JOURNAL OF BACTERIOLOGY, Jan. 1995, p. 144–151 Vol. 177, No. 1
0021-9193/95/$04.00+0

Two Different *Escherichia coli proP* Promoters Respond to Osmotic and Growth Phase Signals

JAY MELLIES,† ARLENE WISE, AND MERNA VILLAREJO*

Section of Microbiology, University of California, Davis, California 95616

Received 20 July 1994/Accepted 26 October 1994

***proP* of *Escherichia coli* encodes an active transport system for proline and glycine betaine which is activated by both hyperosmolarity and amino acid-limited growth. *proP* DNA sequences far upstream from the translational start site are strongly homologous to the promoter of *proU*, an operon that specifies another osmoregulated glycine betaine transport system. Mutation and deletion analysis of *proP* and primer extension experiments established that this promoter, P1, was responsible for *proP*'s strong expression in minimal medium and its response to osmotic signals. When cells were grown in complex medium, expression from a *proP-lacZ* fusion was induced three- to fourfold as growth slowed and cells entered stationary phase. Stationary-phase induction was dependent on *rpoS*, which encodes a stationary-phase sigma factor. Deletion of 158 bp of the untranslated leader sequence between P1 and the *proP* structural gene abolished *rpoS*-dependent stationary-phase regulation. Transcription initiation detected by primer extension within this region was absent in an *rpoS* mutant. *proP* is therefore a member of the growing class of σ^S-dependent genes which respond to both stationary-phase and hyperosmolarity signals.**

The growth rate of *Escherichia coli* in hyperosmotic media is enhanced by the accumulation of a limited number of organic solutes. The preferred solute is glycine betaine (10, 38), which is accumulated by two transport systems, *proP* and *proU*. *proP* encodes an integral membrane protein (7) which transports glycine betaine and proline with low affinity (2, 4, 28). ProP regulation occurs at the level of transcription and through control of transport activity. *proP-lacZ* transcriptional fusions show a two- to fivefold increase in activity when cells are osmotically stressed (11, 31). Transport of glycine betaine and proline by ProP is also increased by osmotic upshock (14, 28) and amino acid limitation (14, 37).

proU encodes a high-affinity glycine betaine uptake system (5, 8, 28). Like ProP, ProU is regulated at the level of transcription and through control of transport activity. Under hyperosmotic conditions, the activity of *proU-lacZ* operon fusions increases 60- to 100-fold (3, 5, 29), and transport of glycine betaine by the *proU* system increases (28). Unlike *proP*, *proU* transcription is not induced in stationary phase, although several other osmotically responsive genes, e.g., *osmY*, *osmB*, *otsB*, and *otsA*, respond to growth phase signals (15, 18, 40).

Certain observations have suggested that *proP* and *proU* may be regulated in a similar manner. First, induction from *proP-lacZ* and *proU-lacZ* operon fusions is rapid upon osmotic shock (16). Second, the *proP* gene sequence was recently reported (7), and a region 190 bp upstream of the translation initiation site is strongly homologous to the promoter of *proU*. In this investigation, we studied the regulation of *proP*, asking whether that putative promoter functions in transcription initiation. We found that this promoter was responsible for strong basal expression and response to osmotic signals, while a second downstream transcription initiation site was involved in *rpoS*-dependent stationary-phase induction. Finally, we have studied in vitro *proP* expression to further understand osmotic regulation of both *proP* and *proU*.

* Corresponding author. Mailing address: Section of Molecular and Cellular Biology, University of California, Davis, CA 95616. Phone: (916) 752-6281. Fax: (916) 752-3085. Electronic mail address: mrvillarejo@ucdavis.edu.

† Present address: Max-Planck-Institut für Biologie, Abteilung Infektionsbiologie, D 72076 Tübingen, Germany.

MATERIALS AND METHODS

Bacterial strains. The *Escherichia coli* strains used are listed in Table 1. All strains containing *lacZ* fusions were derivatives of MC4100. DH5α was used for all cloning purposes. TE2680 was used to transfer promoter-*lacZ* fusions to the *E. coli* chromosome.

Media and growth conditions. M9 minimal medium was supplemented with 0.2% glucose and 1 μg of thiamine per ml. For growth of strains containing *lacZ* fusions inserted into the *trp* locus, 30 μg of tryptophan per ml was added. All strains were grown aerobically at 37°C.

Osmotic regulation was studied in defined minimal medium because it lacks osmoprotectants. To analyze the effect of osmotic shock on *proP* transcription, strains with single-copy *proP-lacZ* fusions were prepared for β-galactosidase assays and RNA isolation by 1:50 dilution of an overnight culture in M9 glucose medium into fresh medium. After reaching exponential phase, selected cultures were shocked by addition of NaCl to 0.3 M. Samples were taken 1 h after salt shock, unless otherwise indicated.

For β-galactosidase analysis of stationary-phase regulation of *proP* transcription, strains containing single-copy *proP-lacZ* fusions were grown overnight in Luria-Bertani medium (henceforth referred to as complex medium) and diluted 1:1,000 into fresh medium. The 1,000-fold dilution into fresh medium was necessary to dilute out β-galactosidase activity carried over from stationary phase, prior to harvest at exponential phase. Samples were harvested at exponential phase (approximately 50 Klett units) and 3 h after the beginning of stationary phase, unless otherwise indicated.

Plasmid constructions. Construction of the 2,000-bp *proP* and 1,700-bp *proU* fragments fused to *lacZ* and integrated into the chromosome of JM2000 and JM1700, respectively, was described previously (29). Additional *proP* promoter fragments were created by PCR and also used to construct single-copy *lacZ* fusions in MC4100. Fragments containing base substitutions and the 3′ deletion constructs were created by using primers which incorporated an *Eco*RI or *Bam*HI restriction site at the 5′ end to allow directed cloning into the pRS551 expression vector (36).

