RpoS-dependent regulation of genes expressed at late stationary phase in *Escherichia coli*

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Received 6 March 1996; revised version received 23 March 1996

Abstract **We have identified 6 *Escherichia coli* genomic genes, including 4 new genes, responsive to the stationary phase. One of them was regulated positively by RpoS at the stationary phase, and the remaining 5 negatively at a late stationary phase, all of them responding to multiple environmental stresses. Nucleotide sequences as well as such multiple responses revealed that those genes may have more than one overlapping-promoter recognized by different σ-factors which regulate gene expressions during their cell growth.**

Key words: Global expressional regulation; *E. coli* gene; Late stationary phase

1. Introduction

As bacterial cells reach stationary phase, a drastic change is seen in the cell physiology, where distinct sets of synthesizing proteins appear to be directed against various environmental stresses such as acidic pH, oxidative stress, and anaerobiosis [1-3]. With the exact mechanisms of adaptation to the stresses not well known, the cells appear to survive in spite of the nutrient limitation, presumably by use of proteins proceeding from the recycling, refolding, or repairing of the proteins damaged by the stresses [4]. Many of these reused proteins, such as heat shock proteins, GroEL, DnaK, and HtpG, are synthesized predominantly during the stationary phase [2].

Sigma factor σ^s, encoded by *rpoS* or *katF* as a subunit of RNA polymerase, is known to direct the global regulatory network in *Escherichia coli*, controlling gene expression under various conditions including heat shock, osmotic shock, nutrient limitation, and stationary phase [5-7]. In this network, σ^{32}, σ^{70}, and a gearbox-binding protein that remains to be identified may also be involved [8], and many genes seem to be under the control of more than one σ-dependent promoter [9]. Such control is not only seen in *E. coli* but also in several bacteria [10-15].

After a promoter library constructed previously [16] was examined under various conditions, the expression of many genes was found to be affected by different environmental stresses (Talukder, Yanai, Kato and Yamada, submitted). Here, we characterized 6 genes responsive to the stationary phase of *E. coli*. Moreover, partial nucleotide sequences of the genes were determined, defining their first, possible promoter sequences and further their genomic locations and functions.

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2. Materials and methods

2.1. Bacterial strains and plasmids

The *E. coli* K-12 strains used in this study were MC1000, *araD139 Δ(ara-leu)7697 ΔlacX74 galU galK rpsL* [17]; JM103, *Δ(lac-pro) thi strA supE endA sbcB hspR4* F′ *traD36 proAB lacI*^{q}*ZΔM15* [18]; UM122, *rpoS (rpoS*::Tn*10)* provided by Akira Ishihama. YU381, MC1000 *rpoS* was constructed from UM122 by P1 transduction [19]. In a promoter library [16], each clone has a *Sau*3AI-digested *E. coli* W3110 genomic DNA fragment inserted into the *Bam*HI site in front of the promoter-less *'lacZ* gene on pMC1396, which produces a fusion protein between a genomic gene and LacZ under the promoter of the genomic gene.

2.2. DNA manipulations, sequencing, and gene mapping

Conventional recombinant DNA techniques [20] were used. The restriction enzymes, T4 DNA ligase, Taq DNA polymerase, and the DNA sequencing kit were purchased from Takara Shuzo (Kyoto, Japan). DNA fragments from each clone were prepared by digesting with the restriction enzymes or by polymerase chain reaction [21] and used for DNA sequencing [22] and determining the genomic location of the gene. The *E. coli* ordered phage library [23] was hybridized with the fragments as a probe, and the detection was carried out using the ECL kit (Amersham, Bucks, UK). For homology searches we used the EMBL, GenBank, SWISS-PROT, and NBRF-PIR databases in GENETYX (Software Development, Tokyo, Japan).

