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Transcriptional Organization and In Vivo Role of the *Escherichia coli* *rsd* Gene, Encoding the Regulator of RNA Polymerase Sigma D

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The regulator of sigma D (Rsd) was identified as an RNA polymerase σ^{70}-associated protein in stationary-phase *Escherichia coli* with the inhibitory activity of σ^{70}-dependent transcription in vitro (M. Jishage and A. Ishihama, Proc. Natl. Acad. Sci. USA 95:4953–4958, 1998). Primer extension analysis of *rsd* mRNA indicated the presence of two promoters, σ^S-dependent P1 and σ^{70}-dependent P2 with the gearbox sequence. To get insight into the in vivo role of Rsd, the expression of a reporter gene fused to either the σ^{70}- or σ^S-dependent promoter was analyzed in the absence of Rsd or the presence of overexpressed Rsd. In the *rsd* null mutant, the σ^{70}- and σ^S-dependent gene expression was increased or decreased, respectively. On the other hand, the σ^{70}- or σ^S-dependent transcription was reduced or enhanced, respectively, after overexpression of Rsd. The repression of the σ^S-dependent transcription in the *rsd* mutant is overcome by increased production of the σ^S subunit. Together these observations support the prediction that Rsd is involved in replacement of the RNA polymerase σ subunit from σ^{70} to σ^S during the transition from exponential growth to the stationary phase.

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 or off the expression of specific sets of genes (17). Bacteria employ several different systems for switching the global pattern of gene expression. The specificity control of the transcription apparatus is a powerful mechanism with which to change the gene expression pattern. In *Escherichia coli*, the RNA polymerase core enzyme with the subunit composition $\alpha_2\beta\beta'$ 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, the σ subunit, is required (7). The promoter recognition specificity of RNA polymerase is conferred by one of the multiple species of σ subunit (21). Replacement of the σ subunit on RNA polymerase is an efficient way of switching the transcription pattern.

Up to the present time, seven different molecular species of the σ subunit have been identified in *E. coli* (21, 25). The major σ subunit, σ^{70}, is responsible for transcription of most, if not all, genes expressed during exponential cell growth (21, 25, 51). The other six species of the σ subunit are required only during certain growth stages or under specific growth conditions. In agreement with their functional roles, the levels of these alternative σ subunits vary, depending on the cell growth conditions (31, 34). In addition to the level control, the activity of at least some *E. coli* σ subunits is also subject to control in various ways (26, 27). For instance, the unused σ subunits are stored in inactive forms by forming complexes with another set of proteins, often designated as anti-σ factors, with the regulatory activity of σ functions (24, 27).

Bacteria use flagella to move away from stressful areas into microenvironments favorable for growth. Subunit σ^{28} (σ^F) is involved in transcription of the genes required for the formation of flagella. The *flgM* gene product is an anti-σ^F factor that acts by directly binding σ^F and thereby preventing its interaction with the core RNA polymerase (40). Subunit σ^{24} (σ^E) is a member of the ECF (extracytoplasmic function) family of σ subunits for transcription of the genes for proteins involved in extracytoplasmic functions (44) as well as those required for survival at high temperature, or thermotolerance (14). The σ^E activity is regulated by the *rseA* (regulator of sigma E, or anti-σ^E factor) gene product, which is associated with the inner membrane and inhibits the activity of σ^E by directly interacting with σ^E (12, 48). FecI, which belongs to the ECF family, is involved in transcription activation of the ferric-citrate transport genes (*fec*) (4). Genetic studies revealed that FecR, an inner membrane protein, negatively regulates the activity of FecI (59). Likewise, DnaK associates with and possibly controls the activity of the heat shock σ^{32} (σ^H) subunit (24), which is induced following heat shock, and is involved in transcription of the genes encoding heat shock proteins, including DnaK, DnaJ, and GrpE (18). After returning from heat shock to normal growth conditions, unused σ^H is temporarily stored as DnaJ-DnaK-σ^H complexes (43), which are then dissociated by the action of GrpE, and the released σ^H is finally degraded by FtsH protease (16).

Recently we discovered a novel *E. coli* protein, referred to Rsd (regulator of sigma D), which forms a complex with σ^{70} and prevents its function (33). Purified Rsd protein formed complexes in vitro with σ^{70}, but not with other σ subunits, and inhibited σ^{70}-dependent transcription in vitro to various extents, depending on the promoters used. Since Rsd is induced in the stationary phase of cell growth, unused excess σ^{70} subunit, without being involved in the transcription cycle, should be trapped by Rsd. Thus, the possibility has arisen that Rsd is an anti-σ factor for the major σ^{70} subunit for its storage in the stationary phase. In order to clarify the in vivo function of Rsd, we analyzed the influence of both depletion and overproduction of Rsd on σ^{70}- and σ^S-dependent transcription in vivo. On the basis of the results herewith described, we propose that Rsd is a regulator that facilitates the switching of σ subunit on RNA polymerase from σ^{70} to σ^S during the transition from exponential growth to stationary phase.

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TABLE 1. Characteristics of bacterial strains used in this study

Strain	Relevant genotype	Source or reference
W3110	Type A	32
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	National Institute of Genetics stock ME8286
JC7623	*argE3 his4 leu6 proA2 thr1 thi1 rpsL31 galK2 lacY1 ara14 xyl15 mtl1 supE44 kdgK51 recB15 recC22 sbcB15*	50
ZK126	W3110 Δ*lacU169 tna2*	6
ZK1000	ZK126 *rpoS*::Km	6
CF1946	CF1943 Δ*relA251*::Km Δ*spoT207*::Cm	62
MH20	F$^-$ Δ*lacU169 rpsL relA thiA flbB*	20
MH513	MH20 λ(*ompF-lacZ*) 16-13	20
MJ57	MH513 *rsd*::Km	This work
MJ83	MH513 *rpoS*::Km	This work
MJ31	MC4100 λ(*bolA-lacZ*)	This work
MJ6	MC4100 λ(*rsdI-lacZ*)	This work
MJ39	MJ6 *spoT*::Cm	This work
MJ19	MC4100 λ(*rsdIII-lacZ*)	This work
MJ30	MC4100 *rsd*::Km	This work
MJ35	MJ30 λ(*bolA-lacZ*)	This work
MJ23	MC4100 *rpoS*::Km	This work
MJ27	MJ23 λ(*rsdI-lacZ*)	This work

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* strains used in this work are listed in Table 1. Cells were grown at 37°C with shaking in either Luria-Bertani (LB) broth or medium M9 (46). For cultures of cells carrying antibiotic resistance markers, the media were supplemented with ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), or kanamycin (50 μg/ml). For induction of the cloned genes under the control of the arabinose-regulated promoter, arabinose was added at a final concentration of 0.02%.

