JOURNAL OF BACTERIOLOGY, Sept. 1995, p. 5222–5231
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology
Vol. 177, No. 18

Fis Activates the RpoS-Dependent Stationary-Phase Expression of *proP* in *Escherichia coli*

JIMIN XU AND REID C. JOHNSON*
Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, California 90095

Received 31 March 1995/Accepted 9 July 1995

Fis is a general nucleoid-associated protein in *Escherichia coli* whose expression is highly regulated with respect to growth conditions. A random collection of transposon-induced *lac* fusions was screened for those which give increased expression in the presence of Fis in order to isolate a ProP-LacZ protein fusion. We find that *proP*, which encodes a low-affinity transporter of the important osmoprotectants proline and glycine betaine, is transcribed from two promoters. *proP*$_1$ is transiently induced upon subculture and is upregulated by increases in medium osmolarity. As cells enter stationary phase, a second promoter, *proP*$_2$, is strongly induced. This promoter can also be induced by high medium osmolarity in exponential phase. The activity of *proP*$_2$ depends on Fis and the stationary-phase sigma factor σ^s. In the presence of Fis, *proP*$_2$ expression is increased over 50-fold, as judged by the LacZ activity of cells carrying the *proP-lacZ* fusion as well as by direct RNA analysis, making this the most strongly activated promoter by Fis that has been described. Two Fis binding sites centered at positions −41 (site I) and −81 (site II) with respect to the transcription initiation site of P2 have been defined by DNase I footprinting. Mutations in site I largely abolish stationary-phase activation, while mutations at site II have a minor effect, suggesting that direct binding of Fis to site I is important for Fis-mediated activation of this promoter. In addition to Fis and σ^s, sequences located over 108 bp upstream of the *proP*$_2$ transcription initiation site are required for efficient expression.

Fis is a small basic DNA-binding protein which belongs to the family of general nucleoid-associated proteins. It binds DNA as a dimer with moderate specificity and bends DNA to different extents, depending on the target sequence (14, 42). The expression of Fis varies enormously with respect to growth conditions (4, 39, 40, 51). Fis levels in cells maintained in stationary phase are very low, but upon a nutrient upshift, levels rapidly increase. In rich media, levels of 25,000 to 50,000 dimers per cell are obtained, making Fis one of the most abundant DNA-binding proteins. Peak levels in poorer growth media, however, are lower and approximately correlate with the growth rate. In standard batch cultures, Fis expression is turned off in mid-exponential phase and levels decline as a function of cell division. While high Fis expression in exponential phase has a modest enhancing effect on cell growth under certain conditions, the shutoff in stationary phase is critical for efficient long-term survival (41).

Fis has been recruited to function in many different biological contexts. Fis was originally identified because of its role in regulating site-specific DNA recombination by the DNA invertases (20, 23). In these reactions, it binds to a recombinational enhancer sequence and both promotes the assembly of the correct synaptic complex and activates the recombinase (19). Fis has also been shown to enhance *oriC*-directed replication (13, 16) and to regulate transcription (37, 38, 44, 46, 54, 55).

Most of the genes that have previously been shown to be regulated by Fis are expressed optimally in exponential phase. These include genes whose transcription is enhanced by Fis and that are expressed in high amounts under rapid growth conditions, such as genes encoding rRNAs, certain tRNAs, and translation factors (10, 37, 38, 44, 46). Fis has also been shown to negatively autoregulate its own synthesis (4, 40). We recently initiated a project to identify other genes controlled by Fis. Twelve additional genes whose expression was inhibited by Fis were identified (54). These included not only genes expressed in exponential phase but also genes expressed maximally in stationary phase. Many of these Fis-regulated genes were positively or negatively controlled by *rpoS*, which encodes a sigma factor (34, 36, 50) that directs expression of a number of stationary-phase genes (for a review, see references 24 and 26).

In this study, we identify another gene, *proP*, that is positively regulated by Fis. *proP* encodes a transporter of proline and glycine betaine, which play an important role in protecting cells from osmotic stress (for a review, see reference 11). Unlike other Fis-activated genes, we found that *proP* expression is virtually dependent on Fis and is maximally expressed in stationary phase under batch culturing conditions in Luria-Bertani (LB) broth. Thus, it was of interest to determine whether Fis stimulated *proP* expression directly or by an indirect mechanism. We find that *proP* expression is regulated by two promoters, with Fis being required for the expression of a σ^s-dependent stationary-phase promoter. Evidence that Fis activates this promoter primarily by binding to a site centered at position −41 with respect to its site of RNA chain initiation is provided.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains used in this study are listed in Table 1. Cells were cultured in LB broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) or M9 salts with 0.4% glycerol as a carbon source (31). In some experiments, cells were grown in LB broth without added NaCl (LBN). Antibiotics were used at the following concentrations in solid and liquid media: ampicillin, 100 μg/ml; kanamycin, 20 μg/ml; and tetracycline, 10 μg/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at a concentration of 40 μg/ml.

General molecular and genetic techniques. P1*vir* transduction and bacterial conjugation were performed according to the protocols described by Miller (31). Standard recombinant DNA methods including plasmid isolation, restriction digestion, gel electrophoresis, DNA ligation, and transformation were performed

* Corresponding author. Mailing address: Department of Biological Chemistry, UCLA School of Medicine, 10833 Le Conte Ave., Los Angeles, CA 90095-1737. Phone: (310) 825-7800. Fax: (310) 206-5272.

Downloaded from http://jb.asm.org/ on September 18, 2019 by guest

TABLE 1. Bacterial strains used

Strain	Relevant characteristics	Source or reference
CAG4000	MG1655 Δ*lacX74*	C. Gross
RJ1801	CAG4000 *fis-985*	4
RJ4000	RJ1801 pRJ823 pRJ4000	54
RJ4015	RJ4000 *proP-104*::Tn*phoA'-4*	This study
RJ4067	CAG4000 *proP-104*::Tn*phoA'-4*	This study
RJ4068	RJ1801 *proP-104*::Tn*phoA'-4*	This study
RJ4099	RJ4067 *katF13*::Tn*10*	This study
RJ4111	CAG4000 *katF13*::Tn*10*	This study
RJ4143	CAG4000 *proP-104*::Tn*phoA'-3*	This study
RJ4145	RJ1801 *proP-104*::Tn*phoA'-3*	This study
RJ4335	CAG4000 λ*proP-104Δ2*	This study
RJ4337	CAG4000 λ*proP-104Δ3*	This study
RJ4342	CAG4000 λ*proP-104Δ1*	This study
RJ4387	RJ4143 *katF13*::Tn*10*	This study
UM122	HfrH *thi-1 katF13*::Tn*10*	35

as described previously (45). Restriction fragments used for cloning were separated in low-melting-point agarose gels (SeaPlaque GTG; FMC BioProducts) and were extracted from the gels as described previously (45). A DNA PolI Klenow fragment (Promega) was used to make blunt ends for cloning as required. Plasmid DNAs used for DNA sequencing were isolated with a Qiagen plasmid isolation kit. DNA sequencing was performed with Sequenase version 2.0 (Amersham) as recommended by the manufacturer. Sequences were analyzed with the programs of the University of Wisconsin Genetics Computer Group, and homology searches were performed with Blastmail (1).

Isolation of LacZ protein fusions whose expression is stimulated by Fis. The procedure used to identify genes regulated by Fis was described previously in detail (54). Briefly, a random collection of LacZ protein fusions to chromosomal genes was generated by infecting *Escherichia coli* K-12 strain RJ4000 *fis-985*(pRJ4000,pRJ823) with phage λTn*phoA'-4* (52). pRJ4000 (Ap^r) contains *fis* under *lacP* control on a pBR322-derived plasmid (54), and pRJ823 (Tc^r) is a derivative of pACYC184 containing the *lacI*^{q} gene. Kanamycin-resistant colonies that were more intense blue in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside) than in its absence were chosen as potential Fis-activated fusions for further study. Recombinational switching from a ProP-LacZ protein fusion (*proP104*::Tn*phoA'-4*) to an operon fusion (*proP104*::Tn*phoA'-3*) was done by a two-step protocol as described previously (52). The *proP-104* fusion was initially cloned onto a mini-Mu cloning vector, pWM11, to give pRJ4030 by the in vivo mini-Mu cloning method (18, 52). This plasmid was used to determine the DNA sequence of the fusion joint and was the starting plasmid for further subcloning.

Additional plasmid and λ constructions. pRJ4031 contains a 0.6-kb *Xho*I-*Bam*HI fragment from pRJ4030 that was cloned into the *Sal*I and *Bam*HI sites of pUC18. This fragment carries the *proP-104–lacZ* fusion junction sequence and the *proP* regulatory sequence from nucleotides (nt) −295 to +127 with respect to the AUG initiation codon (unless otherwise specified, this numbering system is used throughout the text; see Fig. 9B). The promoter region from nt +14 to −295 was further subcloned by PCR to give pRJ4034, which was used for the in vitro Fis binding assays. The PCR was performed with primer JX8 (5′-CTGAAT TCCTTTTCAGCATAGCTTTCCTC-3′, which extends from nt +14 to −10, with five bases added to the end of the oligonucleotide to give an *Eco*RI site at one end of the PCR product) and a *lac* universal sequencing primer (−40) together with pRJ4031 as the template. After digestion with *Eco*RI and *Hin*dIII, the PCR product was cloned into the *Eco*RI and *Hin*dIII sites of pUC18.

