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The *rpoE* Gene of *Escherichia coli*, Which Encodes σ^E, Is Essential for Bacterial Growth at High Temperature

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In vitro transcription analysis has shown that only RNA polymerase containing an alternative sigma subunit, σ^E, activates transcription from one of the *rpoH* promoters and the *htrA* promoter. The location of the *rpoE* gene encoding σ^E on the *Escherichia coli* chromosome has recently been established, but no *rpoE* mutant has yet become available for phenotypic testing. We cloned the *rpoE* gene from the λ-ordered clones of the *E. coli* genome and confirmed that the reconstituted RNA polymerase containing the gene product ($E\sigma^E$) can transcribe *htrA* in vitro. We constructed an *rpoE*-defective strain by gene disruption using the cloned *rpoE* gene. We demonstrate that expression of *htrA* is completely dependent on the *rpoE* gene in vivo and that the *rpoE* gene is essential for bacterial growth at high temperature.

In *Escherichia coli*, RNA polymerase consists of the subunits of 2α, β, β′, and a specific σ. The σ subunit directs RNA polymerase to initiate transcription at promoter sites on the DNA (6). The primary σ factor, σ^{70} encoded by *rpoD*, is responsible for transcription of the majority of the genes expressed during exponential growth. In addition, alternative σ factors direct transcription of sets of genes whose products are needed for specific functions, such as nitrogen fixation, flagellum synthesis, heat shock response, and stationary-phase growth (4).

The activities of two alternative σ factors, σ^{32} and σ^E, increase after the cell is exposed to a high temperature or ethanol (2, 3, 5, 21, 25, 28). RNA polymerase containing σ^{32} ($E\sigma^{32}$) transcribes the heat shock genes whose products are related to chaperones and proteases (29). The functions of many of these heat shock proteins are involved in binding to cytoplasmic proteins and assist the cytoplasmic proteins in folding or unfolding (7, 15, 22, 26) and in protecting the cell from severe stresses such as exposure to 10% ethanol or a 50°C temperature (18). Using an in vitro transcription assay, Erickson and Gross (2) and Wang and Kaguni (25) have independently identified the σ^E subunit that is essential for transcription from one ($rpoHp_3$) of the promoters of *rpoH* encoding σ^{32} and the promoter of *htrA* encoding a periplasmic endopeptidase essential for growth at high temperatures. Mecsas et al. (16) have proposed that the σ^E regulon is involved in the processes that function in the extracytoplasmic compartments. The map position of *rpoE* encoding σ^E on the *E. coli* chromosome has recently been determined by sequence comparisons with other σ genes, especially a subfamily whose products regulate extracytoplasmic functions, and an open reading frame upstream of *nadB* near 55.5 min (1, 11) (GenBank accession no. X01180 and D13169). More importantly, the predicted protein sequence encoded by this open reading frame corresponds to known peptide sequences of σ^E (11).

A 2.8-kb *Eco*RI fragment containing the gene was isolated from Kohara's ordered clone λ4A12 and cloned into the *Eco*RI site of vector pRS415 (19), giving pRPOE1 (Fig. 1). The DNA corresponding to the *rpoE* coding region in pRPOE1 was amplified by PCR (Fig. 2A). After digestion of the PCR products with *Nde*I and *Bam*HI enzymes, the DNA fragment was cloned into a T7 promoter plasmid, pT7-7 (23), and the resultant plasmid was designated pTRPOE (Fig. 2A). The *rpoE* DNA fragment of pTRPOE was further cloned into pQE30 to make pQRPOE for the His-tagged σ^E overproduction (Fig. 2A). The pTRPOE and pQRPOE plasmids complemented the $RpoE^-$ phenotype (see below). Overproduction of the 6×-His-tagged σ^E protein in strain M15 (carrying pREP4, which contains the *lacI*^{q} gene and the Km^r gene) (QIAGEN Inc.) containing pQRPOE was achieved by addition of 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the medium. Figure 3 shows the results of analysis by sodium dodecyl sul-