2.3. Bacterial growth and enzyme assay

The cells harboring a plasmid clone were grown to a late exponential phase at 37°C in LB medium (1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl) containing ampicillin (100 μg/ml). The cells were then diluted 1000-fold in fresh LB medium and grown at 37°C for time course experiments. At the time indicated, part of the cells was taken and diluted to 0.28–0.80 of OD_{600}. To prevent further growth, the sample was then cooled, immersed in an ice bucket, and then it was subjected to β-galactosidase assay [19].

3. Results

3.1. Screening of genes regulated expressionally at stationary phase and their expression along with cell growth in proS background

To identify the genes whose expression was regulated at stationary phase, we screened the *lacZ*-fused promoter library consisting of 84 independent clones by measuring β-galactosidase activity along with cell growth. As a result, it was found that 6 clones showed significantly changed activities at the stationary phase (24–48 h): the activities increasing about 4-fold in the case of pYU12, and decreasing 1.5–2-fold in the case of pYU3, pYU7, pYU16, pYU92, and pYU102, compared with the rest of the clones. These 6 clones were thus tested after having been introduced into a *rpoS* mutant strain.

The time course profiles of β-galactosidase activities and the cell growth from the representative clones are shown in Fig. 1; the profiles of pYU7, pYU16, and pYU102 were similar to that of pYU3 (data not shown). β-Galactosidase activity of