PCR amplification was performed with 0.5 ng of pDC15 (7) template DNA, 0.25 μM each primer, 200 μM deoxynucleoside triphosphates, and 1 U of *Taq* polymerase (Promega). Solutions were heated to 95°C for 5 min and passed through 25 cycles as follows: denaturation for 40 s at 94°C, primer annealing for 40 s at 45°C, and extension at 72°C for 1 min. The *proP* fragment in JM520 was created by using primers 5′ proP#1 (5′-CTCGAATTCGTTTGCTGGTCAGT GAC-3′) and 3′proP#1 (5′-CTCGGATCCACCTAATGCGTAAGCAAC-3′). The 520-bp fragment with base substitution at the putative −12 position was created by two initial reactions using the primer pairs 5′proP#1 with 3′proP#2 (5′-GACCTTTTTACCCTCCCAGT-3′) and 3′proP#1 with 5′proP#2 (5′-ACT GGGAGGGTAAAAAGGTC-3′). A third PCR used the two initial products as self-priming templates together with primers 5′proP#1 and 3′proP#1 to create the 520-bp fragment with a base substitution at the −12 position. The 520-bp

TABLE 1. *E. coli* strains used

Strain	Relevant genotype or description	Reference
MC4100	F⁻ Δ(*argF-lac*)*U169 thiA*	6
RH90	MC4100 *rpoS359*::Tn*10*	21
TE2680	F⁻ λ⁻ IN(*rrnD-rrnE*)1 Δ(*lac*)*X74 rpsL galK2 recD1903*::Tn*10d*-Tet *trpDC700*::*putPA1303*::[Kan^s-Cam^r-*lacZ*]	12
JM1700	F⁻ MC4100 *trpDC700*::[Kan^r 1,700-bp *proU-lacZ*]	29
JM2000	F⁻ MC4100 *trpDC700*::[Kan^r 2,000-bp *proP-lacZ*]	29
JM520	F⁻ MC4100 *trpDC700*::[Kan^r 520-bp *proP-lacZ*]	This study
JM363	F⁻ MC4100 *trpDC700*::[Kan^r 382-bp *proP-lacZ*]	This study
JM210	F⁻ MC4100 *trpDC700*::[Kan^r 210-bp *proP-lacZ*]	This study
JM68	F⁻ MC4100 *trpDC700*::[Kan^r 68-bp *proP-lacZ*]	This study
JM520-12	F⁻ MC4100 *trpDC700*::[Kan^r 520-bp *proP-lacZ*]	This study
JM520-14	F⁻ MC4100 *trpDC700*::[Kan^r 520-bp *proP-lacZ*]	This study
JM200S	JM2000 *rpoS359*::Tn*10*	This study
JM520S	JM520 *rpoS359*::Tn*10*	This study
JM520-12S	JM520-12 *rpoS359*::Tn*10*	This study

proP fragment with a substitution at the −14 position was created by the identical procedure with primer pairs 5′proP#1 with 3′proP#3 (5′-GACCTTTTTAC CCTACAAGTG-3′) and 3′proP#1 with 5′proP#3 (5′-CACTTGTAGGGTA AAAAGGTC-3′). For the deletion analysis, *proP* fragments in JM363, JM210, and JM68 were created by using the 5′ primer 5′proP#4 (5′-CTCGAATTCTG TGTGAAGTTGATCACAA-3′) and 3′ primers 3′proP#1, 3′proP#5 (5′-CTC GGATCCTTGGCCCATTTCCGCAA-3′), and 3′proP#6 (5′-CTCGGATCC CGCCTGAATTGGGCAGT-3′), respectively. Sequences of the cloned fragments were confirmed by DNA sequencing.

Genetic procedures. *proP* promoter fragments fused to the *lacZ* reporter gene of pRS551 (36) were transferred to the chromosome of *E. coli* TE2680 chromosome by homologous recombination (12). P1*vir* was used to transfer these fusions to the *trp* locus of the MC4100 chromosome. P1*vir* was also used to transduce the *rpoS359*::Tn*10* mutation from RH90 into strains JM2000, JM520, and JM520-12.

Primer extension analysis. Preparation of whole-cell RNA from *E. coli* was described previously (1). RNA was isolated from MC4100 cultures which were grown in M9, or M9 supplemented to 0.3 M with NaCl, in the same manner as cells used in the assay of *proP-lacZ* fusions. For primer extension analysis of stationary-phase cells, RNA was isolated 3 h after the initiation of stationary phase from strains grown in complex medium. The primer extension procedure used was that of Yim et al. (40), with the following modifications. Ten picomoles of a synthetic oligonucleotide which anneals to *proP* transcripts at positions 503 to 487 was end labeled by using 16 pmol of [γ-^{32}P]ATP (6,000 Ci/mmol, 10 mCi/ml; Amersham) and 6 U of T4 polynucleotide kinase (U. S. Biochemical [USB]) as instructed by the manufacturer. Unincorporated nucleotides were removed by a G-25 spin column (26) equilibrated with RNase-free hybridization buffer. Approximately 0.4 pmol of end-labeled primer was combined with either 25 or 35 μg of ethanol-precipitated total-cell RNA which had been resuspended in 8 μl of hybridization buffer. The mixture was heated to 95°C for 1 min and then allowed to hybridize for approximately 3 to 10 min at 44°C. Six hundred units of reverse transcriptase was added, and incubation continued at 44°C for an additional 45 min. After RNase treatment, phenol-chloroform extraction, and ethanol precipitation, extended DNA products were resuspended in an appropriate amount of loading buffer (USB). The primer extension products were separated on a 6% polyacrylamide–urea sequencing gel adjacent to a DNA sequence generated with the same primer used in the extension reactions. Sequencing was also performed with the dITP analogs as instructed by the manufacturer (USB) to resolve compressions in the sequence.

Recombinant DNA techniques. Electrophoresis of DNA in agarose, ethanol precipitation of DNA, transformations, and phenol extractions were done by standard procedures (26). The Sephaglas BandPrep kit (Pharmacia) was used for isolation of *proP* promoter fragments created by PCR. Double-stranded DNA sequencing was performed with the Sequenase version 2.0 sequencing kit (USB) as instructed by the manufacturer. DNA for sequencing was isolated with a Plasmid Midi kit (Qiagen).

β-Galactosidase assays. Resuspended cell pellets were diluted in Z buffer and assayed for β-galactosidase activity as previously described (30). Protein quantities were determined by the Lowry assay (23). Specific activity values are expressed in nanomoles per minute per milligram of protein.

