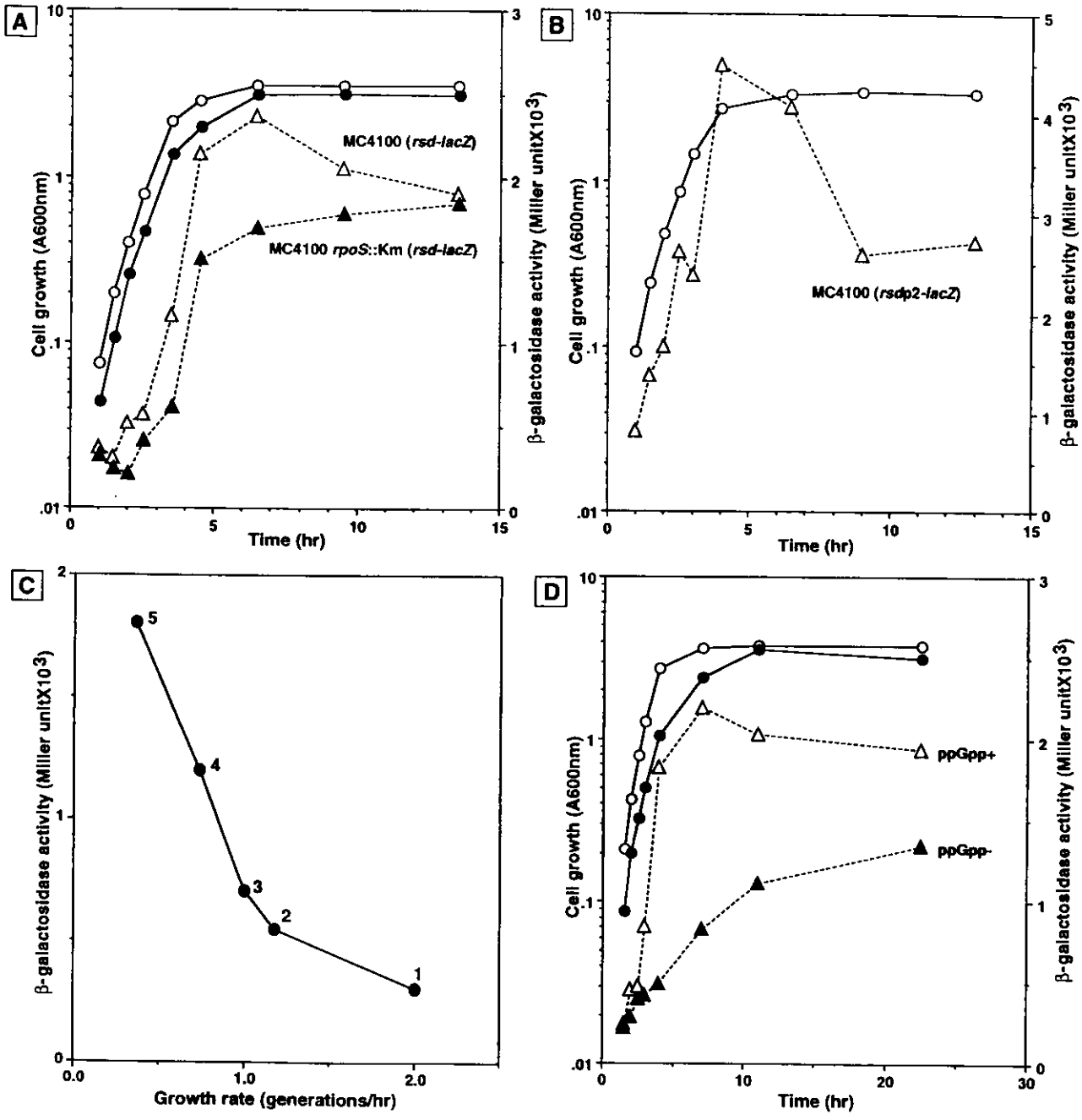
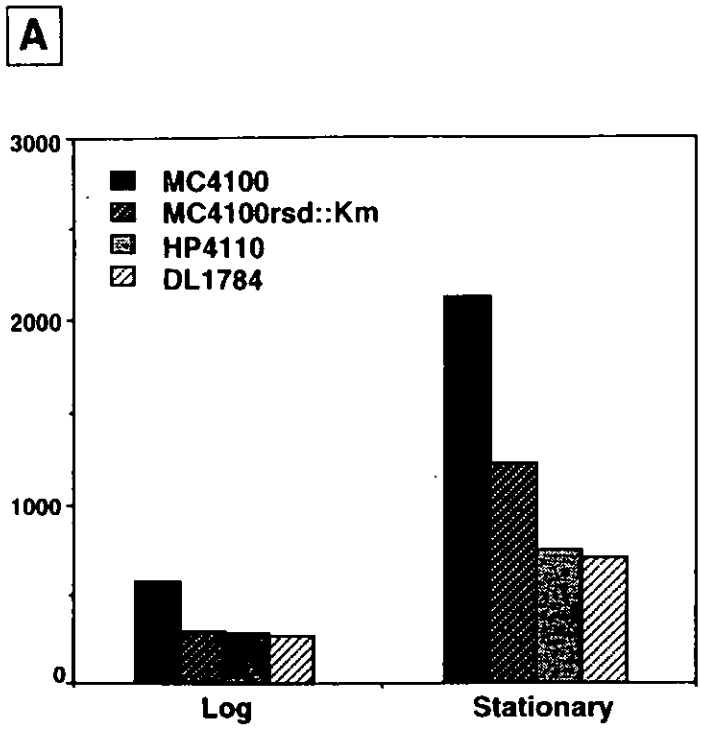