PII S0014-5793(96)00426-7

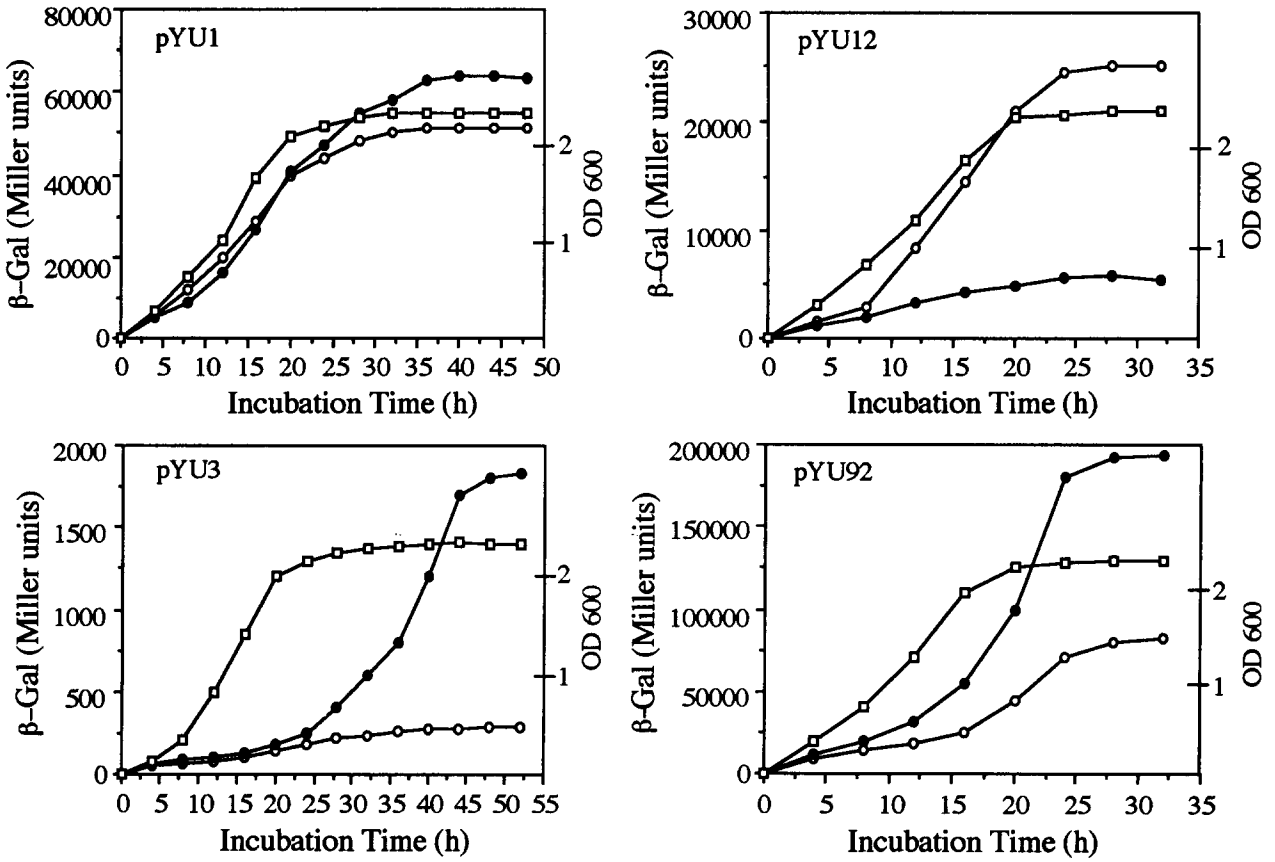

Fig. 1. The effect of *rpoS* mutation on the expression of the genes in 6 pYU clones at the stationary phase. The cells were grown aerobically at 37°C for the indicated times, and β-galactosidase activities of MC1000 (open circles) or isogenic *rpoS* (closed circles) cells harboring each pYU clone were measured. Other growth conditions and measurement of β-galactosidase activity are given in section 2. Growth curves of the cells are represented by rectangles. Incubation time (h), β-galactosidase activity (Miller units), and optical density of the cells are shown on the x-, left y- and right y-axis, respectively. Plasmid pYU1 is a control clone which was not responsive to the stationary phase.

rpoS cells harboring pYU12 was reduced about 4-fold at the stationary phase (24 h) compared with the activities of the wild type cells harboring the same plasmid (Fig. 1 and Table 1). The activities of the other clones except pYU92 were elevated 3–6-fold in the *rpoS* background and were maximal at the late stationary phase (48 h). RpoS thus appears to give either a positive or a negative effect on expression of these genes. The ratio of negative regulation may be not surprisingly high because repression of many genes took place at the stationary phase, accompanying the induction of other specific genes [24]. The activities of pYU3, pYU7, pYU16, and pYU102 were found to be the same in both *rpoS*$^+$ and *rpoS* backgrounds until 24 h, and at 48 h to increase in *rpoS* 3–6 times as much as in *rpoS*$^+$ (Fig. 1 and Table 1), whereas in the case of *rpoS* cells harboring pYU92 was found to be maximally active at 24 h.

3.2. Database searches, genomic location, and promoter of cloned genomic genes

In order to identify the reading frame of the genomic gene

Table 1
Effect of *rpoS* mutation on expression of the *lacZ* fusion genes

Clone	β-Galactosidase activity (Miller units)[a]					
	24 h culture			48 h culture		
	rpoS$^+$	*rpoS*	(%)	*rpoS*$^+$	*rpoS*	(%)
pYU1	24000	26000	110	50000	65000	130
pYU3	170	160	90	370	2000	540
pYU7	10000	11000	110	20000	68000	340
pYU12	24000	6000	25	16000	5000	30
pYU16	400	450	113	300	1500	500
pYU92	72000	190000	260	60000	95000	16
pYU102	110	150	140	180	1100	610

[a]The wild type and *rpoS* cells having each fusion plasmid were grown to late exponential phase at 37°C in LB medium and were then diluted 1000-fold into fresh LB medium and grown until 24 h (stationary phase) or 48 h (late stationary phase) at 37°C under shaking condition. β-Galactosidase activity was measured by the Miller method [19], and the ratio of *rpoS*/*rpoS*$^+$ is expressed as a percentage. Plasmid pYU1 is a control clone which was not responsive to stationary phase.