The *proP-lacZ* fusion sequence carried by pRJ4043 was obtained by PCR amplification with primer JX9 (5′-ACGAATTCAAACACTGGTAGGGTA AAAAG-3′, which extends from nt −211 to −183, with two bases changed [underlined] to construct an *Eco*RI site at one end of the PCR product) and a *lac* reverse primer together with pRJ4031 as the template. After digestion with *Eco*RI and *Bam*HI, the PCR product was cloned into the *Eco*RI and *Bam*HI sites of pUC18 to give plasmid pRJ4043.

pRJ4039 (*proP-104Δ1*), pRJ4047 (*proP-104Δ2*), and pRJ4049 (*proP-104Δ3*) contain the *proP-104–lacZ* fusion with different lengths of regulatory regions extending up to nt −200, −108, and −61, respectively, with respect to the *proP*$_2$ transcription initiation site. pRJ4039 was constructed by cloning a *Sph*I-*Bam*HI fragment from pRJ4031 into the *Eco*RI and *Bam*HI sites of the *lac* protein fusion vector pRS414 (47). pRJ4047 was constructed by cloning an *Eco*RI-*Bam*HI fragment from pRJ4043 into the *Eco*RI and *Bam*HI sites of pRS414. pRJ4049 was constructed by cloning a *Hin*cII-*Bam*HI fragment from pRJ4031 into the *Eco*RI and *Bam*HI sites of pRS414.

The *proP-104–lacZ* fusions carried by plasmids pRJ4039, pRJ4047, pRJ4049, pRJ4045, pRJ4051, and pRJ4055 (see below) were recombined onto λRS45 and lysogenized into CAG4000 as described previously (47). Lysogens expressing the lowest LacZ activities among at least eight independent lysogens were considered as single lysogens and used for further studies.

Site-directed mutagenesis. For site-directed mutagenesis, a two-step PCR method using *Pfu* DNA polymerase (Stratagene) was employed. The template used for the PCR was plasmid pRJ4031. To mutagenize Fis site I, the primers for the first PCR were JX13 (5′-CGGTTAAGGAAGATCTAATCAAACCAGT TGACGC-3′) and a *lac* universal primer (−40). The mutagenic primer is complementary to nt −128 to −161 and contains four mutations (underlined) at positions 1, 2, 4, and 5 in the Fis core recognition sequence (14). The sequence was designed to change all the conserved nucleotides at the left half-site and create a *Bgl*II restriction site. The resulting PCR product was then used together with a *lac* reverse primer for the second PCR. The final PCR product was then cut with *Sph*I and *Bam*HI and cloned between *Eco*RI and *Bam*HI in pRS414 to give plasmid pRJ4051. The fragment was recloned from pRJ4051 into pUC18, resulting in pRJ4052, to facilitate DNA sequencing and subsequently cloning. The introduction of mutations into Fis site II was performed in the same manner as for site I. The mutagenic primer was JX10 (5′-CTGGTAGGGTAAAAAC CGAATTCACTGCCCAATTCAGG-3′, extending from nt −198 to −161 and including five mutations, as underlined, at positions 1, 2, 3, 4, and 8 of the Fis core recognition sequence), and it creates an *Eco*RI site in the PCR product. The final PCR product containing the site II mutations was cut with *Hin*dIII and *Bam*HI and cloned into the corresponding sites of the pUC18 polylinker, resulting in plasmid pRJ4042. Plasmid pRJ4053, which contains both the site I and site II mutations, was obtained by replacing an *Hin*cII-*Bam*HI fragment in pRJ4042 with the same fragment containing the mutations in site I from pRJ4052. pRJ4045 and pRJ4055 were created by cloning the *Sph*I-*Bam*HI fragments of pRJ4042 and pRJ4053 between the *Eco*RI and *Bam*HI sites of pRS414, respectively. All of the expected mutations, along with the sequence of the extended promoter regions, were confirmed by DNA sequencing.

Primer extension and nuclease S1 analysis. Total RNA was prepared from cells growing in LB broth by hot-phenol extraction (8). The primer used in the primer extension reaction was JX8, which is complementary to the coding strand of the *proP* DNA sequence (nt +14 to −10). The primer was end labeled with [γ-^{32}P]ATP (6,000 Ci/mmol; Amersham) by using T4 polynucleotide kinase (Stratagene) and hybridized to 10 μg of total RNA. The extension reactions were performed at 45°C for 1 h with 3 U of avian myeloblastosis virus reverse transcriptase (Promega) as described previously (8). The probe for nuclease S1 analysis was a 307-base-long, single-stranded DNA probe from nt +12 to −295. It was generated by extending ^{32}P-end-labeled primer JX14 (5′-CCTTTTCAG CATAGCTTTCCTC-3′) with pRJ4031 that had been digested with *Hin*dIII as the template. S1 mapping was performed as described previously (45). DNA sequence reference ladders for the primer extension and nuclease S1 analysis were generated with the same 5′-end-labeled primers for each individual experiment.

Gel retardation assay and DNase I footprinting. The labeled DNA fragment used for the gel retardation assay was obtained by digesting pRJ4034 with *Eco*RI and filling in the protruding ends with Klenow and [α-^{32}P]dATP. After further digestion with *Hin*dIII, the 330-bp fragment, including 21-bp flanking sequences from the polylinker of pUC18, was purified by polyacrylamide gel electrophoresis. Binding reactions were performed by incubating the labeled DNA fragments with various amounts of purified Fis protein in a 20-μl solution consisting of 20 mM Tris-HCl (pH 7.5), 80 mM NaCl, 1 mM EDTA, and 2 μg of sonicated salmon sperm DNA (6). Following incubation at room temperature for 10 min, 5 μl of gel loading buffer (20 mM Tris-HCl [pH 7.5], 80 mM NaCl, 10 mM EDTA, 100 μg of sonicated salmon sperm DNA per ml, 7.5% Ficoll, and 0.1% bromophenol blue) was added and the mixtures were electrophoresed in native 8% polyacrylamide gels (60:1) in Tris-borate-EDTA buffer at room temperature. The gels were then dried in a vacuum and autoradiographed.

Fis binding sites at the *proP* regulatory region were identified by DNase I footprinting experiments as described previously (6, 55). The 330-bp *Eco*RI-*Hin*dIII fragment from pRJ4034 was uniquely end labeled either on the coding strand by filling in the *Eco*RI site or on the noncoding strand by filling in the *Hin*dIII site as described above. DNA sequencing specific for GA nucleotides was performed as described previously (28).

β-Galactosidase activity assays. Fourteen-hour overnight cultures in LB broth were diluted 1/100 into 5 ml of LB broth or other medium as specified in the text with appropriate antibiotics and grown at 37°C with shaking. β-Galactosidase activities were assayed at least in duplicate and expressed as Miller units (31). The data shown in each figure are a representative set of at least two separate experiments, and the data presented in the tables represent the means and standard deviations from at least three individual cultures.

RESULTS

Identification of *proP* as a Fis-activated locus. Transposon Tn*phoA'-4* was used to obtain chromosomal LacZ protein fusions as described in Materials and Methods. Fusions whose expression was activated by Fis were initially identified in strain RJ4000, which contains *fis* under the control of the *lacUV5* promoter. Fusion 104 gave as much as a 15-fold greater β-galactosidase activity in the presence of IPTG (Fis^+) than in the absence of IPTG (Fis^-) when grown in LB broth (Fig. 1).

Downloaded from http://jb.asm.org/ on September 18, 2019 by guest

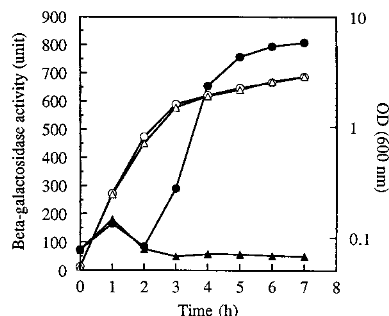


FIG. 1. Effect of *lacP*-controlled Fis on the expression of *proP-104*. Overnight cultures of RJ4015 grown in the absence of IPTG were diluted (1/100) into LB medium containing kanamycin, tetracycline, and ampicillin with (circles) and without (triangles) 1 mM IPTG and incubated at 37°C with agitation. Samples were removed at 1-h intervals, and cell density values (open symbols) and β-galactosidase activities (solid symbols) were measured. In RJ4015, the *fis* gene is carried by plasmid pRJ4000, and because it is under the control of the *lacPUV5* promoter, it is induced by the addition of IPTG. In the presence of IPTG, Fis levels produced by RJ4015 are slightly less than wild-type peak levels (54). OD, optical density.

Thus, fusion 104 identifies a gene that is directly or indirectly activated by Fis.