Plasmids. The plasmids used in this work are listed in Table 2. To create an Rsd expression plasmid, pUCRsd33, carrying the entire *rsd* gene from its own promoters to terminator(s), an 820-bp-long DNA fragment was PCR amplified by using primers Rsd-5 (5′-CGCGGATCCAACCAAACAGGTTCCCCCTGC CAT-3′; *Bam*HI site underlined) and Rsd-6 (5′-AACTGCAGCTCGAGCTCA GCCAGTTAAGGCACTCC-3′; *Pst*I site underlined) (see Fig. 1F for the locations of primer sequences on the *rsd* gene), and the resulting PCR product was cloned into pUC18 between the *Bam*HI and *Pst*I sites to construct pUCRsd33. The *Bam*HI-*Sph*I fragment was isolated from pUCRsd33 and recloned into pACYC184 (9) to create pACYCRsd. The cloned *rsd* gene carries the entire *rsd* sequence downstream from nucleotide −261 (as counted from the ATG initiation codon of Rsd) (see Fig. 1E for the *rsd* sequence).

To construct pBADRsd31-1 for high-level expression of Rsd, an *rsd* fragment containing only the Rsd coding sequence without the promoters was PCR amplified with primers Rsd11 (5′-CCGGAATTCACCATGCTTAACCAGCTCGA TAAC-3′; *Eco*RI site and the *rsd* initiation codon, underlined) and Rsd12 (5′-CATGCATGCTCAAGCAGGATGTTTGACGCGGGC-3′) (see Fig. 1F for the locations of the primer sequences), and cloned into pBAD22A (19) between the *Eco*RI and *Sph*I sites.

Construction of the *rsd* promoter-*lacZ* transcriptional fusions. Two species of *rsd* promoter fragment were PCR amplified with two pairs of primers, Rsd7 (5′-CCGGAATTCAACCAAACAGGTTCCCCCTGCCAT-3′; *Eco*RI site underlined) plus Rsd14 (5′-CGCGGATCCCAGTGAGAAATGTAAAAACCAT GT-3′; *Bam*HI site underlined), and Rsd13 (5′-CCGGAATTCACGACCCAC CAGCCGTGATCTAAT-3′; *Eco*RI site underlined) plus Rsd14 (see Fig. 1F for the locations of these sequences on *rsd*). The PCR products were cloned into pRS551 between the *Eco*RI and *Bam*HI sites, generating pRsd1 or pRsd3, respectively. pRsd1 and pRsd3 contain the *rsd* promoter region sequence from either −261 or −138, respectively, to −34. The sequences of these *rsd* inserts were confirmed by dideoxynucleotide sequencing. The *rsd* gene fusions were then integrated onto phage λRS45, and the recombinant phages were used to lysogenize MC4100 as described previously (56).

As a control, strain MC4100 carrying the *bolA-lacZ* transcriptional fusion on the genome was constructed with λ(*bolA*P1-*lacZ*) (2).

Disruption of the *rsd* gene. Strain MJ30 carrying an internal deletion of the *rsd* gene was constructed as follows. A 1.4-kbp *rsd* gene fragment was PCR amplified with primers f158-1 (5′-CATGCATGCCACAAGATCGAAATTTGCCCGTT C-3′) and f158-2 (5′-CCGGAATTCCATTTCCGGCGTGATGATGCCCTG-3′), which were used for the cloning of *rsd* (33), and subcloned into pUC18 between the *Sph*I and *Eco*RI sites. The *rsd* coding region between the *Bsm*I and *Sna*BI sites was replaced by a *Hinc*II fragment of pUC4K (Pharmacia) carrying the kanamycin resistance gene. The resulting plasmid, pUJC-1, was digested with a mixture of *Bsm*I and *Sna*BI, and the *Bsm*I-*Sna*BI fragment was purified by SUPREC01 (Takara Shuzo Co.). Two micrograms of this linear DNA fragment was transformed into *E. coli* JC7623 (50). Kanamycin-resistant transformants were isolated, which carried the *rsd* deletion mutation integrated in the chromosome. Phage P1*vir* transduction was used to transfer the *rsd* mutation to strain MC4100 for construction of MJ30.

Primer extension analysis. Cells of *E. coli* W3110 type A (32) were grown in LB medium at 37°C. At both the exponentially growing phase and the transition phase from exponential growth to stationary phase, total RNA was prepared by phenol extraction according to the method of Aiba et al. (1). For primer extension reactions, a 25-nucleotide-long primer with the sequence 5′-TGACGC GCTCCGTCAGGTTATCGAG-3′, corresponding to the *rsd* coding sequence between +13 and +37 (as counted from the ATG initiation codon), was ^{32}P-labeled by using MEGALABEL (Takara Shuzo). The reaction mixture, containing 2 pmol of the end-labeled primer and 50 μg of total RNA, was heated for 5 min at 80°C, followed by incubation on ice for 5 min. After addition of 12.5 U of avian myeloblastosis virus reverse transcriptase (Takara Shuzo) in 50 mM Tris-HCl (pH 7.6), 60 mM KCl, 10 mM $MgCl_2$, 1 mM deoxynucleoside 5′-triphosphates, and 1 mM dithiothreitol in a total volume of 20 μl, 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), followed 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 with the appropriate plasmid DNA as the template and the primer used for primer extension. Reaction products were run in parallel with the sequence ladder obtained with a 7-DEAZA sequencing kit (Takara Shuzo, Japan) to determine the end point of extension products.

β-Galactosidase assay. The activity of β-galactosidase was assayed according to the procedure of Miller (46), by using cells which were made permeable by treatment with sodium dodecyl sulfate and $CHCl_3$. The activity assay was repeated at least twice for each sample. The activity is expressed as Miller units: $1{,}000 \times [(A_{420} - 1.75 \times A_{550})/(A_{600} \times \text{reaction time} \times \text{volume})]$.

In vitro single-round transcription assay. RNA polymerase core enzyme was purified from *E. coli* W3350 by passage of the purified RNA polymerase at least three times through phosphocellulose columns (39). Holoenzymes were reconstituted by mixing the core enzyme and threefold molar excess of each σ subunit. Single-round transcription by the reconstituted holoenzymes was carried out under the standard reaction conditions described previously (35).