Table 2
Regulation and map position of the genomic genes responsive to *rpoS* mutation

Clone	Response[a]	Map position (min)	Kohara clone	Gene[b]	Reference
pYU3	(HS, GS, SF)	65.0	6A1, 5B4	*orf-0464*	U28375[c]
pYU7	(GS, SF)	28.6	4F1, 13F9	*sohB*	[34]
pYU12	(GS, SF)	95.3	E1H3	unknown	
pYU16	(AS, SF)	40.6	12B3	*sdaA*	[25]
pYU92	(PS, GS, SF)	87.2	8D12, 10H11	*orf-0169*	[35]
pYU102	(HS, OS, SF)	91.1	1F8, 12B4	unknown	

[a]HS, heat shock; GS, glucose starvation; OS, osmotic shock; SF, stationary phase; AS, ammonium starvation; PS, phosphate starvation (data submitted, Talukder, Yanai, Kato and Yamada).
[b]The *sohB* and *sdaA* genes encode periplasmic protease and L-serine deaminase, respectively. The nucleotide sequences around the genes on pYU12 and pYU102 had been determined by genomic sequencing, but their open reading frames were not reported. *orf-0464* and *orf-0169* had been predicted from genomic sequencing but their functions were not known.
[c]GenBank accession number.

in the 6 clones as well as its product and function, the nucleotide sequences were determined of part of the genes including the junction with *lacZ*. The sequences and the deduced amino acid sequences of their products were compared with those listed in databases (Table 2). Regarding the 6 genes, 2 were found to be functionally known and 4 are still unknown. Interestingly, the ratio of the functionally known genes to the total genes analyzed (2:6) appears to be low. This may be due to the previous lack of characterization of genes expressed preferentially at the stationary phase. The gene in pYU16 was found to be *sdaA*, which encodes L-serine deaminase, and its activity was shown to increase at the stationary phase, the maximum activity of the *sdaA* in the mutant strain being about 14-fold higher than in the wild type [25]. Moreover, the genomic positions of the 6 genes were determined when they were hybridized with the Kohara ordered clones (Table 2), and the positions were consistent with those determined previously.

On the basis of the evidence that the clones responded to the stationary phase (Table 1), possible gearbox-binding sequences as well as −10 hexamer TATACT [26], a putative σ^s sequence, were found at the 5′-flanking regions of the genes in the 6 clones (Fig. 2). The underlined two bases in the hexamer are also highly conserved among the −10 sequences of the σ^{70}-dependent promoters (see Section 4). Those genes were also responsive to other stresses including heat shock, glucose starvation, osmotic shock, ammonium starvation, or phosphate starvation, and some of the sequences related to the stresses were predicted (Talukder, Yanai, Kato and Yamada, submitted).

4. Discussion

From a library consisting of about 10% random *E. coli* genomic promoters [16], 6 genes were found to be expressionally responsive to the stationary phase. Of them, 1 gene appeared to be regulated positively and 5 negatively by RpoS. Despite this dual regulation by RpoS, which has been reported before [27], the detailed mechanism of the negative regulation has not been elucidated. Notably, the negative regulation seemed to stand out remarkably at a late stationary phase (Table 1 and Fig. 1). In the fusion genes on pYU3, pYU7, pYU16, and pYU102, β-galactosidase activity increased only several hours after the stationary phase and