In vitro coupled transcription-translation. Cell-free, coupled transcription-translation was performed with a commercially available kit (Promega) according to the instructions provided. Proteins synthesized in vitro were labeled with 5 μCi of Trans ^{35}S label (1,055 Ci/mmol; ICN) for 60 min at 37°C. Samples were acetone precipitated, denatured, and separated by SDS-PAGE (10% polyacrylamide). The samples in lanes 5 through 9 in Fig. 6 were not denatured prior to electrophoresis to promote tighter banding of ProP. Coomassie blue-stained gels were dried under vacuum on Whatman paper and placed on Amersham Hyperfilm-MP for autoradiography. For quantifying expression, ProV′ and ProP protein bands were excised and placed in vials containing 4 ml of Amersham biodegradable scintillant and counted.

RESULTS AND DISCUSSION

Osmotic induction. A *proP-lacZ* transcriptional fusion was constructed and transferred to the chromosome of strain MC4100 in single copy to facilitate study of *proP* transcriptional regulation. The *proP* fragment in strain JM2000 contains approximately 650 bp of DNA upstream of a putative transcriptional start site at position 251 and downstream DNA to position 1614, located near the 3′ end of the *proP* structural gene (Fig. 1) (7). To compare *proP* and *proU* transcriptional regulation, an analogous *proU-lacZ* fusion was constructed in JM1700.

Figure 1A demonstrates that there was a high basal level of expression (2,000 to 3,000 U) from the 2,000-bp *proP-lacZ* fusion during exponential growth of cells in M9 minimal medium. While *proU* expression is induced 60- to 100-fold upon osmotic shock with 0.3 M NaCl (Fig. 1B) (3), the increase in β-galactosidase activity from the *proP* fusion was modest, between 2- and 3-fold after addition of NaCl. As with *proU*, *proP* expression was reduced as a consequence of intracellular accumulation of its substrate, glycine betaine.

Stationary-phase induction. We monitored the specific activity of the *proP-lacZ* fusion in JM2000 during growth in complex medium to determine whether *proP* transcription was induced during stationary phase. A number of other osmotically regulated genes, *osmY*, *osmB*, *otsB*, and *otsA*, are also induced during stationary phase, independent of medium osmolarity (15, 18, 40). Induction of these genes is dependent on the *rpoS* gene, which encodes the stress-responsive sigma factor σ^S. In contrast, our *proU* fusion is dependent on σ^{70} for expression (17, 40) and shows no stationary-phase induction (our unpublished observation).

β-Galactosidase activity directed from the *proP-lacZ* fusion in JM2000 increased approximately fourfold as growth slowed and cells entered stationary phase (Fig. 2). Stationary-phase induction of the *proP-lacZ* fusion was dependent on a functional *rpoS* gene because an insertional mutation at this locus eliminated induction (Fig. 2). Thus, stationary-phase regulation of *proP* is similar to that of *osmY*, *osmB*, *otsB*, and *otsA* in its dependence on a functional σ^S protein.

Promoter identification. Primer extension analysis was performed to determine whether the putative *proP* promoter, identified by sequence homology with the promoter of *proU* (7), functions in transcription initiation. A ^{32}P-end-labeled

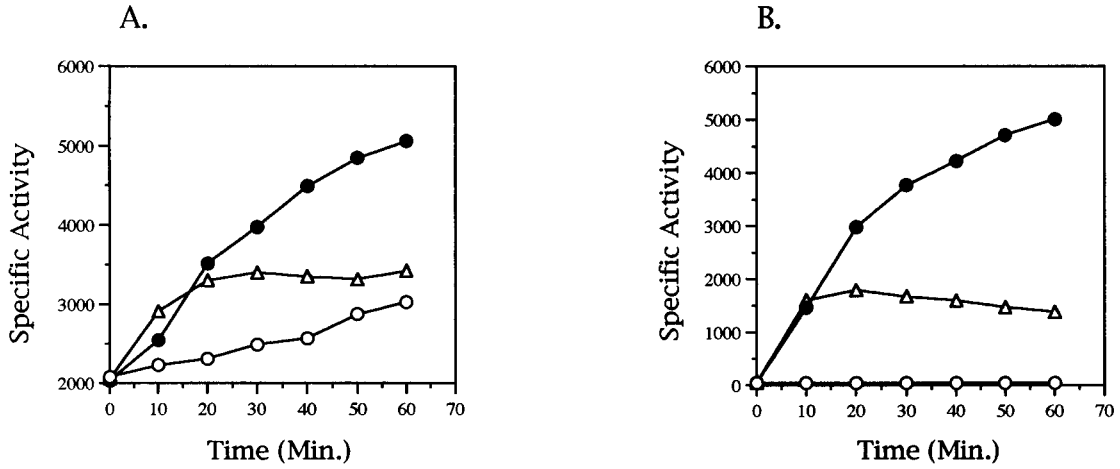


FIG. 1. Osmotic induction of *proP* and *proU*. (A) JM2000 (2,000-bp *proP* promoter fragment); (B) JM1700 (1,700-bp *proU* promoter fragment). Strains were grown in M9 minimal medium to exponential phase and split in three: control with no addition (○), NaCl added to 0.3 M (●), and 0.3 M NaCl and 1 mM glycine betaine added simultaneously (△). Samples were taken after osmotic upshock at the indicated times. β-Galactosidase specific activity values from the *proP*- and *proU-lacZ* fusions (nanomoles per minute per milligram) are shown.

DNA primer directed to *proP* mRNA was extended by using reverse transcriptase. Termination of DNA synthesis occurred when 5′ ends of mRNA were reached or where transcriptase pausing facilitated termination.

Primer extension analysis identified two main transcription initiation sites, responsive to different signals. A band was visualized at position 251 (P1), using mRNA isolated from MC4100 cells grown in M9 minimal medium (Fig. 3A). The G residue at position 251 corresponds to promoter sequences homologous to those of the *proU* promoter (Fig. 4). This band intensified when cells were stressed by the addition of 0.3 M NaCl (Fig. 3A, lane 2).

In minimal medium, prominent bands were also seen at positions 283, 324, and sometimes 333. However, a large, thermodynamically favored RNA hairpin structure from positions 279 to 324 is predicted by software of the Genetics Computer Group. Such a loop might cause reverse transcriptase pausing in this region. Thus, we supposed that bands in this region might represent primer extension termination but not actual sites of transcription initiation.