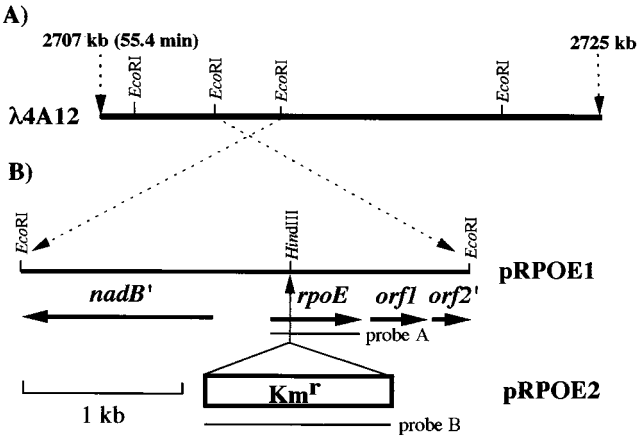

FIG. 1. Physical map of the *rpoE* gene and flanking regions. (A) *Eco*RI restriction map of the λ clone 4A12, as described by Kohara et al. (13). (B) Enlarged map of the 2.8-kb *Eco*RI-*Eco*RI fragment of pRPOE1. The horizontal arrows show the directions of transcription according to the sequence with GenBank accession no. D13169. pRPOE2 was constructed by inserting the kanamycin resistance (Km^r) gene into the *Hin*dIII site of pRPOE1. Probes A and B were used for Southern blot analysis.

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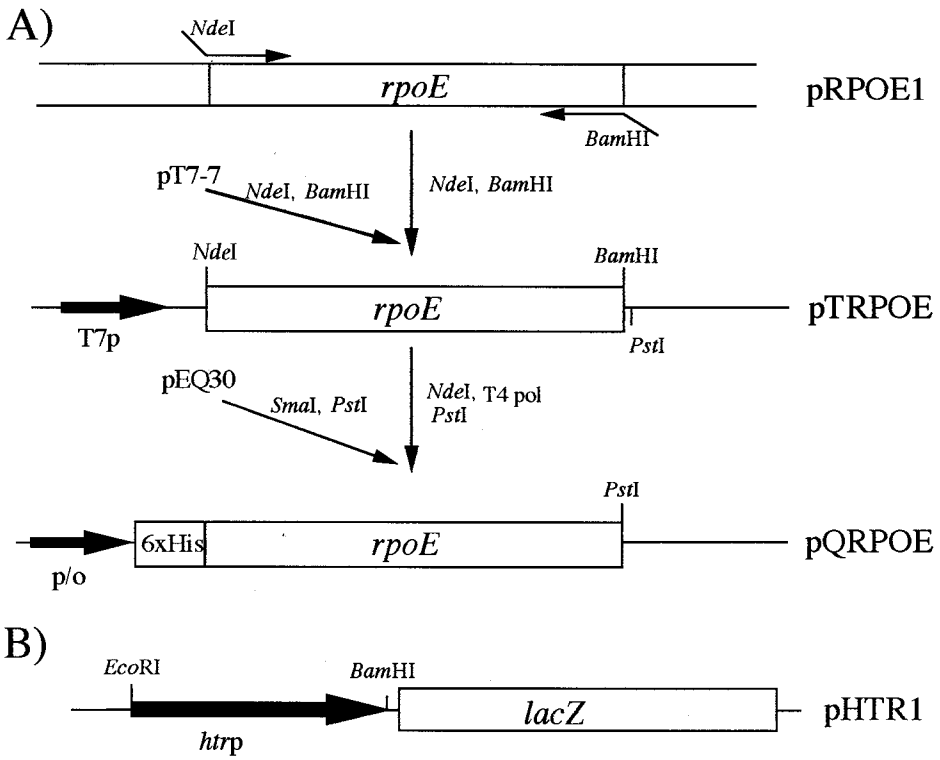