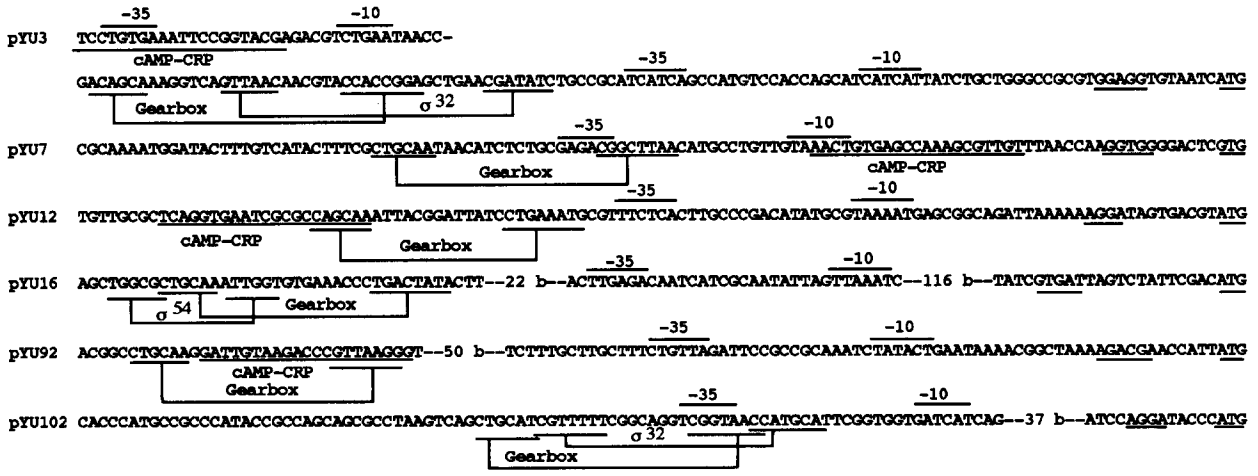

Fig. 2. Partial nucleotide sequences at the 5′-flanking regions of the cloned genomic genes and putative binding sites of factors related to gene expression. In each gene, a putative initiation codon with a sequence homologous to the ribosome-binding sequence at an appropriate distance was predicted before the open reading frame, and a sequence homologous to the σ^{70} promoter sequence [19] was explored. Since the clones responded to multiple stresses (Table 2), sequences homologous to the σ^{32} promoter [19], cAMP-CRP binding [36], σ^{54} promoter sequence [37], gearbox promoter [8], or −10 hexamer (TATACT), possible σ^s-binding consensus sequence [31], were also explored. Overlines and underlines represent putative σ^{70}-dependent promoter sequences and cAMP-CRP binding sequences, respectively. Another two underlines at the right side show possible ribosome-binding sequences and initiation codons. Putative gearbox, σ^{32}, and σ^{54} promoter sequences are represented by underlines with brackets.

was maximal at the late stationary phase (48 h). Such expression patterns have recently been reported to appear in the stationary phase dependent promoters of *bolA* and *fic* [28].

There are several possible mechanisms for the RpoS-related negative regulation. RpoS functions as a σ factor for RNA polymerase, so the negative regulation may function through expressing cognate repressor genes. Alternatively, RpoS may be able to bind DNA without the associated core polymerase as has been shown for RpoN [29], and function directly as a repressor. Another possibility is that the elevated level of RpoS at the stationary phase may result in competition of RpoS and either RpoD (σ^{70}) or other σ factors for binding to RNA polymerase core molecules, whose intracellular level is relatively constant throughout cell growth [30].

The 6 genes responsive to the stationary phase were also found to be sensitive to more than one treatment (Table 2). The nucleotide sequences revealed that the genes may be transcribed by several σ-RNA polymerase core complexes: 2 of them may depend on σ^{32} (encoded by *rpoH*) and σ^s, and 4 on σ^{70}, and σ^s. Such involvement of different σ factors, cAMP-CRP complex, or an unidentified gearbox-binding protein in gene expression at the stationary phase has also been reported previously [8,31]. It may allow genes to be specifically expressed through mutual interactions or competition between the factors.

From mutant analysis, Hiratsu et al. [26] demonstrated that the −10 hexamer, TATACT, may be a σ^s-recognition sequence, especially TATACT. Similarly, the TATACT and TATAAT sequences at the −10 region were reported to be important for promoter recognition by σ^s [32]. In the 6 genes reported here (Fig. 2), the −10 sequences are consistent with the sequences above. The hexamer (TATACT) may therefore be a consensus sequence for σ^s which is similar to that of the major σ factor, σ^{70} [33].

Acknowledgements: We thank Drs. O. Adachi and K. Matsushita for their helpful discussion. We also thank Drs. Y. Kohara, T. Yura, A. Ishihama, and J. Beckwith for providing us with phage clones or bacterial strains. This research was supported by the Sasakawa Scientific Research Grant from The Japan Science Society and by the Ministry of Education, Science, and Culture of Japan.

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