Figure 21. Growth-dependent expression of *rsd-lacZ* promoter. [A] Strains MJ6 (open symbols) and an isogenic *rpoS* mutant MJ27 (closed symbols), both carrying the *rsd*P1P2-*lacZ* fusion integrated in the genomes, were grown in LB medium. Cell growth (circles) was monitored by measuring the turbidity, while the *rsd* promoter activity was determined by measuring β-galactosidase activity (triangles). [B] Strain MJ19 carrying the *rsd*P2-*lacZ* fusion was grown in LB medium.The cell growth (circles) and β-galactosidase activity (triangles) were measured at the indicated times. [C] MJ6 was grown in LB (spot 1), M9-0.4% glucose-0.4% Casamino Acids (spot 2), M9-0.4% glycerol-0.4% Casamino Acids (spot3), M9-0.4% glycerol (spot 4), and M9-0.4% acetate (spot 5). Exponentially growing cells were used for the assay of β-galactosidase activity. [D] Strains MJ6 (open symbols) and its isogenic *spoT* mutant MJ39 (closed symbols) were grown in LB medium. The β-galactosidase activity (triangles) and cell growth (circle) were measured at the indicated times.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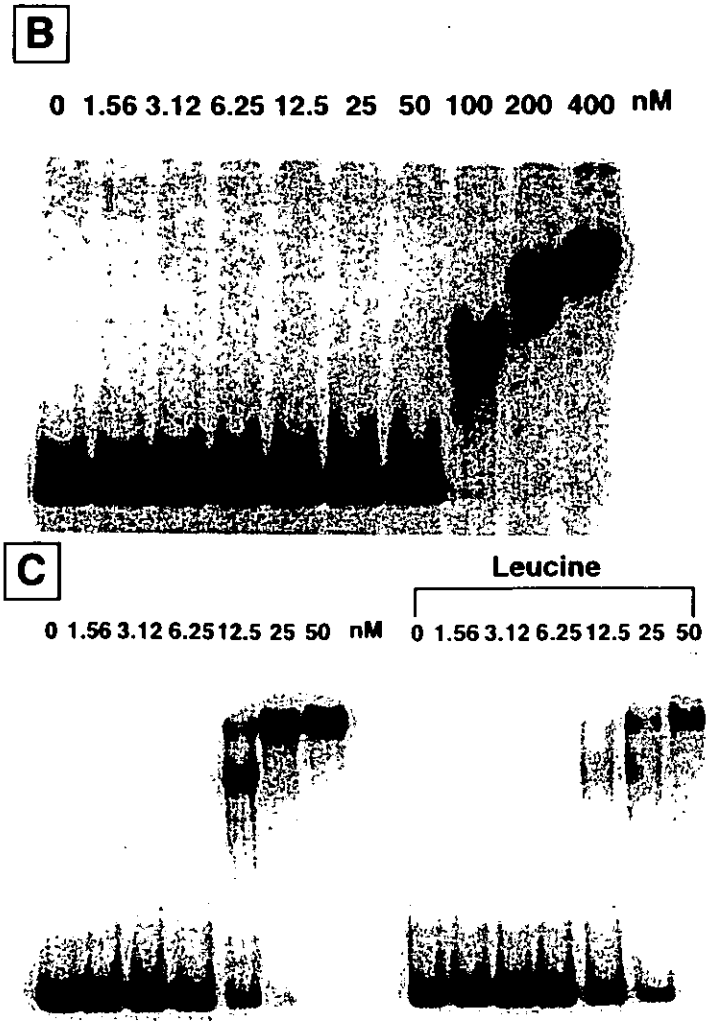
B
0 1.56 3.12 6.25 12.5 25 50 100 200 400 nM
C
Leucine
0 1.56 3.12 6.25 12.5 25 50 nM
0 1.56 3.12 6.25 12.5 25 50