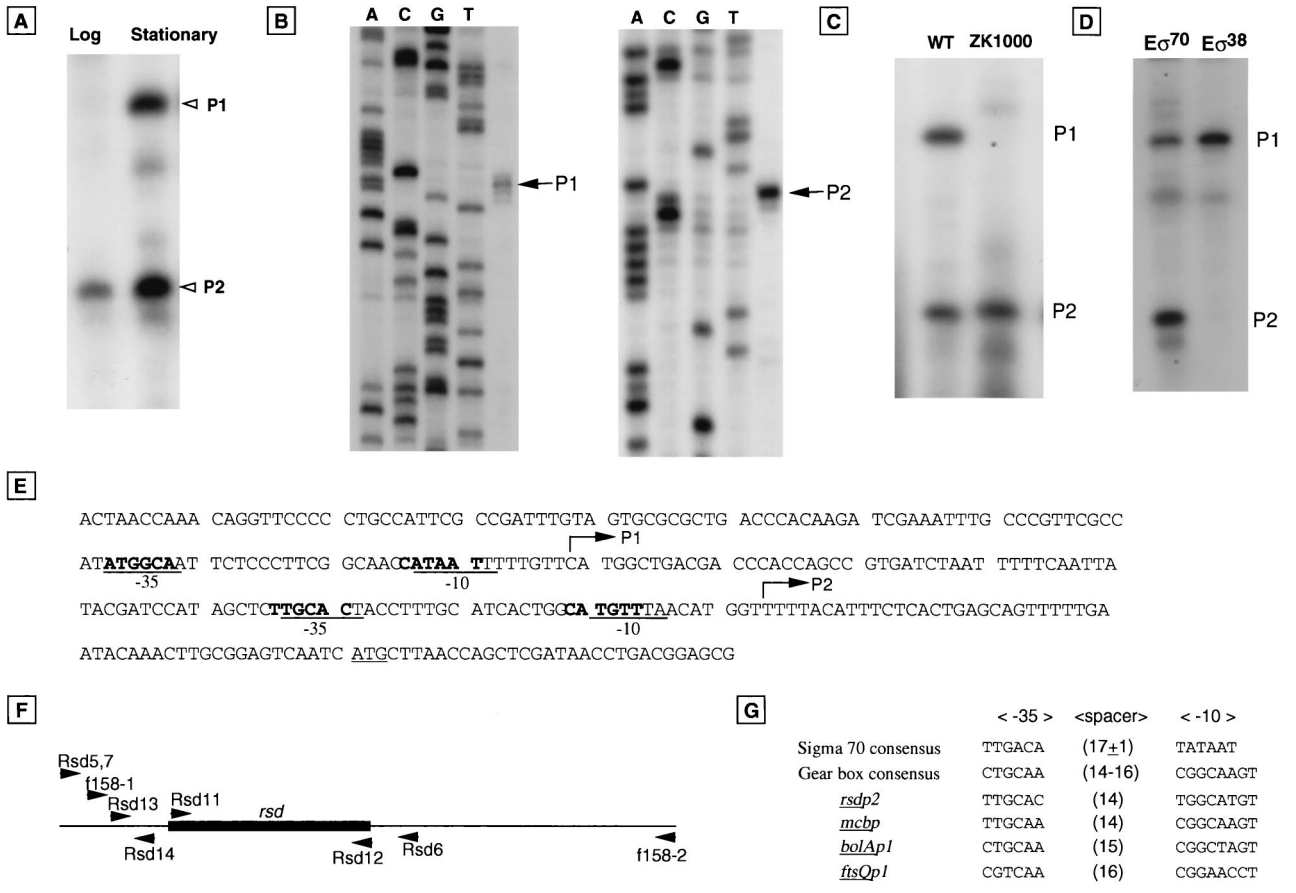
Quantitative Western blot analysis. A quantitative Western blot analysis was carried out according to the method of Jishage and Ishihama (31). Polyclonal antibodies against purified σ^{70}, σ^S, and Rsd were raised in rabbits as described previously (31, 33).

RESULTS

Identification of the transcriptional start sites of *rsd*. The intracellular level of Rsd protein increases during the transition from exponential growth to the stationary phase (33). In order to get insight into the regulatory mechanism underlying growth-dependent expression of the Rsd protein, we determined the transcription start site(s) of the *rsd* gene. For this purpose, total RNA was isolated from both exponentially growing and stationary-phase cells of the type A W3110 strain, which carries the *rpoS* gene in its intact form (32), and was subjected to primer extension analysis. Only one major product (P2) was observed for RNA from the exponential-phase cells (Fig. 1A). Besides the P2 transcript, another product (P1) was identified for RNA from the stationary-phase cells (Fig. 1A). The transcription start point of P1 RNA is located at 148 bp upstream of the translation initiation codon (Fig. 1B), 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 (Fig. 1E).

TABLE 2. Characteristics of plasmids used in this study

Plasmid	Vector	Gene to be expressed	Source or reference
pUJC-1	pUC18	Truncated *rsd*	This work
pUCRsd33	pUC18	Complete *rsd*	This work
pACYCRsd	pACYC184	Complete *rsd*	This work
pBADRsd31-1	pBAD22A	Complete *rsd*	This work
pBF1	pBAD22A	*rpoS*	57a



	< -35 >	<spacer>	< -10 >
Sigma 70 consensus	TTGACA	(17±1)	TATAAT
Gear box consensus	CTGCAA	(14-16)	CGGCAAGT
rsdp2	TTGCAC	(14)	TGGCATGT
mcbp	TTGCAA	(14)	CGGCAAGT
bolAp1	CTGCAA	(15)	CGGCTAGT
ftsQp1	CGTCAA	(16)	CGGAACCT

FIG. 1. Identification of the *rsd* promoters. (A) Primer extension analysis was carried out for RNA extracted from the exponentially growing cells (Log lane) and the stationary-phase cells (Stationary lane). (B) Primer extension products were analyzed by electrophoresis on a denatured polyacrylamide gel together with sequence ladders. P1, transcript from *rsd*P1 promoter; P2, transcript from *rsd*P2 promoter. (C) Primer extension analysis was carried out for RNA extracted from stationary-phase cells of wild-type (WT) strain ZK126 (*rpoS*$^+$) and mutant strain ZK1000 (*rpoS*). (D) In vitro transcription of the *rsd* promoters by Eσ^{70} or Eσ^S holoenzymes. Transcription products were analyzed by polyacrylamide gel electrophoresis in the presence of 8 M urea. (E) Nucleotide sequence of the upstream region of *rsd*. (F) Primers used in this study. (G) Sequence comparison of *rsd*P2 with the known gearbox promoters.