Because of the multiple bands on the primer extension gel, it was important to assess the contribution of transcription initiating at position 251 by a completely independent method. Furthermore, we wished to verify the presence of an *rpoS*-dependent promoter suggested by β-galactosidase assays (Fig. 2). Therefore, we constructed wild-type and mutant versions of a 520-bp *proP* fragment containing DNA from positions 50 to 570 of the *proP* sequence. This construction contains 201 bp of DNA upstream from the transcriptional start site at 251 and 319 bp of downstream DNA. The region includes the 182-bp untranslated leader sequence containing all other putative promoter sites as well as 137 bp of the *proP* structural gene.

Site-directed mutagenesis was used to modify two bases in the proposed P1 promoter region. The base first selected for mutagenesis was position −12 (Fig. 4). The T-to-G mutation at position −12 alters the proposed −10 hexamer of P1 away from the consensus σ^{70} −10 recognition sequence (TATAAT). The corresponding mutation in the *proU* promoter abolishes expression of a *proU-lacZ* fusion (29).

Transcription initiation in the −12 mutant and *rpoS* dependence of *proP* transcription initiation were analyzed in strains containing the 520-bp wild-type and −12 mutant promoters

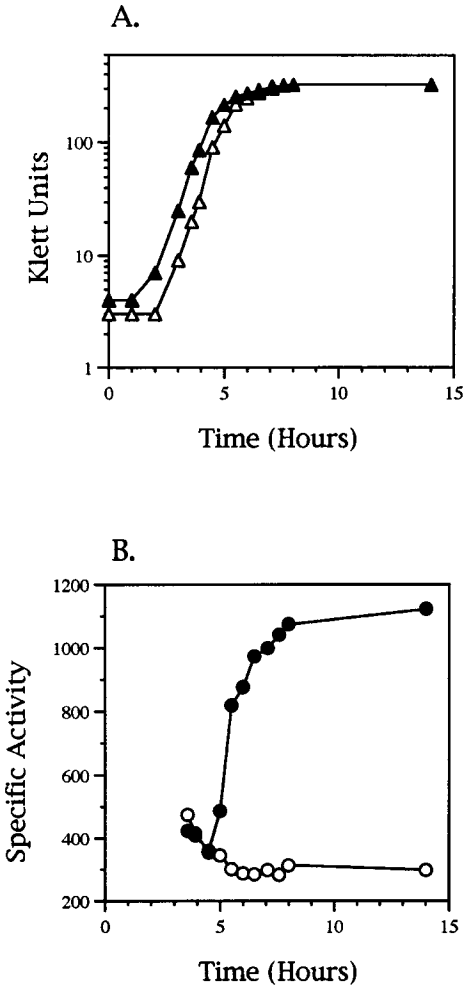


FIG. 2. Induction of *proP* at stationary phase is *rpoS* dependent. Strains JM2000 (*proP*::*lacZ rpoS*$^+$) (closed symbols) and JM2004 (*proP*::*lacZ rpoS*::Tn*10*) (open symbols) were grown in Luria-Bertani medium. (A) Cell growth, Klett units; (B) β-galactosidase specific activity values from the *proP-lacZ* fusion, expressed in nanomoles per minute per milligram.

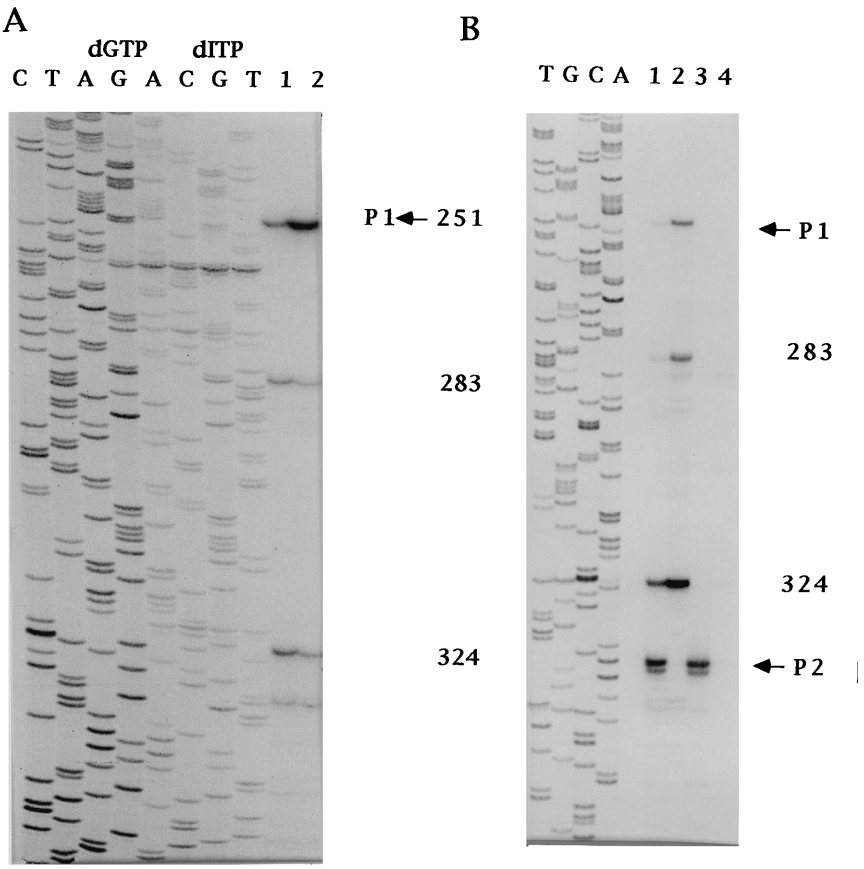


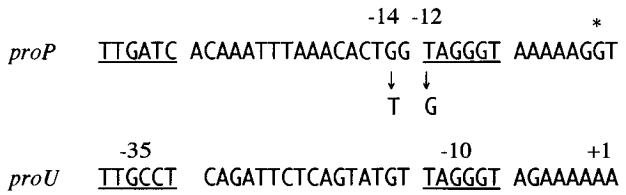
FIG. 3. Primer extension analysis of *proP* transcription initiation sites. The *proP* DNA sequencing ladders generated with dGTP and dITP reagents are shown. Positions of significant bands are noted and correspond to the *proP* sequence reported in reference 7. The two transcriptional start sites at positions 251 and 338 are indicated. (A) For RNA isolation, strain MC4100 was grown in M9 minimal medium to exponential phase. The culture was then split in two: no addition (lane 1) and NaCl added to 0.3 M (lane 2). RNA was harvested 1 h after salt addition. (B) Primer extension products were generated from plasmid-containing cells grown in complex medium and harvested 3 h after the beginning of stationary phase. Lanes: 1, MC4100//pJM520; 2, RH90//pJM520; 3, MC4100//pJM520-12; 4, RH90//pJM520-12.