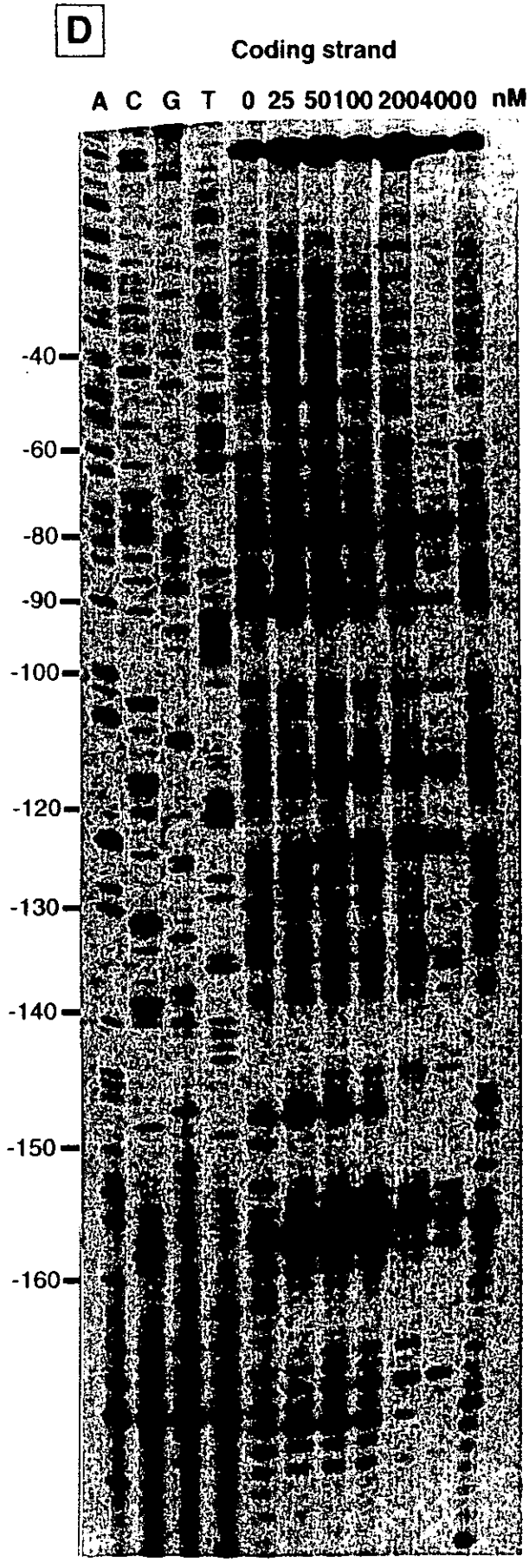
D
Coding strand
A C G T 0 25 50 100 200 400 0 nM
-40
-60
-80
-90
-100
-120
-130
-140
-150
-160

Figure 22. Effects of IHF and Lrp on *rsd* expression. [A] Cells of strain HP4110 and DL1784 were grown in LB medium. β-Galactosedase activity was determined at both log and stationary phases. [B and C] Gel shift assays were performed using a ^{32}P-labeled DNA fragment containing the *rsd* promoter region from -207 to +20 and the indicated amounts of purified IHF [B] or Lrp [C]. [D] DNase I footprinting was performed with the same DNA fragment as used in the gel shift assay and the indicated amounts of purified Lrp.