Fusion 104 was mapped on the *E. coli* chromosome by Hfr crosses and then by P1 transduction with the ordered transposon set of Singer et al. (48). The Tn*phoA'-4* transposon was found to be 44 and 52% cotransducible with *zjd-2231*::Tn*10* and *zje-2241*::Tn*10*, respectively, indicating that it is located at 94 min on the *E. coli* chromosome. To identify the gene containing the transposon insertion, the fusion junction was cloned onto a plasmid by the in vivo mini-Mu procedure and sequenced. The transposon was found to generate an in-frame *lacZ* fusion at codon 43 of *proP* (see Fig. 9B) which encodes a proline and glycine betaine transporter (12). This fusion is therefore referred to as *proP-104.*

Role of Fis in growth phase-dependent expression of *proP*. Analysis of *proP-104* LacZ activity levels during the course of growth in LB broth indicates that *proP* has a distinct two-phased expression pattern. In the absence of Fis (RJ4015 grown without IPTG) (Fig. 1), LacZ levels peaked 1 h after subculturing (phase I expression) and then decreased to a low basal level throughout the rest of the growth cycle. In *fis*$^{+}$ cells (RJ4015 subcultured with 1 mM IPTG), phase I expression in early exponential phase was identical to that in the *fis* mutant cells. However, LacZ levels increased sharply after 2 h of growth in the presence of Fis and reached their peak between 6 and 7 h (phase II or stationary-phase expression) (Fig. 1). At this time, the LacZ activity of the *fis*$^{+}$ cells was about 15-fold greater than that of the *fis* mutant cells.

The results shown in Fig. 1 were obtained under conditions in which Fis expression is artificially controlled by the *lac* promoter. In wild-type strains, the expression of *fis* varies tremendously with growth conditions, being maximal in early to mid-exponential phase and low in early stationary phase (4, 39, 40, 51). To test the true physiological effects of Fis on the expression of *proP*, the *proP-104–lacZ* fusion was transduced into *fis*$^{+}$ (CAG4000) and its isogenic *fis* deletion mutant (RJ1801) by P1 transduction. Fourteen-hour cultures of the transductants were subcultured at 1/100 in fresh LB broth, and β-galactosidase activities were assayed over time.

As was observed in the previous experiment, phase II expression is missing from the *fis* mutant RJ4068 (*fis proP-104*::Tn*phoA'-4*), but the *fis*$^{+}$ strain RJ4067 (*proP-104*::Tn*phoA'-4*) shows strong phase II expression (Fig. 2A). Expression from *proP-104* again increased sharply 2 h after the cells were subcultured but reached its peak level at 4 h, at which time there is about a 10-fold difference in LacZ activity between the *fis*$^{+}$ and *fis* mutant cells. Unlike that observed in Fig. 1, in which *fis* was continuously expressed by *lacP*, phase II expression in wild-type *fis* cells decreased after 4 h. This presumably reflects the decreasing cellular levels of Fis at this time in the growth stage. Unlike that reported on in Fig. 1, for which the starting cultures were grown without IPTG to prevent *fis* expression, the phase I expression in the wild-type Fis background could not be clearly detected in this experiment because of the high carryover of β-galactosidase from the starting cultures. We show below by direct RNA assays that this early burst of expression also occurred in wild-type *fis* cells.

The positive effect of Fis on the phase II expression of *proP-104* was found to be even greater when LacZ levels were measured from *fis*$^{+}$ and *fis* mutant cells growing in LBN. In this medium, in which cells grow slower than in standard LB broth, phase I expression, along with Fis-independent basal level expression during later growth stages, is significantly reduced. However, phase II expression in *fis*$^{+}$ cells remained high, with LacZ activity at the peak level being more than 50-fold higher than that of the *fis* mutant cells (Fig. 2B).

The activation of Fis on *proP* phase II expression was also observed when cells were grown in defined medium (M9 supplemented with glycerol), although the magnitude of activation was lower. Under these conditions, about a fivefold difference in LacZ activity between *fis*$^{+}$ and *fis* mutant cells carrying the *proP-104–lacZ* fusion is obtained when cells enter stationary phase (data not shown). This decreased activation could be due to the lower *fis* expression in this medium (3).

Effects of RpoS on the expression of *proP*. RpoS (or KatF) has been shown to be a sigma factor (34, 36, 50) essential for

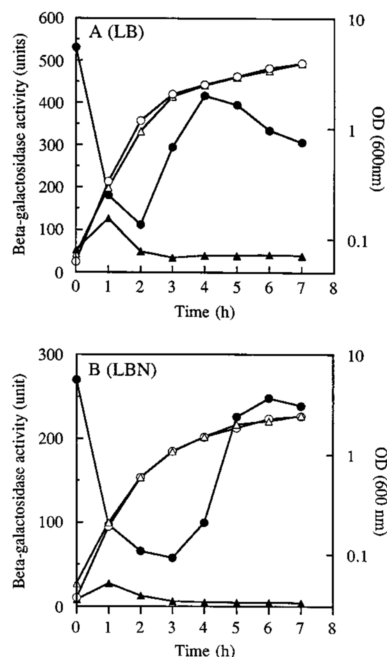


FIG. 2. Effect of chromosomal Fis on the expression of *proP-104*. Overnight cultures of RJ4067 (*proP-104*::Tn*phoA'-4*) cells (circles) and RJ4068 (*proP-104*::Tn*phoA'-4 fis-985*) cells (triangles) were diluted (1/100) into LB medium (A) and LBN (B) with kanamycin and incubated at 37°C with agitation. Samples were removed at 1-h intervals, and cell density values (open symbols) and β-galactosidase activities (solid symbols) were measured. OD, optical density.

Downloaded from http://jb.asm.org/ on September 18, 2019 by guest

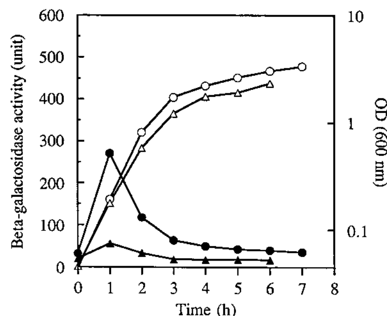


FIG. 3. Effects of RpoS on the expression of *proP-104*. Overnight cultures of RJ4099 (*proP-104*::Tn*phoA'-4 katF13*::Tn*10*) were diluted (1/100) into LB medium (circles) and LBN (triangles) and incubated at 37°C with agitation. Samples were removed at 1-h intervals, and cell density values (open symbols) and β-galactosidase activities (solid symbols) were measured. For a comparison with RpoS$^+$ cells, see Fig. 2. OD, optical density.

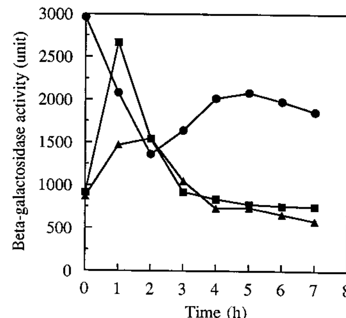


FIG. 4. Effects of Fis and RpoS on the expression of the *proP-104–lacZ* operon fusion. Overnight cultures of RJ4143 (*proP-104*::Tn*phoA'-3*) (circles), RJ4145 (*proP-104*::Tn*phoA'-3 fis-985*) (squares), and RJ4387 (*proP-104*::Tn *phoA'-3 katF13*::Tn*10*) (triangles) were diluted (1/100) into LB medium and incubated at 37°C with agitation. Samples were removed at 1-h intervals, and β-galactosidase activities were measured. Cell density values (optical densities at 600 nm) at the times of the enzyme assay parallel those given in Fig. 2A and Fig. 3 for the corresponding time points.

the expression of many genes expressed in stationary phase (for a review, see references 24 and 26). In addition, we have demonstrated previously that several genes which are negatively regulated by Fis are RpoS dependent (54, 55). Since the phase II expression of *proP-104* occurs during the transition from late exponential to early stationary phase, we tested whether *proP* is also regulated by RpoS. An *rpoS* mutation (*katF13*::Tn*10*) from UM122 (35) was introduced into RJ4067 (*fis*$^+$ *proP-104*::Tn*phoA'-4*) by P1 transduction. β-Galactosidase activities of the resulting *rpoS* mutant were assayed and compared with those of its isogenic *rpoS*$^+$ parental strain. As can be seen from Fig. 3, the expression pattern of *proP* in the *rpoS* mutant is very similar to that of the *fis* mutant reported on in Fig. 2. During the early exponential phase (phase I), LacZ levels in the *rpoS* mutant increased quickly and then dropped to a basal level while phase II expression was totally absent. The difference in LacZ levels at phase II for *rpoS*$^+$ (Fig. 2) and the *rpoS* mutant was about 8- to 10-fold in standard LB broth and 15- to 20-fold in LBN. The combined results from Fig. 2 and 3 indicate that phase II expression of *proP* is dependent upon both Fis and RpoS.

Effects of Fis and RpoS on the expression of a *proP-104–lacZ* operon fusion. The ProP-104–LacZ protein fusion was switched to a *lacZ* operon fusion as described in Materials and Methods. The operon fusion showed an expression pattern similar to that of its corresponding protein fusion in the wild-type *fis* or *rpoS* background (Fig. 4), indicating that regulation is at the transcriptional level. In each case, however, it gave a much higher level of LacZ activity even at the time points when there was very little expression from the protein fusion. These results could imply that *proP* is translated poorly compared with *lacZ* or that the expression of *proP* may also be regulated at the posttranscriptional level.