The P1 start site at −148 is preceded by a sequence, CAT AAT, with a reasonable similarity to the consensus sequence (TATAAT) of σ^{70}-dependent promoter −10 (Fig. 1E). Separated from this −10-like sequence by a 17-bp spacer is an ATGGCA sequence with a reasonable similarity to the consensus sequence (TTGACA) of σ^{70}-dependent promoter −35 (Fig. 1E). On the other hand, the transcription of P2 is initiated at G at −54 bp upstream from ATG (Fig. 1B). Although the −10 hexamer of P2 (CATGTT) is not in good agreement with the σ^{70} promoter −10 consensus sequence, the presence of upstream TG characterizes it as an extended −10 promoter (TGGCATGTT) (36, 47). Such a −10 sequence alignment is a common feature of several gearbox promoters (Fig. 1G) (3, 60). The −35 hexamer (TTGCAC) is separated from the −10 sequence by a 17-bp spacer and is in agreement with the −35 sequence associated with the gearbox promoters (Fig. 1E). Two gearbox promoters, *bolA*p1 and *ftsQ*p1, are known to be recognized by the σ^S subunit (5, 6, 40). To determine whether σ^S is responsible for transcription from the *rsd* P1 and P2 promoters, total RNA was isolated from strain ZK1000, which lacks *rpoS*, and analyzed by primer extension. As shown in Fig. 1C, the P1 product was not detected in the absence of *rpoS*. The results suggest that the P1 promoter is dependent on σ^S, whereas the P2 promoter is transcribed by Eσ^{70}. This conclusion was confirmed by using an in vitro transcription assay. As shown in Fig. 1D, Eσ^S can transcribe only from the P1 promoter, whereas Eσ^{70} is able to initiate transcription from both the P1 and P2 promoters. (Note that the σ^S-dependent promoters are recognized in vitro by both Eσ^{70} and Eσ^S under the conditions employed [58].)

Influence of growth rate and growth phase on the expression of *rsd*. Transcription from the gearbox promoters *bolA*p1 and *ftsQ*p1 increases in the stationary phase of cell growth, and the level of transcription at the growth phase is inversely related to growth rate (3). To determine whether the expression of *rsd* is also dependent on growth phase or growth rate, we constructed an *rsd-lacZ* transcriptional fusion on phage λ and inserted the transducing phage at its normal attachment site on the *E. coli* genome. By using the *rsd-lacZ* transductant thus constructed, the β-galactosidase activity was measured at various time points during the transition from exponential growth to the stationary phase in LB medium at 37°C. The β-galactosidase activity increased seven- to eightfold in the stationary phase compared to that at the exponential phase (Fig. 2A).

To examine whether this activation of the *rsd* promoter in the stationary phase is dependent on σ^S, the *rsd*(P1-P2)-*lacZ* fusion gene was transduced into an *rpoS* null mutant, and the β-galactosidase activity was measured at different growth

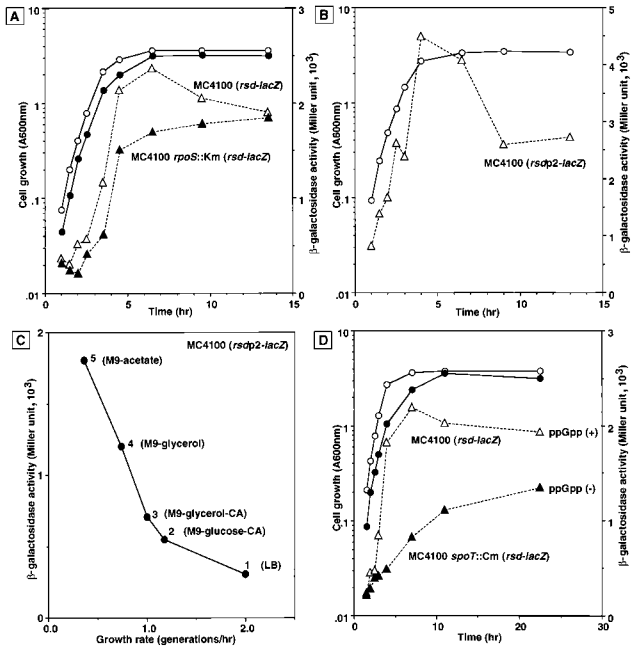


FIG. 2. Growth-dependent expression of the *rsd-lacZ* fusions. (A) Strain MJ6 [MC4100 λ(*rsdI*-lacZ); open symbols] and an isogenic *rpoS* mutant, MJ27 [MC4100 *rpoS*::Km λ(*rsdI*-lacZ); solid symbols], both carrying the *rsd*P1P2-*lacZ* fusion integrated in the genome (Table 1), were grown in LB medium. Cell growth (circles) was monitored by measuring turbidity, while *rsd* promoter activity was determined by measuring β-galactosidase activity (triangles). (B) Strain MJ19 [MC4100 λ(*rsdIII-lacZ*)] carrying the *rsd*P2-*lacZ* fusion was grown in LB medium. The cell growth (circles) and the β-galactosidase activity (triangles) were measured at the indicated times. (C) MJ6 [MC4100 λ(*rsdI-lacZ*)] was grown in LB medium (medium 1), M9–0.4% glucose–0.4% Casamino Acids (medium 2), M9–0.4% glycerol–0.4% Casamino Acids (medium 3), M9–0.4% glycerol (medium 4), and M9–0.4% acetate (medium 5). Exponentially growing cells at A_{600} of 0.4 to 0.5 were used for the assay of β-galactosidase activity. (D) Strain MJ6 [MC4100 λ(*rsdI-lacZ*); open symbols] and its isogenic *spoT* mutant, MJ39 [MC4100 *rpoT*::Cm λ(*rsdI-lacZ*); solid symbols], were grown in LB medium. The β-galactosidase activity (triangles) and cell growth (circle) were measured at the indicated times.

phases. The results, shown in Fig. 2A, indicated that the β-galactosidase activity in the *rpoS* null mutant also increased during the transition from the exponential growth phase to the stationary phase, but the maximum level of expression in the *rpoS* mutant was about 70% the level of the wild-type transductant in the early stationary phase (3 to 4 h after the cessation of cell growth). After prolonged culture in the stationary phase, however, the β-galactosidase activity in the *rpoS* mutant reached the same level as that observed with the wild type, suggesting that the basal level of *rsd* transcription is maintained by using the downstream P2 promoter recognized by $E\sigma^{70}$ RNA polymerase, and transcription from the upstream P1 promoter by $E\sigma^{S}$ takes place only in the early stationary phase.