FIG. 2. Construction of *rpoE* and *htrA-lacZ* plasmids. (A) The *rpoE* coding region was amplified by PCR in the presence of 1 ng of pRPOE1 as a template. The synthetic primers 5′-CTTTACCTCATATGAGCGAGCAGTTAAC and 5′-CCAGGATCCCGCTATCGTCAAC were used. The two primers contain an *Nde*I and a *Bam*HI site (underlined), respectively. We used thermostable Pfu DNA polymerase (Toyobo) with proofreading activity (12). The reaction was carried out according to the manufacturer's recommendations. The *Nde*I-*Bam*HI DNA fragment containing *rpoE* was cloned into pT7-7 to make pTRPOE. pTRPOE was digested with *Nde*I, treated with T4 DNA polymerase (T4 pol), and digested with *Pst*I. The DNA fragment containing *rpoE* was cloned into the *Sma*I and *Pst*I sites of a 6×-His-tagged vector, pQE30 (QIAGEN Inc.), which contains two LacI binding sites in the promoter region, and the resultant plasmid was designated pQRPOE. p/o, promoter and two *lac* operators. (B) The *htrA* promoter region was amplified by PCR in the presence of 1 μg of total genomic DNA of MC4100 with primers 5′-GTAGAATTCTAACCAGGCTTTTGTAA and 5′-AGTGGATCCAGTGCTAATGTGGTTTT. The two primers contain an *Eco*RI and a *Bam*HI site (underlined), respectively. After digestion of the PCR products with *Eco*RI and *Bam*HI, the DNA fragment was cloned into pRS415 to make pHTR1, in which the *htrA* promoter was fused to the *lacZ* gene to give an operon fusion. The *Eco*RI and *Bam*HI sites are located at positions −97 and +68, respectively, from the transcription initiation site of *htrA* (10).

fate-polyacrylamide gel electrophoresis (SDS-PAGE) of the overexpression and purification of His-tagged σ^E. For purification of the 6×-His-tagged σ^E protein, the crude lysate denatured by 8 M urea was applied to a Ni-nitrilotriacetic acid spin column (QIAGEN Inc.). The 6×-His-tagged σ^E protein was eluted with elution buffer (8 M urea, 200 mM imidazole, 0.1 M NaH_2PO_4, 10 mM Tris-HCl, pH 8.0) and purified to near homogeneity as judged by a Coomassie brilliant blue-stained gel (Fig. 3, lane 5). We also tried to overproduce native σ^E using the pTRPOE plasmid and the strain BL21(λDE3) (23), but we could not overproduce the protein to a similar level.

To renature the denatured 6×-His-tagged σ^E protein, it was dialyzed against buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 50% glycerol. To reconstitute holoenzyme, the core RNA polymerase (1 pmol) was mixed with a fourfold molar excess of σ^{70} or His-tagged σ^E and the mixtures were incubated for 20 min at 30°C. The activities of the reconstituted RNA polymerases were assayed by in vitro transcription with linear *tac* and *htrA* DNAs as templates. As shown previously (8), in addition to the core RNA polymerase (E), σ^{70} was required for specific transcription initiated from the *tac* promoter (Fig. 4, lanes 1 and 3). However, the *tac* template was not transcribed by $E\sigma^E$ (Fig. 4, lane 4). In contrast, the *htrA* template was efficiently transcribed by $E\sigma^E$ (Fig. 4, lane 6) but

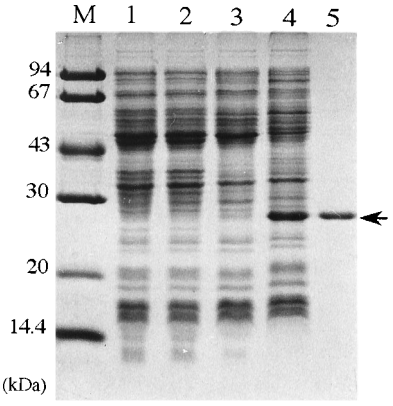

FIG. 3. Overproduction and purification of His-tagged σ^E. Strain M15 (pREP4) carrying the vector pQE30 or the overproduction plasmid pQRPOE was grown at 37°C to mid-exponential phase in L broth containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml). Cells were further grown for 4 h in the absence or presence of 2 mM IPTG. The samples were subjected to SDS–13.5% PAGE and stained with Coomassie brilliant blue. Lane M, molecular mass markers as indicated on the left; lanes 1 and 2, total proteins of cells carrying pQE30 without and with IPTG, respectively; lanes 3 and 4, total proteins of cells carrying pQRPOE without and with IPTG, respectively; lane 5, purified His-tagged σ^E (arrow).