Identification of the *proP* promoters by 5′ RNA analysis. Nuclease S1 and primer extension assays were performed to locate the relevant transcription start sites and directly characterize the promoters responsible for the phase-dependent expression of *proP*. As elaborated below and consistent with the results of Mellies et al. (30), we believe that there are two independently controlled promoters (*proP*$_1$ and *proP*$_2$), though the number of RNA species detected by the different assays complicates the interpretation.

Figure 5A shows the results of an S1 nuclease protection assay of RNA isolated from cells grown for 1 h and 3 h after subculture. The probe was a 307-base-long, single-stranded DNA which was end labeled at nt +12 and which extends to −295 with respect to the initiating AUG of *proP*. RNA isolated from both *fis* and *fis*$^+$ cells after 1 h of growth yielded primarily two protected species corresponding to transcripts with 5′ ends at nt −109 and −182 with respect to the initiating AUG of *proP* (Fig. 5A, lanes 1 and 2). We discuss further below our belief that the RNA beginning at nt −182, which was also

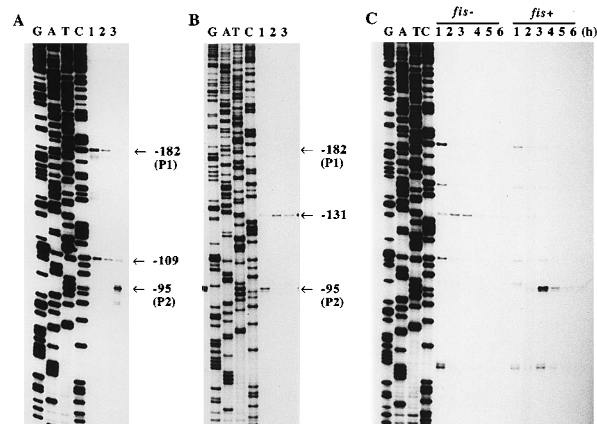
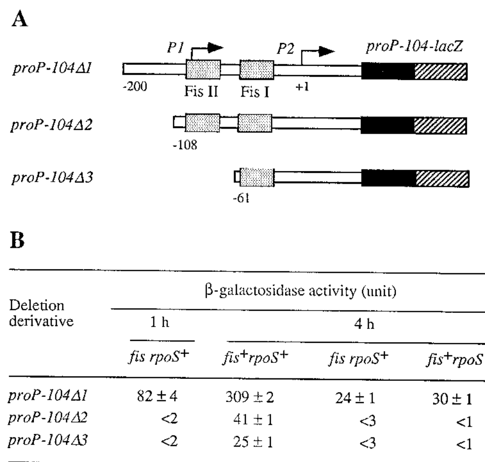


FIG. 5. Mapping of the 5′ end of the *proP* transcripts and their expression over time. (A) S1 mapping. The probe for the S1 mapping is a 307-base-long, single-stranded DNA containing the *proP* sequence from nt +12 to −295. RNA (10 μg) isolated from cells growing for 1 or 3 h in LB broth was used for each reaction. Lanes 1 through 3 represent RNA samples isolated from RJ1801 (*fis-985*) at 1 h, CAG4000 (*fis*$^+$) at 1 h, and CAG4000 at 3 h, respectively. (B) RNA samples (10 μg per reaction) isolated from various strains growing in LB broth for 3 h were analyzed by primer extension with a 24-base, ^{32}P-end-labeled primer (JX8) complementary to the DNA sequence from nt +14 to −10 with respect to the *proP* AUG initiation codon. Lane 1, CAG4000 (*fis*$^+$); lane 2, RJ1801 (*fis-985*); lane 3, RJ4111 (*fis*$^+$ *rpoS*). Two transcription initiation sites indicated by both S1 mapping and primer extension at nt −182 and −95 with respect to the *proP* AUG codon are marked as P1 and P2. Other S1 and primer extension products are denoted on the basis of their locations with respect to the *proP* AUG codon (see text for details). (C) CAG4000 (*fis*$^+$) and RJ1801 (*fis-985* [*fis*-]) were grown in LB medium at 37°C with shaking. Cell samples were taken at 1-h intervals, and total RNA was isolated. RNA samples (10 μg per reaction) isolated from RJ1801 (*fis-985*) and CAG4000 (*fis*$^+$) at various times (in hours) as indicated were analyzed by primer extension as described in the legend for panel B. Cell density values (optical densities at 600 nm) at the time of the RNA isolation parallel those given in Fig. 2A for corresponding time points. DNA sequence ladders (G, A, T, and C) were generated by DNA sequencing of double-stranded DNA templates with the same primer used for the S1 mapping or the primer extension reaction.

Downloaded from http://jb.asm.org/ on September 18, 2019 by guest



Deletion derivative	β-galactosidase activity (unit)			
	1 h	4 h		
	fis rpoS$^+$	*fis*$^+$*rpoS*$^+$	*fis rpoS*$^+$	*fis*$^+$*rpoS*
proP-104Δ1	82 ± 4	309 ± 2	24 ± 1	30 ± 1
proP-104Δ2	<2	41 ± 1	<3	<1
proP-104Δ3	<2	25 ± 1	<3	<1

FIG. 6. Effects of DNA sequence deletions within the *proP* regulatory region on the expression of *proP*. (A) Schematic diagram of the *proP-104* deletion derivatives which are described in Materials and Methods. The two Fis binding sites (Fis I and Fis II), the *proP* coding sequence, and the lacZ sequence are denoted as stippled, black, and hatched boxes, respectively, along with the locations of the P1 and P2 transcription initiation sites. The numbers represent the locations of the upstream ends of the deletions with respect to the $proP_2$ RNA initiation site. (B) λ monolysogens carrying the *proP-104Δ1*–, *-104Δ2*–, and *-104Δ3–lac* fusions were generated in CAG4000 (*fis*$^+$), and the *fis-767* (4) and *katF13*::Tn*10* (*rpoS*) mutations were introduced by transduction. β-Galactosidase activities were determined in the resulting strains after 1 or 4 h of growth in LB broth at 37°C as denoted.

present in the primer extension assays at early time points (Fig. 5C), is the primary RNA initiation site from the promoter responsible for phase I expression ($proP_1$). At 3 h, the most prominent protected species in *fis*$^+$ cells corresponds to an initiation site at nt −95 (Fig. 5A, lane 3). At this time, the −182 band was absent and the −109 band was barely visible. The RNA initiating at nt −95 was also the primary species in primer extension analysis of RNA isolated from wild-type cells grown for 3 h (Fig. 5B, lane 1). This RNA was absent from *fis* (Fig. 5, lane 2) or *rpoS* (lane 3) mutant cells. Thus, the RNA initiating at nt −95 that was detected by both S1 mapping and primer extension must correspond to the start site of the Fis- and RpoS-dependent phase II promoter ($proP_2$).

In addition to the P1 transcript at −182 and the P2 transcript at −95, a number of other products were generated by primer extension assays, especially with RNA isolated from cells at early growth stages (Fig. 5B and C). These are located at nt −65, −97, −109, −131, and −151. The relative abundance of the RNAs at nt −97, −109, and −151 mimics the level of the P1 transcript, and the level of the RNA at −65 corresponds to the combined abundance of the P1 plus P2 transcripts at various times of cell growth (Fig. 5C). The RNA at nt −131 shows a somewhat different temporal expression pattern, the significance of which is not clear. None of these, with the exception of the RNA at nt −109, is present in S1 analysis, leading us to conclude that they likely represent primer extension artifacts that may in part be the result of the palindromic nature of the region. While the RNA species at nt −109 that is detected by both the S1 analysis and the primer extension analysis may represent another transcription start site, it seems more likely that it is a processed RNA product originating from P1 or an artifact resulting from its location at the base of an energetically favorable stem-loop structure. Support for this conclusion comes from analysis of deletions within the *proP* regulatory region (see below) (Fig. 6). *proP-104Δ2*, which has *proP* DNA upstream of nt −203 removed, programs <2% of the LacZ activity of *proP-104Δ1*-containing sequences up to −295 after subculturing for 1 h. This deletion would therefore be expected to inactivate the P1 promoter, since it removes part of its −35 region, but would contain 94 bp of DNA upstream of −109. Finally, we note that Mellies et al. (30) found that a point mutation at nt −12 within the P1 promoter eliminated the transcript from P1 and two other primer extension products equal to our −109 and −151 but not from P2 (−95), which provides further confirmation of the above assignments.

Differential expression of the *proP* P1 and P2 promoters. In Fig. 5C, the *proP* P1 and P2 promoter activities are monitored by primer extension analysis during the course of growth. Levels of the P1 transcript are maximal 1 h after subculture and then decrease sharply, paralleling the phase I expression pattern observed in the *proP-104–lacZ* fusion assays. Its levels are increased by a *fis* mutation (Fig. 5A and C) and are elevated after osmotic upshift (data not shown).

As can be seen from Fig. 5C, the P2 transcript closely follows the phase II expression of the *proP-104–lac* fusion reported on in Fig. 1 and Fig. 2. In *fis*$^+$ cells, it becomes barely detectable 2 h after subculturing, peaks at 3 h around the exponential- to stationary-phase transition, and gradually decreases thereafter (Fig. 5C). The P2 transcript is not detectably synthesized in the *fis* mutant at any time point tested.