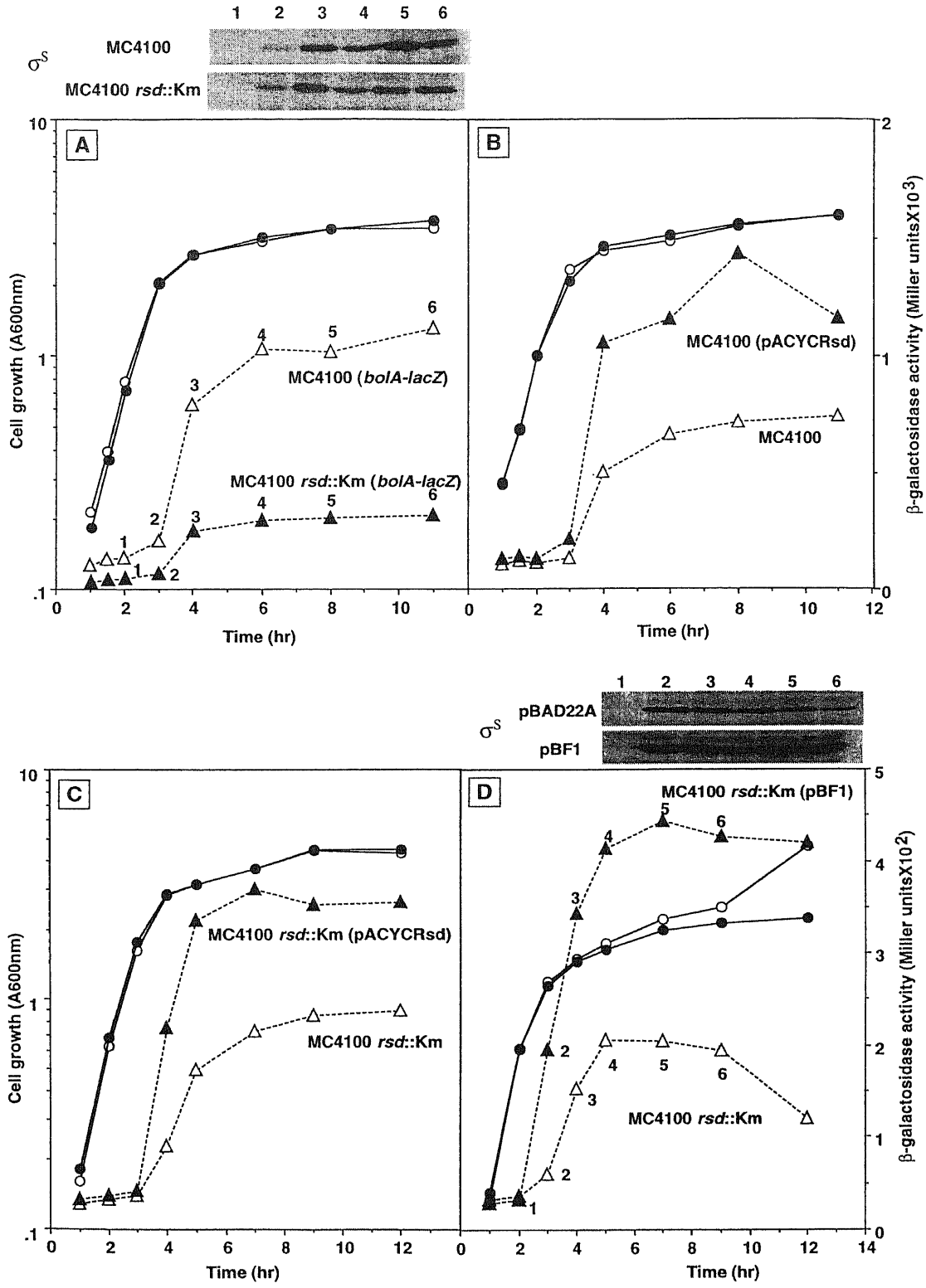

Figure 23. Effects of the *rsd* mutation on *bolA*p1 expression. Cells were grown in LB medium. Growth (circles) was monitored by measuring the turbidity, while the β-galactosidase activity (triangles) was determined at the indicated times. Aliquots containing the same cell numbers were subjected to the quantitative Western blot assay for measurement of σ^{38} levels. [A] Strains MJ31 (open symbols) and its isogenic *rsd* mutant MJ35 (closed symbols), both carrying the *bolA*P1-*lacZ* fusion integrated in the genomes; [B] MJ31 (pACYC184) (open symbols) and MJ31 (pACYCRsd) (closed symbols); [C]]MJ35 (pACYC184) (open symbols) and MJ35 (pACYCRsd) (closed symbols); [D] MJ35 (pBAD22A) (open symbols) and MJ35 (pBF1) (closed symbols).