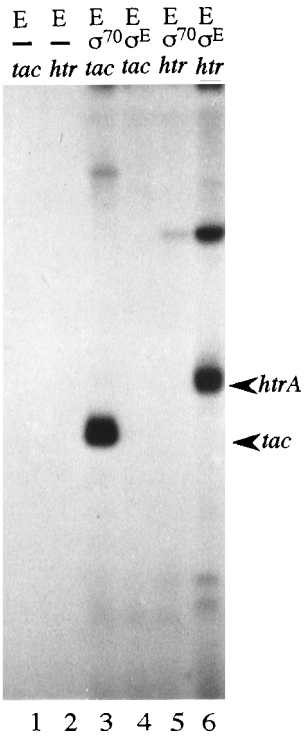

FIG. 4. In vitro transcription by the reconstituted RNA polymerases. Single-round in vitro transcription was carried out as described by Makino et al. (14). A reaction mixture containing the template DNA (0.2 pmol) and the reconstituted RNA polymerase in 35 μl of transcription buffer (50 mM Tris-HCl [pH 7.5], 200 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol) was preincubated for 10 min at 37°C to allow the formation of open complex. The substrate solution (15 μl), containing heparin, ATP, GTP, CTP, and [^{32}P]UTP, was then added to initiate one round of RNA synthesis. After 5 min, labeled transcripts were precipitated with ethanol and analyzed by electrophoresis on a polyacrylamide gel containing 8 M urea. The components of each reaction mixture, RNA polymerase core enzyme (E), sigma subunit (σ^{70} or σ^E), and DNA template (*tac* or *htrA*), are given above the lanes. The templates used were the 299-bp *Bam*HI-*Hin*dIII fragment of pKK233-2 (Pharmacia) carrying the *tac* promoter and the 165-bp *Eco*RI-*Bam*HI fragment of pHTR1 (Fig. 2B) carrying the *htrA* promoter. The predicted lengths of the runoff transcripts from the *tac* and *htrA* promoters are 56 and 68 nucleotides, respectively. Positions of the specific transcripts are indicated by arrows.

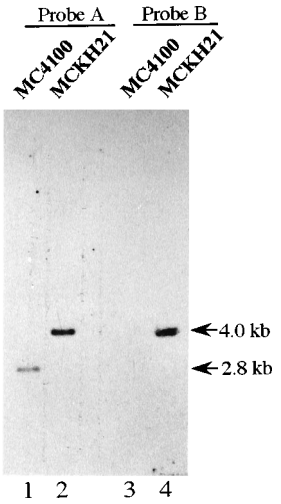

FIG. 5. Southern blot hybridization of chromosomal DNA isolated from MC4100 (wild type) and MCKH21 (*rpoE*::Km^r). Probe A (Fig. 1B) was a 0.6-kb *Nde*I-*Bam*HI DNA fragment containing the *rpoE* gene derived from pTRPOE (Fig. 2A). Probe B (Fig. 1B) was a 1.2-kb *Hin*cII-*Hin*cII DNA fragment containing the Km^r gene of pUC4K. Each probe was labeled with [α-^{32}P]dCTP by nick translation. Lanes 1 and 2, results of hybridization with probe A; lanes 3 and 4, results of hybridization with probe B. In lanes 1 and 3, *Eco*RI-digested chromosomal DNA of MC4100 was analyzed, and in lanes 2 and 4, that of MCKH21 was analyzed. Unlabeled size marker DNA fragments were analyzed in the same gel, and their mobilities are indicated by arrows.

was not transcribed by E and Eσ^{70} (lanes 2 and 5). These in vitro results indicate that the purified protein is the σ factor required for specific transcription from the *htrA* promoter and the renatured σ^E is active for the formation of the active RNA polymerase holoenzyme Eσ^E.