To measure the β-galactosidase activity solely from the P2 promoter, another *rsd*(P2)-*lacZ* transcriptional fusion without the P1 promoter was constructed and transduced into the wild-type strain. Again the maximum activity was observed during the transition from exponential growth to stationary phase (Fig. 2B). Moreover, the maximum level of β-galactosidase activity driven by the P2 promoter alone was twofold higher than the activity from both P1 and P2. One possible explanation for this unexpected observation is that a regulatory signal repressing *rsd* transcription is located upstream of the P2 promoter.

We next investigated the effect of growth rate on *rsd* expression. The expression levels of the *rsd*(P1-P2)-*lacZ* fusion were compared among the exponentially growing cultures in media supplemented with various carbon sources. As shown in Fig. 2C, the β-galactosidase activity was low in cells growing at high rates (media 1 to 3), but substantially increased in cells growing at lower rates (media 4 and 5). This result indicates that *rsd* expression is inversely related to the growth rate. Genes whose expression increase with decreasing growth rate are often under the positive control of ppGpp, the mediator of stringent control (for details see Discussion). To determine whether ppGpp also affects *rsd* expression, we measured the β-galactosidase activity in a *relA1 spoT* strain which does not produce ppGpp. As shown in Fig. 2D, the β-galactosidase activity in the mutant strain MJ39 devoid of ppGpp was reduced to 38% the level of the *relA1 spoT*$^{+}$ strain at the maximum expression, and then the expression increased to 70% in the late stationary phase. These observations suggest that ppGpp is partly involved in stimulation of *rsd* transcription, but the full expression of *rsd* may require an additional factor(s) or condition(s).

Effect of *rsd* mutation on σ^{S}-dependent transcription. Upon entry into the stationary phase, σ^{S} begins to be produced (31, 34) and allows the core polymerase to recognize and transcribe the genes required for stationary-phase survival (22). Previously we showed that Rsd interacts in vitro preferentially with free σ^{70}, but not the core enzyme-bound σ^{70} ($E\sigma^{70}$) (33). If Rsd interacts in vivo with free σ^{70}, the level of functional σ^{70} available for use in the transcription cycle should be reduced, depending on the concentration of Rsd, ultimately leading to the switching of the global transcription pattern from the σ^{70}-dependent genes to those which carry σ^{S}-dependent promoters.

To test the above possibility, we constructed a mutant *E. coli* strain lacking the *rsd* gene and measured the expression of a σ^{S}-dependent *bolA*p1-*lacZ* transcription fusion under the exogenous supply of various levels of Rsd. As shown in Fig. 3A, the expression of *bolA*p1-*lacZ* in the *rsd* mutant strain was reduced to about 30% the level of wild-type strain. However, Western blotting analysis indicated that this reduction in β-galactosidase synthesis was not caused by a decrease or increase in the levels of σ^{S} and σ^{70} proteins, respectively (for σ^{S}, see the Western blot pattern above Fig. 3A [data not shown for the σ^{70} pattern]). Thus, the decrease in Rsd level led to a reduction in the utilization of σ^{S} for expression of the σ^{S}-dependent *bolA*p1-*lacZ* fusion gene. We then tested the opposite case, i.e., the effect of increased expression of Rsd on σ^{S}-dependent gene expression. Expression of Rsd in wild-type cells by using an *rsd* expression vector, constructed with plasmid pACYC, resulted in a transient but significant (about 1.5-fold) increase in the expression level of *bolA*p1-*lacZ* (Fig. 3B). Western blot analysis indicated that the maximum level of Rsd expression was higher than that of σ^{70} (data not shown). To confirm that the observed decrease or increase in *bolA*p1-*lacZ* expression was due to the direct effect of a decrease or increase, respectively, in the Rsd level, we introduced an Rsd expression vector, pACYCRsd, into the *rsd* mutant. As expected, the β-galactosidase activity increased by about twofold after induction of Rsd (Fig. 3C).

The increase in Rsd should lead to a decrease in the concentration of functional σ^{70} subunit, and as a result, the relative amount of $E\sigma^{S}$ holoenzyme may increase because the intracellular level of core enzyme stays constant at a level characteristic of the rate of cell growth (29). Likewise the decrease in Rsd level may result in an increase in $E\sigma^{70}$ holoenzyme, ultimately leading to the reduction in $E\sigma^{S}$ level. In order to further confirm this hypothesis, we next examined