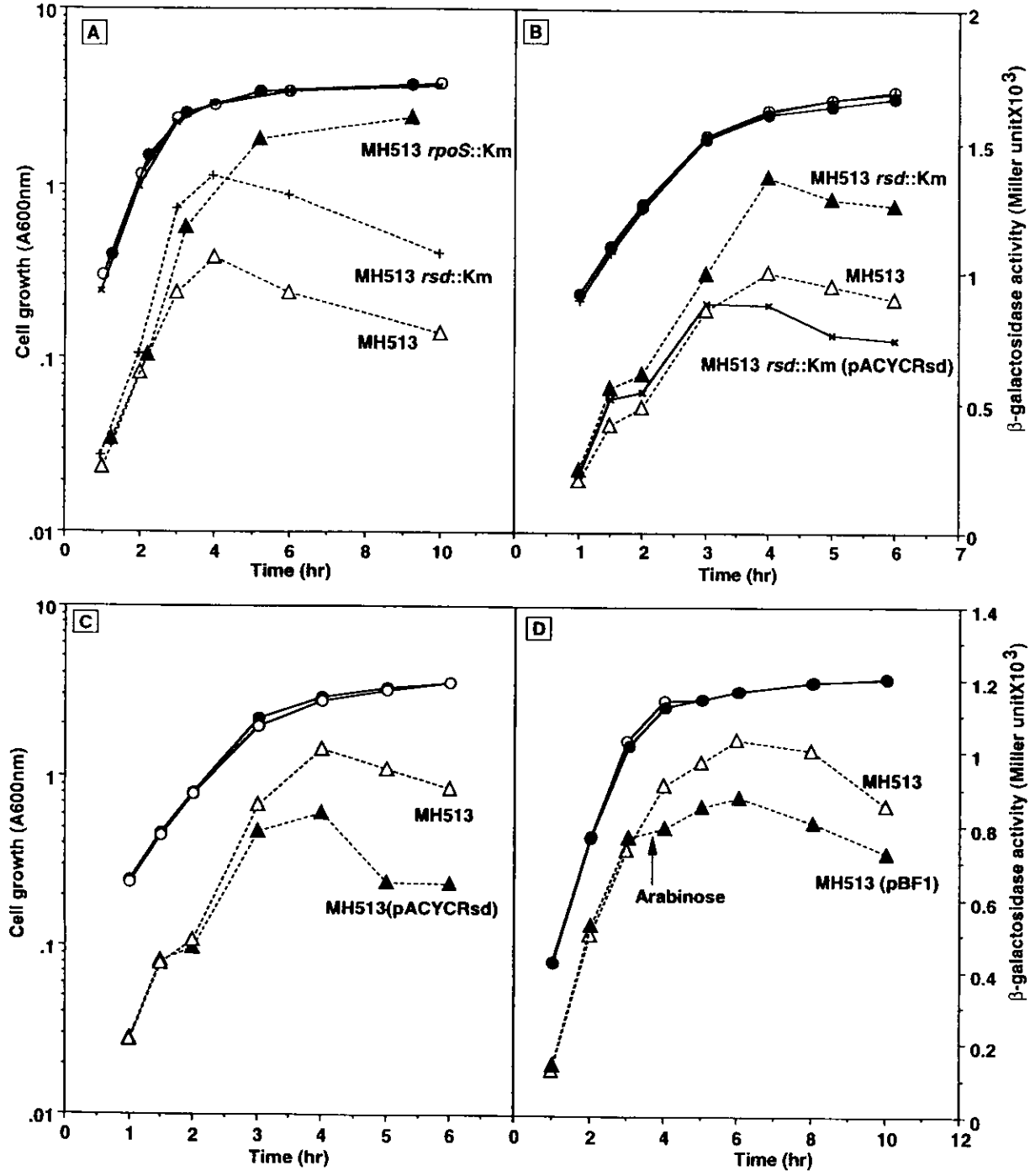

Figure 24. Effects of the *rsd* mutation on *ompF* expression. cells were grown in LB medium Growth (circles) was monitored by measuring the turbidity, while the β-galactosidase activity (triangles) was determined at the indicated times. Expression of σ^{38} was induced at the mid log phase by adding arabinose (0.02%). [A] Strains MH513 (open symbols), its isogenic *rpoS* mutant MJ83 (closed symbols) and a *rsd* mutant MJ57 (cross symbols); [B] MJ513 (pACYC184) (open symbols), MJ57 (pACYC184) (closed symbols), and MJ57 (pACYCRsd) (cross symbols); [C] MH513 (pACYC184) (open symbols) and MH513 (pACYCRsd) (closed symbols); [D] MH513 (pBAD22A) (open symbols) and MH513 (pBF1) (closed symbols).