fused to *lacZ* of multicopy plasmids. Cells containing plasmids were used in primer extension analysis to give a clearer picture of the low level of transcription initiation at the *proP* −12 mutant promoter. Additionally, use of strains with chromosomal versions of the −12 mutant promoter-*lacZ* fusions at the *trp* locus contain a second wild-type promoter at the *proP* locus, which might obscure results obtained with the mutant promoter. β-Galactosidase assays of the plasmid-containing cells confirmed that activity from these cells (data not shown) reflected the same regulatory patterns as single-copy chromosomal constructs (Fig. 4).

Figure 3B shows the results of primer extension analysis of cells grown to stationary phase in complex medium. Lane 1 (MC4100//pJM520) represents wild-type promoter usage. The intensity of the bands at P2 relative to the band at P1 suggested that an A at position 338 was an important initiation site during stationary phase in complex medium. A less intense band at position 339 (G) may represent a secondary initiation site. Analysis in the *rpoS*::Tn*10* strain RH90 (lane 2) showed a complete absence of RNA initiation at P2. The increased intensity of bands at 251, 283, and 324 in lane 2 reflects the fact that 40% more RNA was used for reactions loaded in lanes 2 and 4 than in lanes 1 and 3. The absence of transcription initiation at P2 in the RH90 background indicates that this promoter is *rpoS* dependent.

In lane 3 (MC4100//pJM520-12), the T-to-G transversion at −12 prevented transcription initiation at P1. The −12 mutation also eliminated bands at 283 and 324, indicating that they did not represent transcription initiation sites but represented either degradation products derived from RNA initiating at 251 or sites of reverse transcriptase pausing. In lane 4 (RH90//pJM520-12), only very light bands were visible near 283 and at 324. Presumably, these bands are derived from position 251 of the wild-type chromosomal copy of *proP*.

In vivo assays of strains which contain chromosomal copies of wild-type and mutant promoter-*lacZ* fusions (Fig. 4) confirmed and extended the results of the primer extension experiments shown in Fig. 3. We compared induction of the 2,000-bp *proP-lacZ* fusion in JM2000 (wild type) with induction in JM2000S (*rpoS*::Tn*10*). Osmotic shock increased specific activity directed by the 2,000-bp fragment from approximately 2,000 to 4,000 U. Specific activity at stationary phase in complex medium was approximately 3.5 times higher than activity during exponential growth. The *rpoS*::Tn*10* mutation did not prevent salt induction of the 2,000-bp fusion. Slightly lower basal activity from the fusion in the *rpoS* mutant may be due to loss of σ^S-dependent transcription from the P2 promoter in M9 medium without salt. Stationary-phase induction was not observed in the mutant background. Therefore, stationary-phase induction on the 2,000-bp promoter fragment was σ^S dependent, but osmotic induction was not. Regulation of the 520-bp fragment was similar to that of the 2,000-bp wild-type frag-



Relevant Genotype	ß-Galactosidase Specific Activity: Minimal Medium, No Addition	Minimal Medium, + 0.3 M NaCl	Complex Medium, Exp.	Complex Medium, Stat.
2000 bp Φ(*proP-lacZ*) (wt)	1,809	4,130	341	1164
2000 bp Φ(*proP-lacZ*) (wt) *rpoS*::Tn10	1,069	4,259	394	295
520 bp Φ(*proP-lacZ*) (wt)	2,102	4,000	388	995
520 bp Φ (*proP-lacZ*) (wt) *rpoS*::Tn10	1051	4006	457	354
520 bp Φ(*proP-lacZ*) -12 T to G	143	252	7	345
520 bp Φ(*proP-lacZ*) -12 T to G *rpoS*::Tn10	13	48	5	16
520 bp Φ(*proP-lacZ*) -14 G to T	677	1,686	58	834

FIG. 4. Homologous promoters of *proP* and *proU*. The base substitutions on the 520-bp *proP* fragment in strains JM520-12 and JM520-14 at the putative −12 and −14 positions, respectively, are indicated. The putative transcriptional start site at position 251 (7) is noted by an asterisk. The *proU* −10 and −35 regions and the start of transcription (+1) were previously identified (13, 27, 29). β-Galactosidase specific activity values from the *proP-lacZ* fusions are expressed in nanomoles per minute per milligram. Values are from an assay typical of four separate experiments in which the same regulatory trends were observed. wt, wild type.

ment, indicating that all necessary regulatory information is contained in the 520-bp construct.

The T-to-G substitution at the −12 position, which apparently prevents transcription initiation from P1 (Fig. 3B), significantly reduced both basal and salt-stressed expression of the 520-bp fusion when cells were grown in M9 (Fig. 4). The decrease in overall expression directed by the mutated *proP* promoter indicates that P1 was responsible for the majority of *proP* transcription in minimal medium. The remaining activity (6 to 7% that from the wild-type promoter) was *rpoS* dependent. This result implies a low level of σ^S-dependent transcription from P2 in minimal medium.

Expression from the JM520-12 mutant promoter during exponential phase in complex medium was also severely reduced but still strongly inducible in response to stationary-phase signals. The −12 mutation reduced expression during exponential growth in complex medium by 98%, and expression during stationary phase was *rpoS* dependent (compare JM520-12 and JM520-12S). This finding indicates that P1 is also used during exponential phase in complex media but is not required for stationary-phase induction.

The absence of transcription initiation at P2 in the RH90 background (Fig. 3B) and the absence of stationary-phase induction in JM520-12S clearly show that stationary-phase induction of *proP* in complex media depends on a functional σ^S protein and that this sigma factor is required for transcription from P2. These data, however, do not indicate whether *rpoS* acts directly or indirectly at P2. No obvious σ^{70} promoter sequence corresponding to an initiation site at position 338 was found. Perhaps this indicates a direct action by σ^S. RpoS increases four- to sixfold as cells reach stationary phase (22), close to the magnitude of *proP* stationary-phase induction. Nonetheless, an additional factor, dependent on $E\sigma^S$ for expression, may mediate *proP* stationary-phase induction.