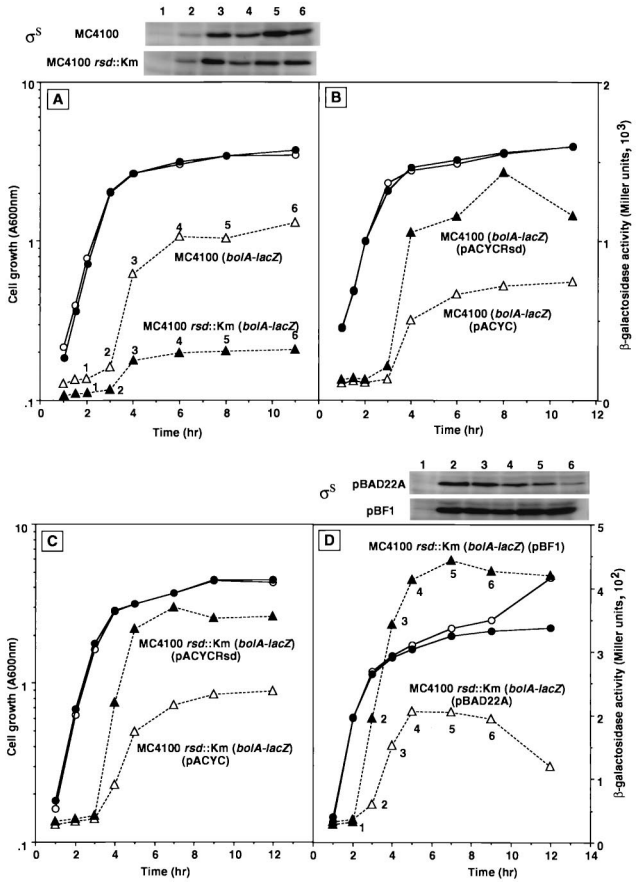


FIG. 3. Effect of the *rsd* mutation on *bolA*P1-directed transcription. Cells were grown in LB medium. Growth (circles) was monitored by measuring turbidity, while β-galactosidase activity (triangles) was determined at the indicated times. Aliquots containing the same cell numbers were subjected to a quantitative Western blot assay for measurement of σ^S levels. (A) Strain MJ31 [MC4100 λ(*bolA-lacZ*); open symbols] and its isogenic *rsd* mutant, MJ35 [MC4100 *rss*::Km λ(*bolA-lacZ*); solid symbols]. (B) MJ31 [MC4100 λ(*bolA-lacZ*)] carrying either pACYC184 (open symbols) or pACYCRsd (solid symbols). (C) MJ35 [MC4100 *rsd*::Km λ(*bolA-lacZ*)] carrying either pACYC184 (open symbols) or pACYCRsd (solid symbols). (D) MJ35 [MC4100 *rsd*::Km λ(*bolA-lacZ*)] carrying either pBAD22A (open symbols) or pBF1 (solid symbols). Arabinose was added at 2 h of culture (time point 1).

possible effect of the exogenous supply of σ^S on the expression of *bolA*p1-*lacZ* fusion. For this purpose, σ^S was overexpressed by using the σ^S expression vector under the control of an arabinose-inducible promoter (see the Western blot pattern shown above Fig. 3D). The activity of *bolA*p1-*lacZ* indeed increased more than twofold (Fig. 3D), reaching a level as high as that observed when Rsd was expressed in the *rsd* mutant strain (Fig. 3C).

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

Effect of *rsd* mutation on σ^{70}-dependent transcription. The total number of core enzyme molecules, which are not involved in the transcription cycle, is close to the combined number of seven species of the σ subunit (25, 27). Thus, the intracellular concentrations of seven species of the σ subunit should be the major determinant of the relative amount of the seven forms of the holoenzyme. If this is the case, an increase or decrease in the amount of one σ subunit should affect not only the level of the holoenzyme containing that particular σ subunit, but also the levels of other holoenzymes containing different σ subunits. To monitor the change in the intracellular level of σ^{70} with a high sensitivity, we used the *ompF* promoter as a test promoter. The *ompF* gene encoding an outer membrane porin protein is transcribed by $E\sigma^{70}$ and regulated by OmpR. The level of *ompF* transcription is known to be directly correlated to the level of $E\sigma^{70}$ (15, 54). Thus, the *rsd* null mutation may lead to an increase in functional σ^{70} (and a decrease in $E\sigma^S$ level) and ultimately to induction of *ompF* transcription. To test this possibility, we next measured the β-galactosidase activity encoded by the *ompF-lacZ* transcriptional fusion.

As shown in Fig. 4A, the expression of the *ompF-lacZ* fusion significantly increased in the mutant *E. coli* strain lacking the *rsd* gene (Fig. 4A), but the high-level expression of *ompF-lacZ* in the *rsd* mutant was suppressed by the supply of Rsd protein by an expression plasmid (Fig. 4B). The inhibitory effect on β-galactosidase synthesis by the overexpressed Rsd was also observed with the wild-type *E. coli* (Fig. 4C). All of these observations are consistent with the prediction that the Rsd protein forms complexes with σ^{70}, and thereby the concentration of holoenzyme $E\sigma^{70}$ decreases, leading to the reduction in σ^{70}-dependent transcription.

A decrease in $E\sigma^{70}$ may also take place with an increase in other σ subunits. To test this possibility, attempts were made to change the level of the σ^S subunit. In the *rpoS* mutant, the level

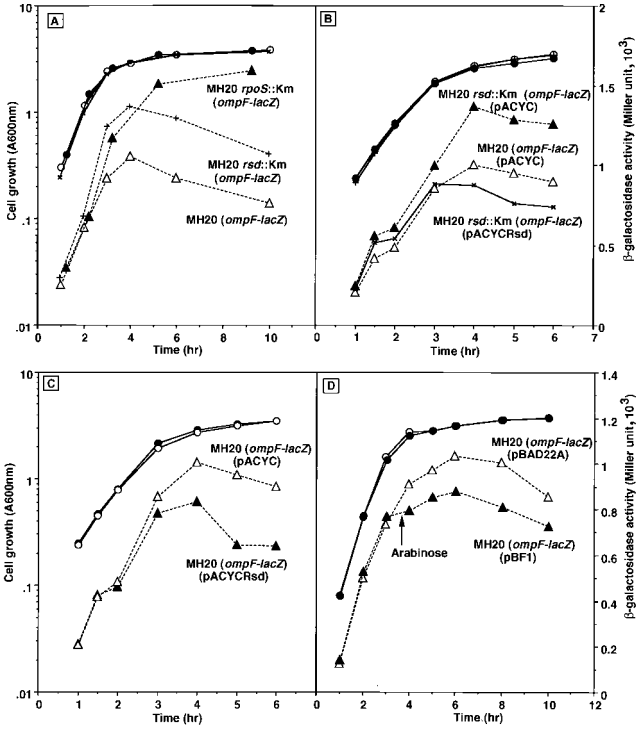


FIG. 4. Effect of the *rsd* mutation on *ompF* promoter-directed transcription. Cells were grown in LB medium. Growth (circles) was monitored by measuring turbidity, while β-galactosidase activity (triangles) was determined at the indicated times. Expression of σ^S was induced by adding arabinose (0.02%) at the middle of the exponential phase. All *E. coli* strains carried the *ompF-lacZ* fusion integrated in the genomes. (A) Strain MH513 [MH20 λ(*ompF-lacZ*); open symbols], its isogenic *rpoS* mutant, MJ83 [MH20 *rpoS*::Km λ(*ompF-lacZ*); solid symbols], and an *rsd* mutant, MJ57 [MH20 *rsd*::Km λ(*ompF-lacZ*); crosses]. (B) MJ513(pACYC184; open symbols), MJ57(pACYC184; solid symbols), and MJ57 (pACYCRsd; crosses). (C) MH513(pACYC184; open symbols) and MH513 (pACYCRsd; solid symbols). (D) MH513(pBAD22A; open symbols) and MH513 (pBF1; solid symbols).