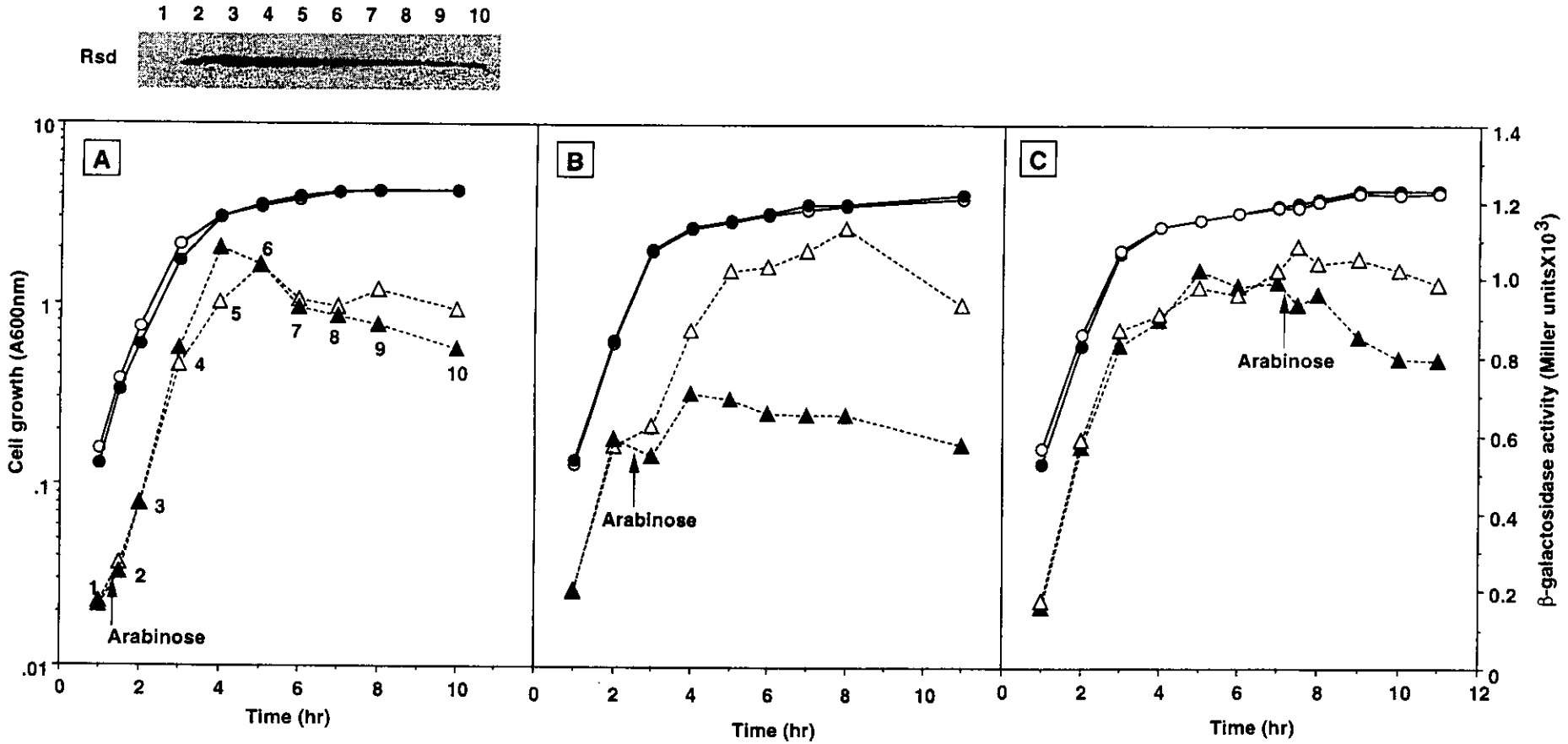

Figure 25. Effect of Rsd production on the expression of *ompF-lacZ*. Cells of MH513 (pBAD22A) (open symbols) or MH513 (pBADRsd) (closed symbols) were grown in LB medium. Growth (circles) was monitored by measuring the turbidity, while the β-galactosidase activity (triangles) was determined at the indicated times. Overproduction of Rsd was induced by adding arabinose (0.02%) at the early exponential phase [A], at the middle of exponential phase [B] and after entry into the stationary phase [C]. Aliquots containing same cell numbers were subjected to the quantitative Western blot for measurement of Rsd levels.

11 ACKNOWLEDGMENT

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