Upstream DNA sequence is required for P2 expression. To determine the sequence requirements for optimal expression of phase II expression, we constructed several upstream deletion derivatives of *proP-104*. The three deletion derivatives *proP-104Δ1*, *proP-104Δ2*, and *proP-104Δ3* contain 200, 108, and 61 bp of the *proP* DNA sequence upstream of the P2 RNA start site, respectively, and share the identical *proP-lacZ* fusion site with the original chromosomal *proP-104–lacZ* fusion (Fig. 6A). The constructs were recombined onto lambda, and single-copy lysogens were obtained. *fis*$^+$ lysogens were allowed to grow in LB broth for 4 h and were assayed for LacZ activity. *proP-104Δ2* and *proP-104Δ3* were found to express only 13 and 8% of the *proP-104Δ1* activity, respectively, during phase II (Fig. 6B). While poorly expressed, the activities of *proP-104Δ2* and *proP-104Δ3* were still dependent on both Fis and RpoS. Their LacZ activities decreased more than 8-fold in a *fis* background and more than 30-fold in an *rpoS* background. These results suggest that the DNA sequence upstream of nt −108 with respect to the initiation site of P2 is required for optimal activity of P2. However, a minimal P2 promoter consisting of 61 bp of the upstream sequence can be activated up to 10-fold by Fis.

Fis binding to the regulatory region of *proP*. Potential interactions between the Fis protein and the promoter region of *proP* were investigated in vitro. A 330-bp *Hin*dIII-*Eco*RI fragment carrying the *proP* sequence from nt +14 to −295 was used for gel retardation assays with purified Fis protein. Two Fis-DNA complexes could be detected (Fig. 7), indicating two Fis binding sites present in the *proP* regulatory region. Complex I, formed with the lowest amount of Fis, has an apparent K_d of 3×10^{-9} M, while complex II, which presumably has an additional Fis dimer bound to a second site, requires about twofold more protein to convert 50% of the DNA into this form. The precise locations of the two Fis sites were further defined by DNase I footprinting. As can be seen from Fig. 8A and B, the stronger Fis binding site, site I, is located from nt −48 to −34 and site II is located from −88 to −74 with respect to the transcription initiation site of P2. Sites I and II are centered at nt −41 and −81, respectively. Figure 9 provides a summary. As is often observed in other regions of multiple Fis binding sites (4, 41, 55), weaker protected segments can also be seen at higher Fis concentrations (data not shown).

Downloaded from http://jb.asm.org/ on September 18, 2019 by guest

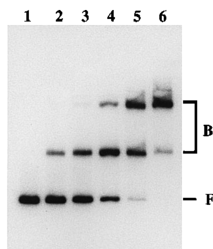


FIG. 7. Electrophoretic mobility shift of *proP* promoter DNA by Fis. The *Eco*RI-*Hin*dIII DNA fragment from pRJ4034 containing the *proP* promoter region (from nt −295 to +14) was end labeled with ^{32}P at the *Eco*RI site. The fragment was incubated with various amounts of Fis protein in a 25-μl final reaction volume, and the Fis-DNA complexes were separated by electrophoresis in an 8% polyacrylamide gel. Free (F) and Fis-bound (B) DNA bands after autoradiography are indicated. Lanes 1 through 6: 0, 0.5, 1, 2, 4, and 8 ng of Fis, respectively.

Effects of Fis site I and II mutations on P2 activity. To test whether direct binding of Fis to site I or site II is required for P1 activation, multiple mutations were introduced into each site by site-directed mutagenesis (Fig. 10). Site I is expected to partially overlap the RNA polymerase binding site. Accordingly, mutations at site I were introduced into the upstream half of the 15-bp core sequence at positions −44, −45, −47,

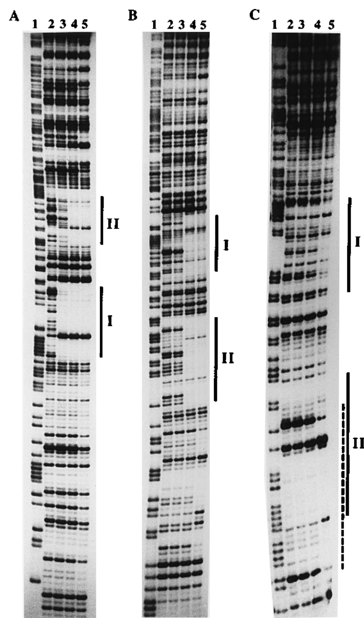


FIG. 8. Footprint of Fis on the wild-type and the mutant *proP* promoter regions. (A and B) The *Eco*RI-*Hin*dIII (nt −295 to +14) DNA fragments from pRJ4034 carrying the wild-type *proP* promoter region were 3′ labeled with ^{32}P at the *Eco*RI end (coding strand, panel A) and at the *Hin*dIII end (noncoding strand, panel B). (C) To examine the effects of the mutations in Fis site I and site II on Fis binding, DNase I footprinting was carried out with a 264-bp DNA fragment (from nt −250 to +14) containing the mutated binding sites. The fragment was obtained by PCR with primers JX11 (5′-GTGAATTCAAT GAGTCCTAAACGAAATCC-3′, extending from nt −250 to −238, with 6 bases added to the 5′ end of the oligonucleotide to give an *Eco*RI site at one end of the PCR product; the primer was 5′ end labeled with ^{32}P) and JX8, with pRJ4053 (with Fis site mutations) serving as the template. The DNA fragments were incubated with 0, 10, 20, and 40 ng of purified Fis (lanes 2 to 5 in each panel, respectively), treated with DNase I, and electrophoresed in a 6% sequencing gel. The positions of the two primary Fis binding sites are indicated with bars and marked with I and II, respectively. The dashed line in panel C designates a new protected region created by the introduction of mutations in site II. Lane 1 in each panel contains a GA sequencing ladder.

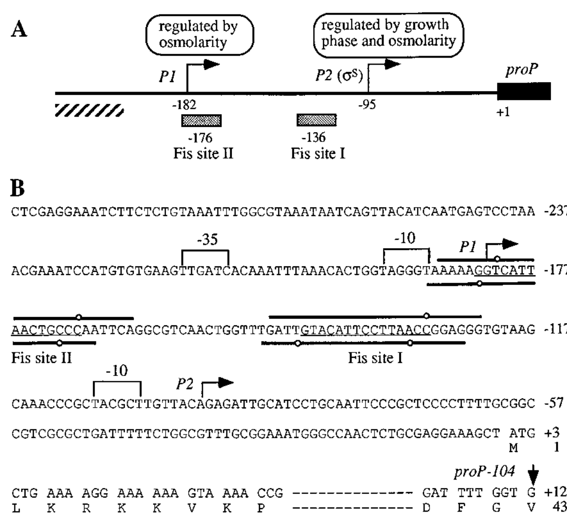


FIG. 9. Features of the *proP* regulatory region. (A) Schematic representation of the *proP* regulatory region. The locations of the transcription initiation sites of the P1 and the σ^S-dependent P2 promoters relative to the start of the *proP* coding sequence are depicted. Also shown are the two high-affinity Fis binding sites centered at nt −136 and −176, respectively, and the relative location of the upstream DNA sequence (hatched region) that is required for efficient P2 expression as defined by *proP-104*Δ*2* (Fig. 6). (B) The DNA sequence for the regulatory region of *proP* was originally determined by Culham et al. (12). The nucleotides protected from DNase I attack by Fis on the top and bottom strands (sites I and II) are denoted with bars that are interrupted with open circles at nucleotides that are hypersensitive or remain sensitive. The predicted Fis core recognition sequences are underlined. Transcription initiation sites determined by S1 nuclease mapping and primer extension for the P1 and P2 promoters are indicated with arrows, and potential RNA polymerase recognition sequences are denoted with horizontal brackets. The *proP-104–lacZ* fusion site at amino acid 43 is indicated with a vertical arrow.

and −48 with respect to the P2 RNA start site in order to avoid affecting RNA polymerase binding. A derivative with both site I and site II mutations was generated by combining restriction fragments which carried the mutated sites I and II as described in Materials and Methods. DNase I footprint assays indicated that the site I mutations reduced the binding of Fis by greater than fourfold, but weak binding could still be detected at high Fis concentrations (Fig. 8C). The site II mutations abolished Fis binding at site II but apparently created a new binding site 7 bases upstream of site II with an affinity about twofold lower than that of the wild-type site II.

The effects of the mutations on the expression of P2 were determined by a LacZ activity assay of lysogens grown in LBN

Sequence (Fis II / Fis I)	Mutation	Strain	LacZ activity
GGTCATTA / GTACAT (Fis II, Fis I, P2)	Wild type	RJ4406	139 ± 2
ccgaATTc	Fis II	RJ4403	74 ± 2
agAtcT	Fis I	RJ4401	11 ± 1
ccgaATTc / agAtcT	Fis I+II	RJ4408	9 ± 1

FIG. 10. Effects of mutations in Fis binding sites on the activity of P2. The mutations introduced into the Fis sites are illustrated. The first nucleotide in each group of underlined DNA sequences corresponds to the first nucleotide in the proposed Fis core recognition sequences, and nucleotides which have been changed are shown in lowercase letters. Single-copy lysogens containing the wild-type and mutant Fis binding sites were grown for 5 h in LBN after the subculturing, and LacZ activities (mean numbers of units ± standard deviations) were determined.