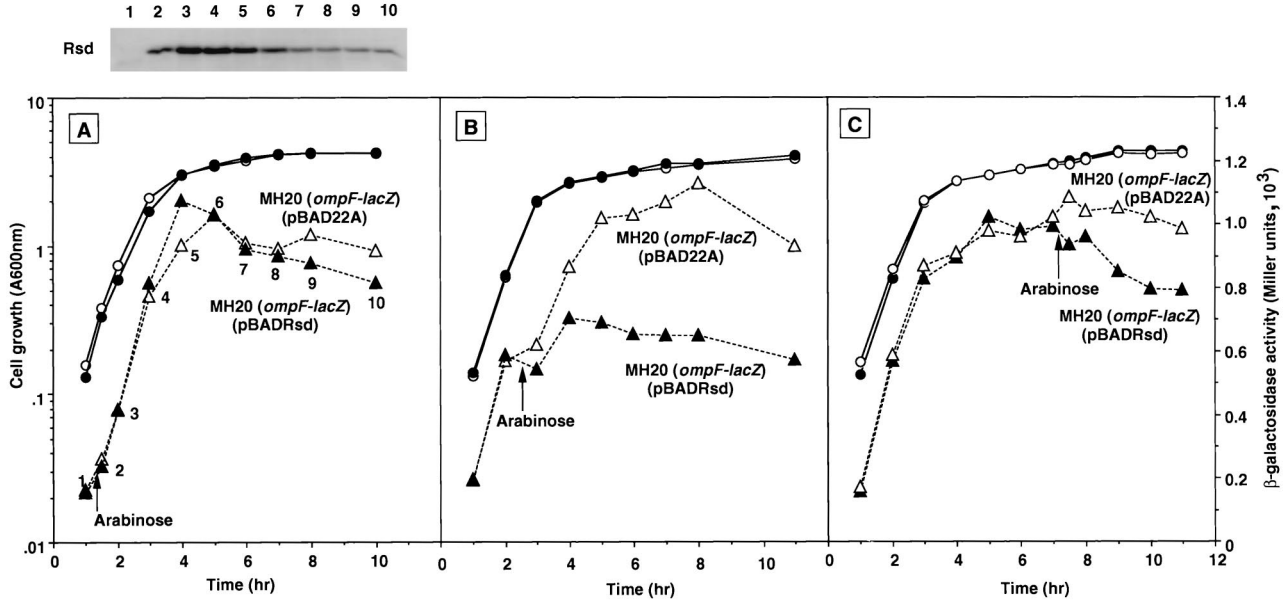


FIG. 5. Effect of Rsd production on *ompF*-directed transcription. Cells of MH513 [MH20 λ(*ompF-lacZ*)] carrying either pBAD22A (open symbols) or pBADRsd (closed symbols) were grown in LB medium. Growth (circles) was monitored by measuring turbidity, while β-galactosidase activity (triangles) was determined at the indicated times. Overproduction of Rsd was induced by adding arabinose (0.02%) at the early exponential phase (A), at the middle of the exponential phase (B), and after entry into the stationary phase (C). Aliquots containing the same cell numbers were subjected to quantitative Western blotting for measurement of the Rsd level.

of *ompF-lacZ* expression increased about twofold compared with that in wild-type cells (Fig. 4A). On the other hand, the expression of *ompF-lacZ* was significantly reduced upon induction of the expression of the σ^S subunit under control of the arabinose-inducible promoter (Fig. 4D). In agreement with our observations, it has been reported that *rpoS* mutations result in overproduction of OmpF (49), whereas the expression of σ^S leads to repression of *ompF* expression (54).

Influence of Rsd expression on σ^{70}-dependent transcription. If the competition in core binding among various σ subunits is so critical for the determination of the global pattern of transcription, the level of σ^{70}-dependent transcription should be influenced by the presence or absence of other minor σ subunits. The influence of the high-level expression of Rsd on σ^{70}-dependent *ompF* transcription was examined in the presence and absence of the σ^S subunit. The high-level expression of Rsd protein could be achieved by inserting the *rsd* gene into an expression vector under the control of the arabinose-inducible promoter. When Rsd was induced in the early exponential phase (in the absence of σ^S), the expression level of *ompF-lacZ* fusion, as measured by β-galactosidase activity, was essentially the same as that in the absence of Rsd induction (Fig. 5A). On the other hand, the expression of Rsd in the late exponential phase or the early stationary phase (in the presence of σ^S) significantly inhibited the expression of the *ompF-lacZ* fusion, down to about 60% of the level in the absence of Rsd expression (Fig. 5B). In the late stationary phase, the inhibitory effect of Rsd on the *ompF-lacZ* induction again became weaker, giving 80% of the activity without Rsd expression (Fig. 5C).

The fluctuation of the inhibition level of *ompF-lacZ* expression by the expressed Rsd could be observed if the level of Rsd expression is altered, depending on the cell growth phase. By checking the expression level of plasmid-encoded Rsd by quantitative Western blotting, however, the level of Rsd expression was found to stay almost constant, at least during the growth transition period analyzed in this study (data not shown). Since the pattern of growth phase-dependent variation in the inhibition of *ompF-lacZ* expression by the overexpressed Rsd correlates with the change in σ^S production level, we assumed that the effect of Rsd is observed only in the situation in which the σ^S subunit is present for competition with σ^{70} in binding to the core enzyme. This is consistent with the expectation deduced from our proposal that the presence of functional σ^S represses the expression of σ^{70}-dependent genes such as *ompF*.

DISCUSSION

The *rsd* gene was found to carry two transcriptional start sites, *rsd*P1 and *rsd*P2. Transcription from the upstream P1 promoter is dependent on σ^S, while the downstream P2 is driven by σ^{70}. The sequence of P2 shows a strong similarity to that of the gearbox promoters, such as *bolA*P1 and *ftsQ*P1, which are both induced in the stationary phase (2, 3, 45) and are transcribed by the $E\sigma^S$ holoenzyme (5, 6, 41). On the basis of sequence similarity and unique expression patterns, such as the inverse relationship with the growth rate (Fig. 2C), the *rsd*P2 promoter can be classified as a member of the gearbox family promoters, but it cannot be transcribed by $E\sigma^S$ in vivo (Fig. 1C) and in vitro (Fig. 1D). The sequence TGGCATGT of the *rsd*P2 promoter −10 region is slightly different from the gearbox consensus sequence, CGGCAAGT (Fig. 1G). The gearbox promoter for *mcb* also does not require σ^S for its stationary-phase induction (6, 42), but instead, the σ^{70}-dependent *mcb* transcription requires transcription factors such as OmpR and EmrR (45). Thus, the possibility should be tested that an as yet unidentified transcription factor(s) is involved in σ^{70}-dependent transcription from *rsd*P2. The mediator ppGpp for stringent control is one candidate for the transcription factor for transcription activation from the *rsd*P2 promoter (see below).