Because of the sequence similarity between P1 of *proP* and the *proU* promoter (Fig. 4 and reference 7), we explored the effect of a second mutation at P1. A mutation at the −14 position of the *proU* promoter effectively abolishes transcription (29). Position −14 in the *proU* spacer is part of an extended −10 sequence important for maximal gene expression (19). The same G-to-T substitution at position −14 of the 520-bp *proP* fragment was constructed in JM520-14. In this case, basal expression of the *proP-lacZ* fusion expression was reduced by 70% and salt-induced expression was reduced by 60%. The substitution at −14 reduced stationary-phase expression by only 15%. Therefore, although the mutation at −14 reduces *proP* expression, an extended −10 sequence is a less important factor in transcription initiation at P1 of *proP* than at the *proU* promoter.

Deletion analysis. A deletion analysis was undertaken to assess the contribution of flanking DNA sequences to overall expression (Fig. 5; Table 2). Promoter fragments constructed by PCR were fused to *lacZ* and transferred to the chromosome of strain MC4100 for assay.

The minimal P1 promoter could be studied in isolation on a 68-bp fragment in JM68. Expression from P1 was strong and salt inducible in minimal medium, but this construct showed little activity during exponential growth in complex medium and no induction at stationary phase. Therefore, sequences outside the minimal P1 promoter region are required for nor-

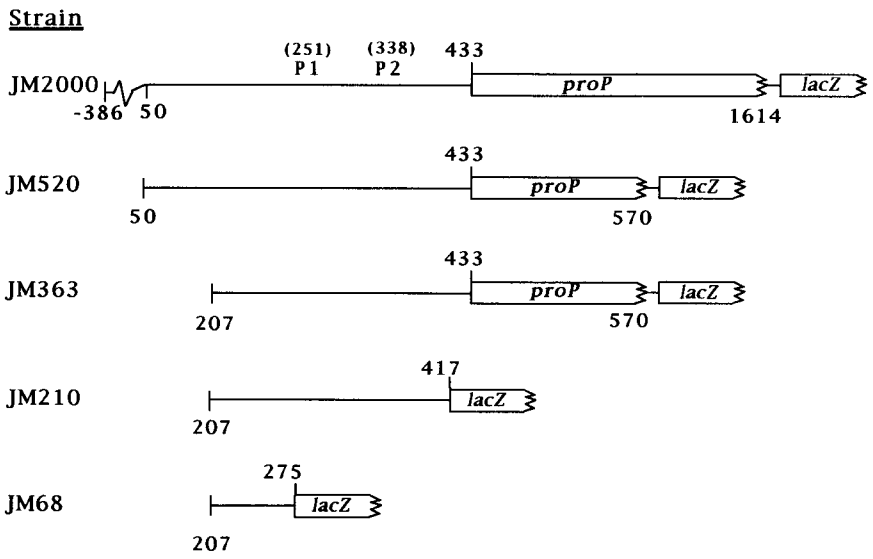


FIG. 5. Deletion analysis of the *proP* regulatory region. Constructions of *proP-lacZ* fusion strains are described in Materials and Methods. Position 433 is the start of translation of the *proP* structural gene (7). The positions of P1 and P2 are indicated relative to positions 433 and 50. Positions corresponding to termini of *proP* fragments are also noted.

mal expression during exponential growth and for stationary-phase induction.

The fusion in JM210, which contains both P1 and P2, showed a sevenfold induction of β-galactosidase activity in stationary phase. Therefore, stationary-phase induction was dependent on DNA sequences between positions 275 and 417 (Table 2; Fig. 5).

Maximum expression of *proU* requires an upstream activating sequence. Although not necessary for osmotic inducibility, it contributes approximately threefold to expression (24). If *proP* contained a similar element, it would be present in JM520 and deleted in JM363, which contains no upstream DNA beyond position −44 of P1. Comparison of the β-galactosidase activities from these two fusions showed that removal of the upstream sequences caused an approximate twofold reduction in specific activity in minimal medium, with or without the addition of salt. Activity was also reduced two to threefold during exponential phase in complex medium. However, osmotic and stationary-phase induction still occurred. Since primer extension analysis did not detect a promoter in this region, we conclude that the region between positions 50 and 207 contains an upstream activating sequence which contributes approximately twofold to *proP* expression.

TABLE 2. Deletion analyses of the *proP* regulatory region

Relevant genotype	β-Galactosidase sp act[a]			
	Minimal medium		Complex medium	
	No addition	+0.3 M NaCl	Exponential phase	Stationary phase
2,000-bp Φ(*proP-lacZ*)	1,809	4,130	341	1,164
68-bp Φ(*proP-lacZ*)	1,067	1,594	57	49
210-bp Φ(*proP-lacZ*)	867	2,184	98	729
363-bp Φ(*proP-lacZ*)	983	2,193	159	886
520-bp Φ(*proP-lacZ*)	2,102	4,000	388	995

[a] Values from the *proP-lacZ* fusions (nanomoles per minute per milligram) are from an assay typical of four separate experiments in which the same regulatory trends were observed.

The deletion analysis also searched for a downstream regulatory element in *proP* analogous to the negative regulatory sequences located within *proV*, the first structural gene of the *proU* operon (9, 32). Removal of this element causes an approximate 10-fold increase in basal *proU* expression. Removing sequences encoding the *proP* structural gene did not significantly alter expression. β-Galactosidase activity from the fusion in JM210, which contains none of the *proP* structural gene, was very similar to that from the fusion in JM363. Both of these *proP-lacZ* fusions exhibited osmotic and stationary-phase induction. Therefore, *proP* differs from *proU* in that there appeared to be no negative regulatory element in the *proP* structural gene.

In vitro *proP* expression. The increase in intracellular potassium concentration resulting from osmotic stress is thought to signal *proU* expression (38). In vitro studies consistent with this conclusion demonstrated that *proU* expression was stimulated by elevated potassium glutamate concentration both in coupled transcription-translation systems (34) and in purified transcription systems containing only RNA polymerase and nucleotides (33, 39). To determine whether *proP* expression responds to similar signals, we compared *proP* and *proU* expression in an S-30 coupled transcription-translation system. Plasmids pDC15 and pBP1, which contain *proP* and *proU′* (*proV′*) genes, respectively, and a functional *bla* gene were used to program ^{35}S-labeled protein synthesis.