Downloaded from http://jb.asm.org/ on September 18, 2019 by guest

TABLE 2. Effects of osmotic shock on the expression of *proP-104*[a]

Strain (genotype)	Fusion	β-Galactosidase activity (U) in[b]:			
		LBN	LBN + NaCl	M9	M9 + NaCl
RJ4067 (*fis*$^+$ *rpoS*$^+$)	ProP-104–LacZ	300 ± 10	1,323 ± 126	97 ± 2	928 ± 36
RJ4068 (*fis rpoS*$^+$)	ProP-104–LacZ	4.6 ± 0.2	286 ± 6	30 ± 1	914 ± 39
RJ4099 (*fis*$^+$ *rpoS*)	ProP-104–LacZ	32 ± 3	995 ± 124	42 ± 1	1,496 ± 26
RJ4335 (*fis*$^+$ *rpoS*$^+$)	ProP-104Δ2–LacZ	4.4 ± 0.5	37 ± 1	13 ± 1	27 ± 1
RJ4337 (*fis*$^+$ *rpoS*$^+$)	ProP-104Δ3–LacZ	2.9 ± 0.4	18 ± 1	8 ± 1	15 ± 1
RJ4369 (*fis*$^+$ *rpoS*)	ProP-104Δ3–LacZ	<1	<1	nd[c]	nd
RJ4372 (*fis rpoS*$^+$)	ProP-104Δ3–LacZ	<1	<1	nd	nd

[a] Overnight cultures grown in M9 with 0.4% glycerol (M9) and in LBN were subcultured into the same medium and incubated at 37°C with shaking. Cells carrying the ProP-104–LacZ protein fusion were grown in M9 for 6 h and in LBN for 3 h, and NaCl at 0.3 M and 0.4 M was added to half of the parallel cultures growing in M9 and LBN, respectively. Cells were allowed to grow for an additional 3 h, and β-galactosidase activities were determined. Cells containing the ProP-104Δ2–LacZ fusion and the ProP-104Δ3–LacZ fusion were grown in M9 and LBN and were treated in the same manner as those described above, except that the culturing times in LBN before and after NaCl treatment were reduced to 1.5 h each.

[b] Data are means ± standard deviations.

[c] nd, not determined.

carrying the *proP-104Δ1* fusion with or without the mutations. Results shown in Fig. 10 indicate that the site I mutations reduced LacZ activity by up to 12-fold. However, mutations in site II alone reduced LacZ activity by less than 2-fold. There is no additional decrease in activity when site II mutations are combined with mutations in site I. Expression from P2 in the presence of the site I or site I plus site II mutations is reduced another 3.8-fold when assayed in a *fis* mutant strain (data not shown). These results suggest that the activation by Fis of *proP* expression from P2 is primarily mediated through binding at site I, although an effect of the site I mutations on promoter interactions by the σ^s form of RNA polymerase cannot be excluded (see Discussion). The residual Fis-dependent activity produced from the site I mutant can be accounted for by the weak binding to site I, even with the 4-bp substitutions.

Response of *proP* to increased medium osmolarity. Given that *proP* is positively regulated by medium osmolarity (21, 30, 32, 33), we wished to determine the relative contributions of the P1 and P2 promoters along with those of the transcription factors Fis and RpoS under different conditions. As described below, our results indicate that both *proP* promoters can be induced by an increase in medium osmolarity. Osmoregulation of the P1 promoter does not require Fis or RpoS and occurs efficiently in rich or minimal medium. The P2 promoter responds to an increase in medium osmolarity when exponentially growing cells are cultured in rich medium, and this increase in expression requires both RpoS and Fis.

The ability of the *proP* P2 promoter to respond to an increase in medium osmolarity was determined with the *proP-104Δ2–* and *proP-104Δ3–lac* fusions, which are deleted for the P1 promoter. Growth was for a total of 3 h in LBN (1.5 h before and 1.5 h after the addition of 0.4 M NaCl) to minimize the effects of the stationary-phase signal. At this time, P2 expression in cells not subjected to osmotic shock is very low, presumably because of the limiting amount of RpoS (Table 2 and Fig. 2). In osmotically shocked cells, however, P2 activity was increased by 6.3-fold with *proP-104Δ3* and 8.5-fold with *proP-104Δ2* (Table 2), with the overall LacZ activity levels approaching those induced by stationary-phase signals (Fig. 6B). The low levels of P2 activity obtained by these derivatives are due to the deletion of the upstream activation sequence as described above. As was observed with stationary-phase expression, osmotically induced P2 expression was Fis and RpoS dependent. An increase in the level of the P2 RNA transcript programmed by the wild-type *proP* region after an upshift in medium osmolarity in LBN has also been observed by primer extension assays (data not shown). The ability of the P2 promoter to respond to medium osmolarity was also assayed in cells containing the *proP-104Δ2–* or *proP-104Δ3–lacZ* fusion that were grown in M9 plus 0.4% glycerol. Cells were grown to an optical density at 600 nm of ~0.8, and a portion of the culture was osmotically shocked by adding NaCl to 0.3 M (not including the NaCl present in the growth medium). LacZ activities were assayed after an additional 3 h of growth. Under these conditions, expression from P2 was increased only about twofold (Table 2). The poor response of P2 to osmotic shock under these conditions is probably due to the low cellular levels of Fis present under the poor growth conditions.

Strains with the full-length *proP-104–lacZ* fusion, which is under the control of both P1 and P2, were also assayed under different conditions of osmotic induction. In the first case, cells were grown in LBN for 3 h, which was followed by the addition of NaCl to 0.4 M and growth of the cells for an additional 3 h prior to the β-galactosidase assays. Induction ratios of 4-, 62-, and 31-fold in wild-type, *fis*, and *rpoS* cells, respectively, were obtained (Table 2). Contrary to the results with the deletion derivatives, efficient induction in strains containing the *proP-104* fusion was also obtained in M9 medium under the conditions described above, with induction ratios of 10-, 31-, and 36-fold for the wild type, the *fis* mutant, and the *rpoS* mutant, respectively. Because the P2 promoter requires Fis and RpoS under both hyperosmotic and stationary-phase conditions, we conclude that the osmotically induced expression from *proP-104* in the *fis* and *rpoS* mutants is directed primarily from P1, and therefore these proteins are not required for P1 to respond to medium osmolarity.

DISCUSSION

Proline and glycine betaine have been shown to be important osmoprotectants in enterobacteria (for a review, see reference 11). Growth inhibition occurring under increased medium osmolarity has been shown to be alleviated by exogenous proline or glycine betaine, which accumulate in the cell in high amounts (5, 9, 43). Proline can be transported into the cell to high levels by three independent systems: PutP, ProP, and ProU (53). While proline auxotrophy can be satisfied by the function of any of these systems, ProP and ProU are responsible for the high-level accumulation of both proline and glycine betaine in cells subjected to high osmotic conditions. ProP is a low-affinity transporter (29). The predicted amino acid sequence of ProP suggests that it belongs to an extended su-

Downloaded from http://jb.asm.org/ on September 18, 2019 by guest

perfamily of integral membrane-associated solute-ion cotransporters (12). The activity of the ProP system can be increased by osmotic shock by enhancing *proP* expression (17, 21, 30, 32, 33) and by directly increasing the activity of the permease. In the latter case, in vitro experiments employing membrane vesicles have shown that the increase in ProP activity by osmotic upshift is very rapid (32).

Regulation of *proP* transcription. Results from Mellies et al. (30) and this paper demonstrate that *proP* is expressed from two independently controlled promoters. A promoter, P1, initiating transcription 182 bp upstream of the start of the *proP* coding sequence is regulated by medium osmolarity. The location of P1 was also suggested by Culham et al. (12) on the basis of an analysis of several different *proP* clones. They found that only plasmid clones carrying the *proP* DNA sequence that included the P1 region could alleviate the salt sensitivity of a *putP proU proP* triple mutant. We have demonstrated that in complex medium (LB broth), the expression from this promoter is transiently activated shortly after subculturing, perhaps because cells sense a small change in osmolarity. This promoter can be activated at different growth stages and in a variety of growth media by an increase in osmolarity (reference 30 and this paper). As was pointed out previously (12), the sequence of the *proP* P1 promoter displays a striking similarity to that of the osmotically regulated *proU* P2 promoter region, with the sequences sharing a 59% identity in a 46-bp stretch. However, the *proU* promoter appears to show a greater response to an increase in medium osmolarity than does $proP_1$ (21, 30). Neither Fis nor RpoS is necessary for P1 to respond to increased medium osmolarity. We consistently find that P1 promoted RNA levels that are somewhat increased in *fis* mutant cells (Fig. 5), which may be the result of Fis binding at site II, which overlaps the promoter. However, this effect is not reflected in the ProP-LacZ fusion activities. In addition, we have recently found that cyclic AMP (cAMP) receptor protein (CRP)-cAMP complex has a strong inhibitory effect on $proP_1$, perhaps by binding to the almost perfect consensus CRP binding site located within its −35 region (56). The importance of CRP-cAMP for the osmolarity control of P1 activity is under investigation.