Since no strain with a mutation in *rpoE* has been reported, we constructed an *rpoE* mutant strain by gene disruption using the cloned gene as described by Winans et al. (27). pRPOE1 has only one *Hin*dIII site in the *rpoE* coding region (Fig. 1B). pRPOE1 linearized by *Hin*dIII was treated with T4 DNA polymerase and ligated with the *Hin*cII fragment of pUC4K that contains the Km^r gene (24). The resultant plasmid, pRPOE2 (Fig. 1B), was digested with *Eco*RI, and the DNA fragment that contained the *rpoE* gene interrupted by the Km^r gene was introduced into strain JC7623 (*recBC sbcB*). Km^r transformants were selected. The Km^r marker was transferred into the MC4100 strain by transduction with P1 *vir* phage. As shown in Fig. 5, we confirmed the insertion of the Km^r gene into the *rpoE* gene in one of the transductants (MCKH21) by Southern blot hybridization (20). Chromosome DNA was extracted from MC4100 and MCKH21, digested with *Eco*RI, separated by electrophoresis, transferred to a filter membrane, and hybridized with radioactive probe DNA. Probe A (Fig. 1B), which contained the entire *rpoE* gene, hybridized with the 2.8-kb fragment of MC4100 (Fig. 5, lane 1) but hybridized with the 4.0-kb fragment of MCKH21 (lane 2). Probe B (Fig. 1B), which contained the 1.2-kb Km^r gene, did not hybridize with any DNA fragment of MC4100 (lane 3) but hybridized with the same 4.0-kb fragment as the one in lane 2 (lane 4). These results show that the 2.8-kb *Eco*RI fragment of the chromosome that contains the *rpoE* gene is disrupted by the Km^r gene.

We examined the β-galactosidase activities of strain MCKH21 (*rpoE*::Km^r) and its parental strain, MC4100, both containing a single-copy *htrA-lacZ* gene on the chromosome. For this purpose, we made a λ*htrA-lacZ* phage by using pHTR1 (Fig. 2B) and λRS45 according to the method of Simons et al. (19). The phage was introduced into the attachment sites of MC4100 and MCKH21 to make MC4100λΦ[*htrA-lacZ*] and MCKH21λΦ[*htrA-lacZ*], respectively. The β-

TABLE 1. Effect of *rpoE* mutation on *htrA-lacZ* expression

Strain	Plasmid	β-Galactosidase activity[a] (Miller units)
MC4100λΦ[*htrA-lacZ*]	None	1,840
MCKH21λΦ[*htrA-lacZ*]	None	3
MCKH21λΦ[*htrA-lacZ*]	pT7-7 (vector)	5
	pTRPOE	4,540
	pQE30 (vector)	4
	pQRPOE	9,130

[a] Cultures were grown overnight at 30°C in L broth. Ampicillin (100 μg/ml) and kanamycin (50 μg/ml) were added when required. Aliquots were diluted 1,000-fold into the same fresh medium and grown for approximately 12 h. β-Galactosidase activity was assayed as described by Miller (17). The data are the averages for three experiments.