The proposed amino acid sequence of ProP predicts a highly hydrophobic protein with molecular mass of 54.8 kDa (7). Typical of hydrophobic membrane proteins, ProP migrated as a diffuse band with greater mobility than would be predicted by its molecular mass (Fig. 6). Migration of the ProV′ protein corresponded to a molecular mass of 42 kDa, as previously observed (34). To quantify expression, ProU′ and ProP ^{35}S-labeled protein bands were excised and counted (Table 3). ProP expression was maximal with the addition of 0.1 M potassium glutamate but showed reduced expression, from 2,738 to 1,693 cpm, with the addition of this salt to 0.3 M. ProU′ expression increased approximately fivefold in the presence of 0.3 M potassium glutamate, in a manner consistent with pre-

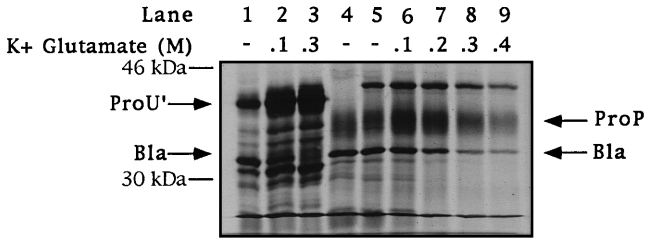


FIG. 6. In vitro *proP* expression. Plasmid DNA containing either *proP* or *proU′*, in vectors also containing a functional *bla* gene, was added to a purified in vitro transcription-translation system extract. Samples were incubated at 37°C for 1 h with the concentration of potassium glutamate indicated above each lane. Lanes 1 to 3 contain ProU′ (ProV′) samples, denatured. Lane 4 contains a denatured ProP sample, and lanes 5 to 9 contain ProP samples which were not denatured prior to electrophoresis.

vious reports (17, 33, 34, 39). The inhibition of ProP expression by potassium glutamate was similar to the inhibition of *bla* (Fig. 6), which is known to be inhibited in vivo and in vitro by hyperosmolarity (29, 33).

These in vitro experiments were useful in understanding the regulatory differences between *proP* and *proU*. Current evidence suggests that osmotic induction of *proU* occurs partly through the potassium-mediated relief of repression by the DNA-binding protein H-NS (25, 29, 39). H-NS interacts largely within the first structural gene of the *proU* operon, *proV*, to strongly repress operon expression. Deletion of sites within *proV* derepresses *proU* about 10-fold. Our deletion experiments with *proP* demonstrated that this gene lacked a negative regulatory element in its structural gene. Furthermore, *proP* basal expression is higher than that of *proU*, and mutations at the *hns* locus do not affect *proP* expression in vivo (29). If the model describing *proU* induction as due to the potassium-induced relief of H-NS repression is correct, then the absence of potassium glutamate induction of *proP* is not surprising. Rather, it correlates with the absence of HN-S repression and the lack of a downstream regulatory element in the *proP* system.

Concluding remarks. *proP* utilizes two different promoter sites to respond to hyperosmotic and stationary-phase signals. This differs from genes whose induction in response to both stationary-phase and hyperosmosis requires σ^S (15, 18, 40). We have determined that the P1 promoter contributes significantly to overall expression in minimal medium and is central to the osmotic response. However, osmotic induction of *proP* did not rely on σ^S. Moreover, the P1 initiation site was not required for stationary-phase induction in complex medium. Rather, *proP* stationary-phase induction occurred at a second downstream promoter (P2), and transcription from P2 was σ^S dependent.

TABLE 3. Quantification of ProP and ProU′ in vitro expression

Plasmid	Added K^+ glutamate (M)	cpm
pDC15 (*proP*)	0.0	2,738
	0.1	4,325
	0.2	2,747
	0.3	1,693
	0.4	1,659
pBP1 (*proU′*)	0.0	5,784
	0.1	22,222
	0.3	32,426

proP can now be added to the growing list of dual-signal genes responsive to both osmotic and stationary-phase signals.

proP and *proU* perform a common function during osmotic stress, the transport and accumulation of glycine betaine. These multiple systems are comparable to the inducible *kdp* (20, 35) and constitutive *trk* (35) systems for the transport of potassium, an ion important in the initial response to osmotic stress (10). P1 of *proP* is similar to the *proU* promoter both in sequence and in the presence of upstream activating sites. However, downstream sequences which repress *proU* under nonactivating conditions are absent in *proP*. Therefore, while *proU* provides an inducible system for the uptake of glycine betaine, *proP* mediates a partly constitutive system.

ACKNOWLEDGMENTS

We are grateful to Janet Wood and Doreen Culham for providing strains and access to unpublished data.

This study was supported by NIH grant GM337788 awarded to M.V.