Under standard batch culturing conditions in LB broth, *proP* expression is very low in mid-exponential phase but rapidly increases as cells begin to enter stationary phase (Fig. 1–3). This stationary-phase-dependent increase in *proP* expression reflects a corresponding increase in RNA levels that is initiated from the P2 promoter (Fig. 5C). Transcription from the P2 promoter is absolutely dependent on σ^s (Fig. 3 and 5B), whose levels increase as cells approach stationary phase (15, 50). Thus, the availability of σ^s is probably the primary determinant responsible for the increase in stationary-phase expression at $proP_2$. This conclusion is consistent with the finding that P2 expression increases in response to an upshift in medium osmolarity even in exponential phase. This induction is dependent upon RpoS as well as Fis. While RpoS is normally limiting in exponential phase, its levels have been shown to be increased by high osmolarity (25). Under poor growth conditions in which Fis is limiting, P2 is not efficiently induced by an osmotic upshift.

As was found for other σ^s-dependent promoters, the sequence around the −10 region of P2 displays a similarity to those of σ^{70} promoters, but there is no resemblance in the −35 region (50). In this context, P2 is likely to be controlled directly by σ^s, although this remains to be determined. It is interesting to note that the isolated P1 promoter of *proU* is also positively regulated by σ^s (27). Unlike *proP*, however, the overall expression of *proU* with its complete set of regulatory elements (P1, P2, and negative regulatory element) is largely unaffected by σ^s (27), reflecting the complexity of this system. A number of other osmoregulated genes, including *otsBA* that encodes enzymes responsible for the synthesis of trehalose which also functions as an important osmoprotectant, are positively regulated by σ^s (11, 22, 26).

Fis directly activates the *proP* P2 promoter by binding at nt −41. In addition to its dependence on RpoS, expression from the *proP* P2 promoter is essentially dependent on Fis, making it the most strongly activated promoter by Fis that has been described. Fis has been shown to stimulate transcription of operons encoding rRNAs, tRNAs, and ribonucleotide reductase (2, 37–39, 44, 46, 49), but the presence of Fis in these cases is not essential for promoter activity, and its effects are relatively smaller. These known Fis-activated genes are all highly expressed by the σ^{70} form of RNA polymerase during exponential growth under conditions of rapid growth rates, which are precisely the conditions under which Fis is maximally expressed. In contrast, the *proP* P2 promoter is expressed by the σ^s form of RNA polymerase and is expressed in the late exponential and early stationary phase, when Fis levels are rapidly decreasing. The wild-type levels of Fis present at this stage of the growth cycle appear to be limiting $proP_2$ activity since significantly higher ProP-104 LacZ levels are obtained when Fis is constitutively expressed from the *lac* promoter (compare Fig. 1 and Fig. 2). In addition, ProP-104–LacZ levels gradually decrease after about 4 h of growth in wild-type cells but remain high when Fis is expressed from *lacP*. Late-exponential-phase expression of *proP-104* in cells grown in minimal glycerol medium is lower presumably because of the reduced cellular levels of Fis (56).

Overall, positive control by Fis and σ^s will generate a burst of *proP* expression in late exponential and early stationary phases, resulting in an accumulation of the low-affinity transporter of osmoprotectants. This would be advantageous to cells exposed to hyperosmotic conditions later in stationary phase when they may not be able to rapidly respond by increasing de novo gene expression. Moreover, Fis, which is abundantly present in exponential phase under rich growth conditions, may be an appropriate activator for the *proP* system, because osmoprotectants like proline and glycine betaine may be more likely available when cells have been under these growth conditions versus in a nutrient-poor environment.

How does Fis activate the *proP* P2 promoter? Two high-affinity Fis sites are present in the regulatory region (Fig. 9 gives a summary). Fis site I displays the strongest affinity and is centered at nt −41. Unlike most Fis binding sites (14), site I contains a CC at the center of the core recognition sequence, and the right flanking sequence is very G rich. The G-rich flanking sequence extends from nt −26 to −33 in the P2 promoter and thus may constitute part of the σ^s binding site. In order to disrupt Fis binding to this site but not interfere with RNA polymerase recognition, several nucleotide changes (−44, −45, −47, and −48) were introduced into the left half of the Fis core sequence. These mutations reduced Fis binding to site I (Fig. 8C) and severely decreased P2 activity, suggesting that binding to this site is critical for Fis activation (Fig. 10). On the basis of the upstream boundaries of σ^{70} promoters (57) and the high similarity of σ^{70} and σ^s (26, 34), it is likely that these changes may be outside the region involved in σ^s-DNA interactions, though this has not been confirmed. A second lower-affinity Fis binding site (site II) centered at nt −81 does not appear to play a major role in controlling P2 activity. Mutations that eliminate Fis binding at this site only slightly decrease P2 expression and do not further decrease expression in combination with the site I mutations (Fig. 10). The minor

Downloaded from http://jb.asm.org/ on September 18, 2019 by guest

effect of Fis binding to the site II region is tempered, however, by the fact that the site II mutations created a weak Fis binding site shifted 7 bp upstream of site II (Fig. 8C).

There appears to be an additional undefined upstream *cis*-acting sequence that is beyond 109 bp upstream of the transcription initiation site from P2 and that is required for optimal P2 expression. Without this sequence, P2 expression in early stationary phase is reduced more than 10-fold, as a comparison of LacZ activities programmed by *proP-104Δ3* and *proP-104Δ1* shows. Substrates without this upstream sequence still display growth phase regulation (Fig. 6), however, which is consistent with σ^s being the primary stationary-phase activator of P2. Preliminary data indicate that this additional activating element may be CRP-cAMP binding at the consensus position mentioned above (56). It is possible that Fis functions indirectly by facilitating the function of this activator. This could occur by Fis-mediated bending of the DNA at site I, which could aid in the interactions of the upstream activating element with RNA polymerase. Though Fis may function in this manner, it also appears to directly activate the polymerase at P2, since a greater than eightfold activation by Fis occurs on substrates missing the upstream activating sequence (e.g., *proP-104Δ3*) (Fig. 6). The location of Fis site I at nt −41 is analogous to the positions of other activators, such as the λ repressor, MalT, AraC, and CRP (in some cases), in which direct contact with σ^{70} has been implicated (7). We are currently testing the ability of mutant Fis proteins to stimulate *proP* transcription as a first step towards elucidating the mechanism by which Fis is directly activating σ^s RNA polymerase. The large effect of Fis at this promoter makes this an attractive system for determining the mechanism of transcriptional activation by Fis.

ACKNOWLEDGMENTS

We thank Barry Wanner for providing bacterial strains and Sarah Cramton for critical reading of the manuscript.