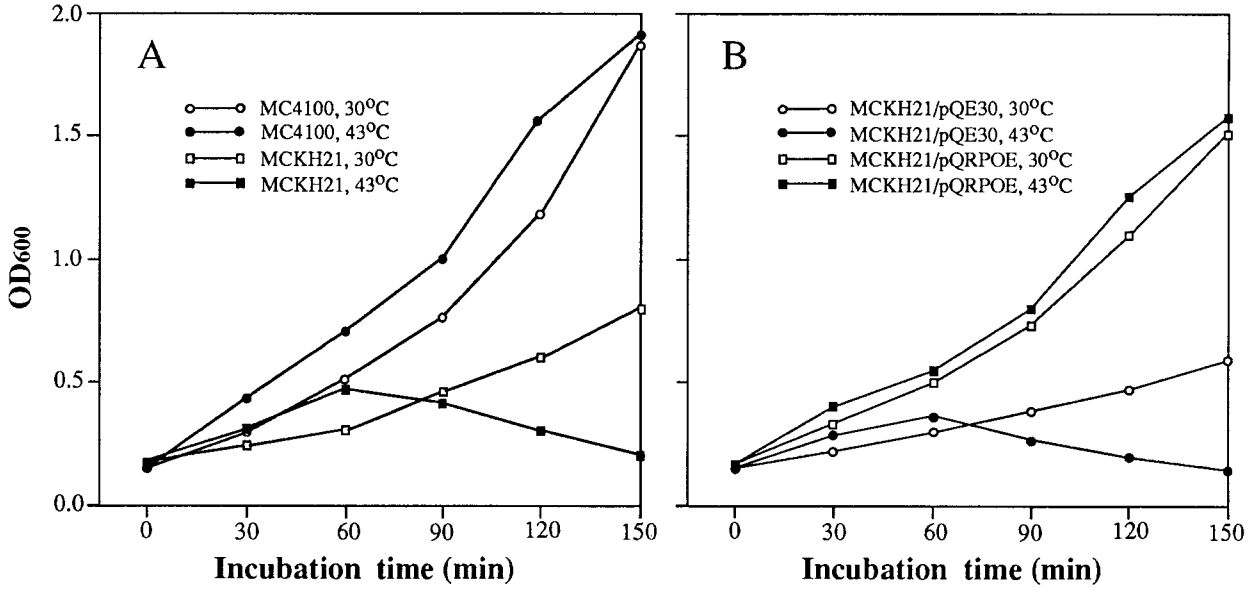

FIG. 6. Growth curves of MC4100 (wild type) and MCKH21 (*rpoE*::Km^r) (A) and MCKH21 containing pQE30 or pQRPOE (B). Cultures were grown overnight at 30°C in L broth. Ampicillin (100 μg/ml) and kanamycin (50 μg/ml) were added when required. Aliquots were diluted 1,000-fold into fresh L medium. The L broth cultures were either incubated continuously at 30°C or preincubated at 30°C and shifted to 43°C at time zero. OD_{600}, optical density at 600 nm.

galactosidase activities of these cells were completely dependent on the intact *rpoE* gene (Table 1). Moreover, when the plasmid pTRPOE or pQRPOE (Fig. 2A), containing the *rpoE* gene, was introduced into the *rpoE* mutant strain MCKH21λΦ [*htrA-lacZ*], the β-galactosidase activity was restored. These results demonstrate that the *rpoE* gene is responsible for the expression of the *htrA* gene.

The *htrA* mutants were originally isolated as mini-Tn*10* transposon insertion mutants which could grow at 30°C but could not grow at temperatures above 42°C (9, 10). Since expression of the *htrA* gene was very strictly regulated by σ^E in vitro and in vivo, we thought that the *rpoE*-deficient strain could not grow at high temperature. The growth curves of isogenic MC4100 (wild type) and MCKH21 (*rpoE*::Km^r) in L broth at 30 and 43°C are shown in Fig. 6A. MCKH21 cells began to lyse at 60 min after the shift to 43°C, as observed with the *htrA* mutant cells (9). The colony-forming ability of the *rpoE* mutant cells began to decline as the optical density of the cells decreased (data not shown). It is noted that *rpoE* disruption certainly has a deleterious effect on growth at 30°C. Moreover, when pQRPOE, containing the *rpoE* gene, was introduced into MCKH21, these RpoE$^-$ phenotypes were complemented both at 30 and 43°C (Fig. 6B). These results show that the *rpoE* gene is essential for bacterial growth at high temperature.

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