Even though the *rsd* gene carries the σ^S-dependent P1 and σ^{70}-dependent P2 promoters, the contribution of σ^S in the expression of *rsd* seems to be transient for a short period

during the transition from exponential growth to the stationary phase (Fig. 2A and 5). Under certain circumstances, the upstream sequence including the P1 promoter exerts an inhibitory effect on the P2 function, because the deletion of the P1 region resulted in an increase in transcription from the downstream P2 promoter (Fig. 2B). The mechanism of inhibition by the upstream sequence remains unsolved.

The expression of several genes encoding stationary-phase proteins or stress-response proteins, including *rmf* (63), *cspD* (64), *lrp* (41), and *sspA* (61), shows an inverse relationship to the growth rate, even though the promoters of these genes do not share a common sequence. The *rmf* gene encoding a protein associated with 100S ribosome dimers is expressed in the stationary phase, but does not require σ^S (63). Disruption of *rmf* results in loss of the formation of ribosome dimers and reduction in cell viability during the stationary phase (63). A stationary-phase protein, CspD, with the predicted function of an RNA and/or DNA chaperone has a high level of sequence similarity to the cold shock protein CspA, but *cspD* expression is not induced by cold shock (64). Stringent starvation protein A (SspA) is one of the RNA polymerase-associated proteins (28). The synthesis of SspA is induced by starvation for glucose, nitrogen, phosphate, or amino acids (61). Lrp is also induced under starvation conditions and plays an enhancing role in transcription of some stress-response genes (41). The stress-response genes under the control of gearbox promoters are also inversely proportional to the growth rate. Detailed analysis is needed to define the promoter element(s) that is present in these promoters, including *rsd*P2, and is critical for the 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 (8). Direct interaction of ppGpp with the RNA polymerase has been demonstrated both in vitro (11, 55) and in vivo (8, 23). The ppGpp-associated RNA polymerase loses transcription activity of the growth-related genes such as those for rRNA, ribosomal proteins, and tRNA. The promoter activity of the genes, *rsd* (this study), *cspD* (64), *lrp* (41), and *sspA* (61), all showing an inverse relationship with the growth rate, is positively regulated by ppGpp, suggesting that the putative DNA signal must be recognized by the ppGpp-bound RNA polymerase. At present, however, an indirect effect of ppGpp has not been excluded.

To gain insight into the function of Rsd in vivo, we constructed an *rsd* null mutant strain. However, the mutant showed apparently no distinct phenotype from the wild-type parental strain, as analyzed by measuring its growth curve and its viability in various media. Several lines of evidence, however, indicated that the variation in Rsd level influenced the level of σ^{70}- and σ^S-dependent transcription of at least some specific genes. (i) The level of σ^{70}-dependent *ompF-lacZ* and σ^S-dependent *bolA-lacZ* fusions increases and decreases, respectively, in the absence of Rsd (Fig. 3), but (ii) the expression of *ompF-lacZ* and *bolA-lacZ* decreases and increases, respectively, in the presence of overexpression of Rsd (Fig. 4).

The intracellular concentration of the RNA polymerase core enzyme stays constant (25, 29), while the levels of seven species of the σ subunit vary, depending on the rate and phase of cell growth (see references 30 and 34 and also reviewed in references 26 and 27). In exponentially growing *E. coli* cells, only three species of the σ subunit, σ^{70}, σ^N, and σ^F, are present at detectable levels (34), but upon entry into the stationary phase, the levels of both σ^S and σ^H increase markedly, while the levels of the other σ do not change significantly (30, 34). Under the steady state of cell growth, the vast majority of core enzyme is associated with the nucleoid and is involved in the dynamic cycle of transcription (29, 53, 57). The level of free core enzyme, not involved in transcription, is considered to be 10 to 30% of the total number of RNA polymerase molecules (29). Thus, a competition must take place between the seven species of the σ subunit for binding to a fixed number of core enzyme molecules. 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 $E\sigma^S$ holoenzymes. The prediction is supported by the observations that (i) the reduction of σ^S-dependent gene expression by the *rsd* null mutation is suppressed by the expression of σ^S (Fig. 3D), (ii) overexpression of σ^{70} results in a reduction in σ^S-dependent transcription (15), and (iii) an *rpoS* mutant strain exhibits increased expression of the σ^{70}-dependent genes (15, 54). Similar situations have been observed between σ^{70} and σ^H (σ^{32}). For instance, the decrease in the intracellular level of σ^{70} results in superinduction of σ^H-dependent genes (51). On the other hand, overexpression of σ^{70} leads to a reduction in the expression of some σ^H-dependent genes (65).

The inhibitory effect of σ^{70}-dependent transcription by the overexpressed Rsd was observed only at certain stages of the cell growth, although the levels of Rsd expression were not much different between the early and the late exponential phases (see the Western blot pattern above Fig. 5A). Possible mechanisms to explain the apparent lack of inhibitory effect of Rsd on the σ^{70} function in the early exponential growth phase include the following. (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 that of the σ^S subunit (38). However, transcription in vitro by the $E\sigma^S$ holoenzyme is markedly enhanced in the presence of high concentrations of glutamate (13), trehalose (37), and polyphosphate (38). (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. (iv) It has also not been excluded that an additional factor present only in the stationary-phase cells is required for Rsd binding to σ^{70}.

A number of anti-σ factors have been discovered in both *Bacillus subtilis* and *E. coli* (for a review, see reference 24). The T4 AsiA protein binds to the *E. coli* σ^{70} subunit and inhibits σ^{70}-dependent transcription, but AsiA is a positive factor for transcription of the middle class of T4 genes (52). *E. coli* FlgM can interact with $E\sigma^F$ and induces the dissociation of σ^F from the core enzyme (9). 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 (24). 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 (24). Rsd has 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 regulatory protein which promotes σ switching from σ^{70} to other minor σ subunits under stress conditions.

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