This work was supported by NIH grant GM38509. J.X. was a recipient of a Markey Postdoctoral Fellowship for Cellular Biochemistry, and R.C.J. is the recipient of an American Cancer Society Faculty Research award.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myer, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Augustin, L. B., B. A. Jacobson, and J. A. Fuchs.** 1994. *Escherichia coli* Fis and DnaA proteins bind specifically to the *nrd* promoter region and affect expression of an *nrd-lac* fusion. J. Bacteriol. **176:**378–387.
3. **Ball, C. A., K. C. Ferguson, and R. C. Johnson.** Unpublished data.
4. **Ball, C. A., R. Osuna, K. C. Ferguson, and R. C. Johnson.** 1992. Dramatic changes in Fis levels upon nutrient upshift in *Escherichia coli*. J. Bacteriol. **174:**8043–8056.
5. **Britten, R. J., and F. T. McClure.** 1962. The amino acid pool in *Escherichia coli*. Bacteriol. Rev. **26:**292–335.
6. **Bruist, M. F., A. C. Glasgow, R. C. Johnson, and M. I. Simon.** 1987. Fis binding to the recombinational enhancer of the Hin DNA inversion system. Genes Dev. **1:**762–772.
7. **Busby, S., and R. H. Ebright.** 1994. Promoter structure, promoter recognition, and transcription activation in prokaryotes. Cell **79:**743–746.
8. **Case, C. C., S. M. Roels, J. E. Gonzalez, E. L. Simons, and R. W. Simons.** 1988. Analysis of the promoters and transcripts involved in IS*10* anti-sense RNA control. Gene **72:**219–236.
9. **Cayley, S., B. A. Lewis, and M. T. Record, Jr.** 1992. Origins of the osmoprotective properties of betaine and proline in *Escherichia coli* K-12. J. Bacteriol. **174:**1586–1595.
10. **Condon, C., J. Phillips, Z.-Y. Fu, C. Squires, and C. L. Squires.** 1992. Comparison of the expression of the seven ribosomal RNA operons in *Escherichia coli*. EMBO J. **11:**4175–4185.
11. **Csonka, L. N.** 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53:**121–147.
12. **Culham, D. E., B. Lasby, A. G. Marangoni, J. L. Milner, B. A. Steer, R. W. van Nues, and J. M. Wood.** 1993. Isolation and sequencing of *Escherichia coli* gene *proP* reveals unusual structural features of the osmoregulatory proline/betaine transporter, ProP. J. Mol. Biol. **229:**268–276.
13. **Filutowicz, M., W. Ross, J. Wild, and R. L. Gourse.** 1992. Involvement of Fis protein in replication of the *Escherichia coli* chromosome. J. Bacteriol. **174:** 398–407.
14. **Finkel, S. E., and R. C. Johnson.** 1992. The Fis protein: it's not just for DNA inversion anymore. Mol. Microbiol. **6:**3257–3265.
15. **Gentry, D. R., V. J. Hernandez, L. H. Nguyen, D. B. Jensen, and M. Cashel.** 1993. Synthesis of the stationary-phase sigma factor σ^s is positively regulated by ppGpp. J. Bacteriol. **175:**7982–7989.
16. **Gille, H., J. B. Egan, A. Roth, and W. Messer.** 1991. The Fis protein binds and bends the origin of chromosomal DNA replication, *oriC*, of *Escherichia coli*. Nucleic Acids Res. **19:**4167–4172.
17. **Gowrishankar, J.** 1986. *proP*-mediated proline transport also plays a role in *Escherichia coli* osmoregulation. J. Bacteriol. **164:**434–445.
18. **Groisman, E. A., and M. J. Casadaban.** 1986. Mini-Mu bacteriophage with plasmid replicons for in vivo cloning and *lac* gene fusing. J. Bacteriol. **168:** 357–364.
19. **Johnson, R. C.** 1991. Mechanism of site-specific DNA inversion in bacteria. Curr. Opin. Genet. Dev. **1:**404–411.
20. **Johnson, R. C., M. F. Bruist, and M. I. Simon.** 1986. Host protein requirements for *in vitro* site-specific DNA inversion. Cell **46:**531–539.
21. **Jovanovich, S. B., M. Martinell, M. T. Record, Jr., and R. R. Burgess.** 1988. Rapid response to osmotic upshift by osmoregulated genes in *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **170:**534–539.
22. **Kaasen, I., P. Falkenberg, O. B. Styrvold, and A. R. Strøm.** 1992. Molecular cloning and physical mapping of the *otsBA* genes, which encode the osmoregulatory trehalose pathway of *Escherichia coli*: evidence that transcription is activated by KatF (AppR). J. Bacteriol. **174:**889–898.
23. **Koch, C., and R. Kahmann.** 1986. Purification and properties of the *Escherichia coli* host factor required for inversion of the G segment in bacteriophage Mu. J. Biol. Chem. **261:**15673–15678.
24. **Kolter, R., D. A. Siegele, and A. Tormo.** 1993. The stationary phase of the bacterial life cycle. Annu. Rev. Microbiol. **47:**855–874.
25. **Lange, R., and R. Hengge-Aronis.** 1994. The cellular concentration of the σ^s subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. Genes Dev. **8:**1600–1612.
26. **Loewen, P. C., and R. Hengge-Aronis.** 1994. The role of the sigma factor σ^s (KatF) in bacterial global regulation. Annu. Rev. Microbiol. **48:**53–80.
27. **Manna, D., and J. Gowrishankar.** 1994. Evidence for involvement of proteins HU and RpoS in transcription of the osmoresponsive *proU* operon in *Escherichia coli*. J. Bacteriol. **176:**5378–5384.
28. **Maxam, A., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74:**560–564.
29. **May, G., E. Faatz, M. Villarejo, and E. Bremer.** 1986. Binding protein dependent transport of glycine betaine and its osmotic regulation in *Escherichia coli* K-12. Mol. Gen. Genet. **205:**225–233.
30. **Mellies, J., A. Wise, and M. Villarejo.** 1995. Two different *Escherichia coli proP* promoters respond to osmotic and growth phase signals. J. Bacteriol. **177:**144–151.
31. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Milner, J. L., S. Grothe, and J. M. Wood.** 1988. Proline porter II is activated by a hyperosmotic shift in both whole cells and membrane vesicles of *Escherichia coli* K-12. J. Biol. Chem. **263:**14900–14905.
33. **Milner, J. L., and J. M. Wood.** 1989. Insertion *proQ220*::Tn*5* alters regulation of proline porter II, a transporter of proline and glycine betaine in *Escherichia coli*. J. Bacteriol. **171:**947–951.
34. **Mulvey, M. R., and P. C. Loewen.** 1989. Nucleotide sequence of *katF* of *Escherichia coli* suggests KatF protein is a novel σ transcription factor. Nucleic Acids Res. **17:**9979–9991.
35. **Mulvey, M. R., P. A. Sorby, B. L. Triggs-Raine, and P. C. Loewen.** 1988. Cloning and physical characterization of *katE* and *katF* required for catalase HPII expression in *Escherichia coli*. Gene **73:**337–345.
36. **Nguyen, L. H., D. B. Jensen, N. E. Thompson, D. R. Gentry, and R. R. Burgess.** 1993. *In vitro* functional characterization of overproduced *Escherichia coli katF*/*rpoS* gene product. Biochemistry **32:**11112–11117.
37. **Nilsson, L., and V. Emilsson.** 1994. Factor for inversion stimulation-dependent growth rate regulation of individual tRNA species in *Escherichia coli*. J. Biol. Chem. **269:**9460–9465.
38. **Nilsson, L., A. Vanet, E. Vijgenboom, and L. Bosch.** 1990. The role of Fis in *trans* activation of stable RNA operons of *E. coli*. EMBO J. **9:**727–734.
39. **Nilsson, L., H. Verbeek, E. Vijgenboom, C. van Drunen, A. Vanet, and L. Bosch.** 1992. FIS-dependent *trans* activation of stable RNA operons of *Escherichia coli* under various growth conditions. J. Bacteriol. **174:**921–929.
40. **Ninneman, O., C. Koch, and R. Kahmann.** 1992. The *E. coli fis* promoter is subject to stringent control and autoregulation. EMBO J. **11:**1075–1083.
41. **Osuna, R., D. Lienau, K. Hughes, and R. C. Johnson.** 1995. Sequence, regulation, and function of *fis* in *Salmonella typhimurium*. J. Bacteriol. **177:** 2021–2032.
42. **Pan, C. Q., J.-A. Feng, S. E. Finkel, R. Landgraf, D. Sigman, and R. C. Johnson.** 1994. Structure of the *Escherichia coli* Fis-DNA complex probed by protein conjugated with 1,10-phenanthroline copper(I) complex. Proc. Natl.

Downloaded from http://jb.asm.org/ on September 18, 2019 by guest

Acad. Sci. USA **91:**1721–1725.

43. **Perroud, B., and D. LeRudulier.** 1985. Glycine betaine transport in *Escherichia coli*: osmotic modulation. J. Bacteriol. **161:**393–401.
44. **Ross, W., J. F. Thompson, J. T. Newlands, and R. L. Gourse.** 1990. *E. coli* Fis protein activates ribosomal RNA transcription *in vitro* and *in vivo*. EMBO J. **9:**3733–3742.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
46. **Sander, P., W. Langert, and K. Mueller.** 1993. Mechanisms of upstream activation of the *rrnD* promoter *P1* of *Escherichia coli*. J. Biol. Chem. **268:** 16907–16916.
47. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
48. **Singer, M., T. A. Baker, G. Schnitzler, S. M. Deischel, M. Goel, W. Dove, K. J. Jaacks, A. D. Grossman, J. W. Erickson, and C. A. Gross.** 1989. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbiol. Rev. **53:**1–24.
49. **Slany, R. K., and H. Kersten.** 1992. The promoter of the *tgt/sec* operon in *Escherichia coli* is preceded by an upstream activation sequence that contains a high affinity Fis binding site. Nucleic Acids Res. **16:**4193–4198.
50. **Tanaka, K., Y. Takayanagi, N. Fujita, A. Ishihama, and H. Takahashi.** 1993. Heterogeneity of the principal σ factor in *Escherichia coli*: the *rpoS* gene product, σ^{38}, is a second principal σ factor of RNA polymerase in stationary-phase *Escherichia coli*. Proc. Natl. Acad. Sci. USA **90:**3511–3515.
51. **Thompson, J. F., L. Moitoso de Vargas, C. Koch, R. Kahmann, and A. Landy.** 1987. Cellular factors couple recombination with growth phase: characterization of a new component in the lambda site-specific recombination pathway. Cell **50:**901–908.
52. **Wilmes-Riesenberg, M. R., and B. L. Wanner.** 1992. Tn*phoA* and Tn*phoA'* elements for making and switching fusions for study of transcription, translation, and cell surface localization. J. Bacteriol. **174:**4558–4575.
53. **Wood, J. M.** 1988. Proline porters affect the utilization of proline as a nutrient or osmoprotectant for bacteria. J. Membr. Biol. **106:**183–202.
54. **Xu, J., and R. C. Johnson.** 1995. Identification of genes negatively regulated by Fis: Fis and RpoS comodulate growth-phase-dependent gene expression in *Escherichia coli*. J. Bacteriol. **177:**938–947.
55. **Xu, J., and R. C. Johnson.** 1995. *aldB*, an RpoS-dependent gene in *Escherichia coli* encoding an aldehyde dehydrogenase that is repressed by Fis and activated by Crp. J. Bacteriol. **177:**3166–3175.
56. **Xu, J., and R. C. Johnson.** Unpublished data.
57. **Yu, X.-M., and W. S. Reznikoff.** 1986. Deletion analysis of RNA polymerase interaction sites in the *Escherichia coli* lactose operon regulatory region. J. Mol. Biol. **188:**545–553.

Downloaded from http://jb.asm.org/ on September 18, 2019 by guest