REFERENCES

1. **Aiba, H., S. Adhya, and B. de Crombrugghe.** 1981. Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. J. Biol. Chem. **256:**11905–11910.
2. **Anderson, R. R., R. Menzel, and J. M. Wood.** 1980. Biochemistry and regulation of a second L-proline transport system in *Salmonella typhimurium*. J. Bacteriol. **141:**1071–1076.
3. **Barron, A., G. May, E. Bremer, and M. Villarejo.** 1986. Regulation of envelope protein composition during adaptation to osmotic stress in *Escherichia coli*. J. Bacteriol. **167:**433–438.
4. **Cairney, J., I. R. Booth, and C. F. Higgins.** 1985. *Salmonella typhimurium proP* gene encodes a transport system for the osmoprotectant betaine. J. Bacteriol. **164:**1218–1223.
5. **Cairney, J., I. R. Booth, and C. F. Higgins.** 1985. Osmoregulation of gene expression in *Salmonella typhimurium*: *proU* encodes an osmotically induced betaine transport system. J. Bacteriol. **164:**1224–1232.
6. **Casadaban, M.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104:**541–555.
7. **Culham, D. E., B. Lasby, A. G. Marangoni, J. L. Milner, B. A. Steer, R. W. van Nues, and J. M. Woods.** 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.
8. **Dattananda, C. S., and J. Gowrishankar.** 1989. Osmoregulation in *Escherichia coli*: complementation analysis and gene-protein relationships in the *proU* locus. J. Bacteriol. **171:**1915–1922.
9. **Dattananda, C. S., K. Rajkumari, and J. Gowrishankar.** 1991. Multiple mechanisms contribute to osmotic inducibility of *proU* operon expression in *Escherichia coli*: demonstration of two osmoresponsive promoters and of a negative regulatory element within the first structural gene. J. Bacteriol. **173:**7481–7490.
10. **Dinnbier, U., E. Limpinsel, R. Schmid, and E. P. Bakker.** 1988. Transient accumulation of potassium glutamate and its replacement by trehalose during adaptation of growing cells of *Escherichia coli* K-12 to elevated sodium chloride concentrations. Arch. Microbiol. **150:**348–357.
11. **Dunlap, V. J., and L. N. Csonka.** 1985. Osmotic regulation of L-proline transport in *Salmonella typhimurium*. J. Bacteriol. **163:**296–304.
12. **Elliott, T.** 1992. A method for constructing single-copy *lac* fusions in *Salmonella typhimurium* and its application to the *hemA-prfA* operon. J. Bacteriol. **174:**245–253.
13. **Gowrishankar, J.** 1989. Nucleotide sequence of the osmoregulatory *proU* operon of *Escherichia coli*. J. Bacteriol. **171:**1923–1931.
14. **Grothe, S., R. L. Krogsurd, D. J. McClellan, J. L. Milner, and J. M. Wood.** 1986. Proline transport and osmotic response in *Escherichia coli* K-12. J. Bacteriol. **166:**253–259.
15. **Hengge-Aronis, R., W. Klein, R. Lange, M. Rimmele, and W. Boos.** 1991. Trehalose synthesis genes are controlled by the putative sigma factor encoded by *rpoS* and are involved in stationary-phase thermotolerance in *Escherichia coli*. J. Bacteriol. **173:**7918–7924.
16. **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.
17. **Jovanovich, S. B., M. T. Record, Jr., and R. R. Burgess.** 1989. In an *Escherichia coli* coupled transcription-translation system, expression of the osmoregulated gene *proU* is stimulated at elevated potassium concentrations and by an extract from cells grown at high osmolality. J. Biol. Chem. **264:**7821–7825.
18. **Jung, J. U., C. Gutierrez, F. Martin, M. Ardourel, and M. Villarejo.** 1990.

Transcription of *osmB*, a gene encoding an *Escherichia coli* lipoprotein, is regulated by dual signals. Osmotic stress and stationary phase. J. Biol. Chem. **265:**10574–10581.

19. **Kumar, A., R. A. Malloch, N. Fufita, D. A. Smillie, A. Ishihama, and R. S. Hayward.** 1993. The minus 35-recognition region of *Escherichia coli* sigma 70 is inessential for initiation of transcription at an "extended minus 10" promoter. J. Mol. Biol. **232:**406–418.
20. **Laimins, L. A., D. B. Rhoads, and W. Epstein.** 1981. Osmotic control of *kdp* operon expression in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **78:**464–468.
21. **Lange, R., and R. Hengge-Aronis.** 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. J. Bacteriol. **5:**49–59.
22. **Lange, R., and R. Hengge-Aronis.** 1994. The cellular concentration of the σ^S subunit of RNA-polymerase in *Escherichia coli* is controlled on the levels of transcription, translation and protein stability. Genes Dev. **8:**1600–1612.
23. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
24. **Lucht, J. M., and E. Bremer.** 1991. Characterization of mutations affecting the osmoregulated *proU* promoter of *Escherichia coli* and identification of 5′ sequences required for high-level expression. J. Bacteriol. **173:**801–809.
25. **Lucht, J. M., P. Dersch, B. Kempf, and E. Bremer.** 1994. Interactions of the nucleoid-associated DNA-binding protein H-NS with the regulatory region of the osmotically controlled *proU* operon of *Escherichia coli*. J. Biol. Chem. **269:**6578–6586.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **May, G., E. Faatz, J. M. Lucht, M. Haardt, M. Bolliger, and E. Bremer.** 1989. Characterization of the osmoregulated *Escherichia coli proU* promoter and identification of ProV as a membrane-associated protein. Mol. Microbiol. **3:**1521–1531.
28. **May, G., E. Faatz, M. Villarejo, and E. Bremer.** 1986. Binding protein dependent transport of glycine betaine and its osmotic regulation in *Escherichia coli* K12. Mol. Gen. Genet. **205:**225–233.
29. **Mellies, J., R. Brems, and M. Villarejo.** 1994. The *Escherichia coli proU* promoter element and its contribution to osmotically signaled transcription activation. J. Bacteriol. **176:**3638–3645.
30. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **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.
32. **Overdier, D. G., and L. N. Csonka.** 1992. A transcriptional silencer downstream of the osmotically controlled *proU* operon of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **89:**3140–3144.
33. **Prince, W. S., and M. R. Villarejo.** 1990. Osmotic control of *proU* transcription is mediated through direct action of potassium glutamate on the transcription complex. J. Biol. Chem. **265:**17673–17679.
34. **Ramirez, R. M., W. S. Prince, E. Bremer, and M. Villarejo.** 1989. In vitro reconstitution of osmoregulated expression of *proU* of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **86:**1153–1157.
35. **Rhoads, D. B., F. B. Waters, and W. Epstein.** 1976. Cation transport in *Escherichia coli*. VIII. Potassium transport mutants. J. Gen. Physiol. **67:**325–341.
36. **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.
37. **Stalmach, M. E., S. Grothe, and J. M. Wood.** 1983. Two proline porters in *Escherichia coli* K-12. J. Bacteriol. **156:**481–486.
38. **Sutherland, L., J. Cairney, M. J. Elmore, I. R. Booth, and C. F. Higgins.** 1986. Osmotic regulation of transcription: induction of the *proU* betaine transport gene is dependent on accumulation of intracellular potassium. J. Bacteriol. **168:**805–814.
39. **Ueguchi, C., and T. Mizuno.** 1993. The *Escherichia coli* nucleoid protein H-NS functions directly as a transcriptional repressor. EMBO J. **12:**1039–1046.
40. **Yim, H. H., R. L. Brems, and M. Villarejo.** 1994. Molecular characterization of the promoter of *osmY*, an *rpoS*-dependent gene. J. Bacteriol. **176:**100–107.