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Negative Regulation of DNA Repair Gene (*ung*) Expression by the CpxR/CpxA Two-Component System in *Escherichia coli* K-12 and Induction of Mutations by Increased Expression of CpxR

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In *Escherichia coli* K-12 overexpressing CpxR, transcription of the *ung* gene for uracil-DNA glycosylase was repressed, ultimately leading to the induction of mutation. Gel shift, DNase I footprinting, and in vitro transcription assays all indicated negative regulation of *ung* transcription by phosphorylated CpxR. Based on the accumulated results, we conclude that *ung* gene expression is negatively regulated by the two-component system of CpxR/CpxA signal transduction.

The *cpxRA* two-component system of *Escherichia coli*, consisting of CpxA sensor kinase or phosphatase and CpxR cognate response regulator, regulates biofilm formation, motility, chemotaxis, host cell invasion, and virulence (5, 8, 13, 21). By using the generally accepted DNA sequence for binding CpxR (two tandem GTAAA motifs separated by a 5-bp linker, the CpxR box), DeWulf et al. (6) performed 15-bp weighted matrix analysis for CpxR recognition sites on the entire *E. coli* genome and identified new target genes (*ung*, *ompC*, *psd*, *mviA*, *aroK*, *rpoE*, *secA*, and *aer*) that might be regulated positively or negatively by CpxR/CpxA. The expression of *cpxRA* increases sharply at the onset of the stationary growth phase (5), suggesting the involvement of the Cpx system in stationary phase survival.

Uracil-DNA glycosylase (Ung), the most abundant type among all of the glycosylases in bacterial cells (16, 20), excises uracil residues from DNA, which arise as a result of either misincorporation of dUTP by DNA polymerase or deamination of DNA cytosine (9, 10, 11, 16, 27). The *ung* mutants show the specific mutator effect of a G · C-to-A · T transition as Duncan and Weiss reported previously (9). The *ung* gene expression remains constant up to the early stationary phase of *E. coli* but declines in the late stationary phase (26). However, the mechanisms underlying these phenomena are not understood well.

In order to clarify the possible involvement of the Cpx system in repression of *ung* expression, the effects of CpxR overexpression on *ung* expression are investigated in this study.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* strains used are listed in Table 1. They were grown at 37°C in a Luria-Bertani (LB) medium (pH 7.5) containing 1% Bacto Tryptone (Difco), 0.5% Bacto Yeast Extract (Difco), and 1% NaCl. The following antibiotics were added to the medium: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; tetracycline, 12.5 μg/ml; and kanamycin, 25 μg/ml.

Construction of plasmids. The plasmids used are listed in Table 1. A DNA fragment (538 bp) containing the *ung* promoter region was prepared by PCR using *E. coli* BW25113 genome DNA as template and a pair of primers, ung-EcoRI-F (5′-TGGAACTTCACGGAATTCAATGTCA-3′) and ung-BamHI-R (5′-AAAATAGGGATCCTGCTTCTCTTCA-3′). After digestion with EcoRI and BamHI, the PCR-amplified fragment was inserted at the corresponding site of pRS551 to generate the plasmid pRSung. A DNA fragment (751 bp) containing the *nlpE* coding region was prepared by PCR using *E. coli* BW25113 genome DNA as a template along with a pair of primers, NLPEAF (5′-ATGCGCGGC AGAATTCGCAGCGGTCGGGAA-3′) and NLPEAR (5′-TGCGTTTGTTTC TAGATCAAGACGGGTTAC-3′). After digestion with EcoRI and BamHI, the PCR-amplified fragment was inserted at the corresponding site of pBAD18 to generate the plasmid pBADnlpE.

Preparation of the labeled probe for S1 nuclease, gel shift, and DNase I footprinting assays. Probe A was generated by PCR amplification of the *ung* promoter region with the primers ung-S1F (5′-TGATGCCTCCCCGGCAAAA T-3′) and ^{32}P-labeled ung-S1R (5′-TAGGGTTGCTGCTTCTCTTC-3′), and *E. coli* BW25113 genome DNA (100 ng) was used as the template for the Ex *Taq* DNA polymerase. The PCR product with ^{32}P at its terminus was recovered from

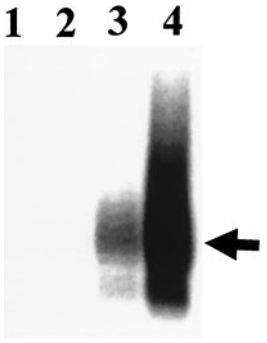

FIG. 1. Overexpression of *cpxR*. The arrow indicates *cpxR* transcript from the *lac* promoter on the plasmid p41-5ΔGFP. BW25113(pCA24ΔGFP) (lanes 1 and 2) and BW25113(p41-5ΔGFP) (lanes 3 and 4) were grown to mid-log phase (OD_{600}, 0.6) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1 mM IPTG. The S1 nuclease assay for the *cpxR* transcript was performed as described in Materials and Methods.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics[a]	Source or reference
E. coli strains		
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	4
BW27559	BW25113 Δ(*cpxR*)*623*	23
BL21	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal* (λcI*857 ind1 Sam7 nin5 lac*UV5-T7 *gene1*) *dcm* (DE3)	Novagen
BD2314	*ung-152*::Tn*10 lacY1 gal-6 trpC45 his-68 purC50 tyrA2 rpsL125 malA1 xylA7 mtl-2 thi-1* [*fluA2 tsx-70 supE44*(AS)]	9
BWung	BW25113 *ung-152*::Tn*10*	BW25113 × P1(BD2314) → Tc^r
CC102[b]	P90C *ara* Δ(*lac proB*)$_{XIII}$/F′ *lacIZ proB*$^+$	2
CC102ung	CC102 *ung-152*::Tn*10*	CC102 × P1(BD2314) → Tc^r
Plasmids		
pCA24N	Cm^r, expression vector	H. Mori[c]
pCA24ΔGFP	ΔGFP, pCA24N	H. Mori[d]
pET-21a (+)	Ap^r, expression vector	Novagen
pBAD18	Ap^r, a vector containing the arabinose P$_{BAD}$ promoter	12
pRS551	Ap^r, Km^r, *lacZ* operon fusion vector	R. W. Simons
p41-5ΔGFP	SfiI fragment (698 bp) containing *cpxR* was cloned into the corresponding sites of pCA24ΔGFP	H. Aiba
pKH50-3	BamHI-NotI fragment (698 bp) containing *cpxR* was cloned into the corresponding sites of pET-21a(+)	K. Yamamoto
pRSung	pRS551, EcoRI-BamHI (538 bp) fragment containing *ung* promoter	This study
pBADnlpE	EcoRI-XbaI fragment (751 bp) containing *nlpE* was cloned into the corresponding site of pBAD18	This study

[a] Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; Tc, tetracycline.
[b] G · C-A · T tester strain.
[c] http://ecoli.aist-nara.ac.jp/gb4/resources/archive/AC1.gif.
[d] NotI digestion and self ligation of pCA24N.

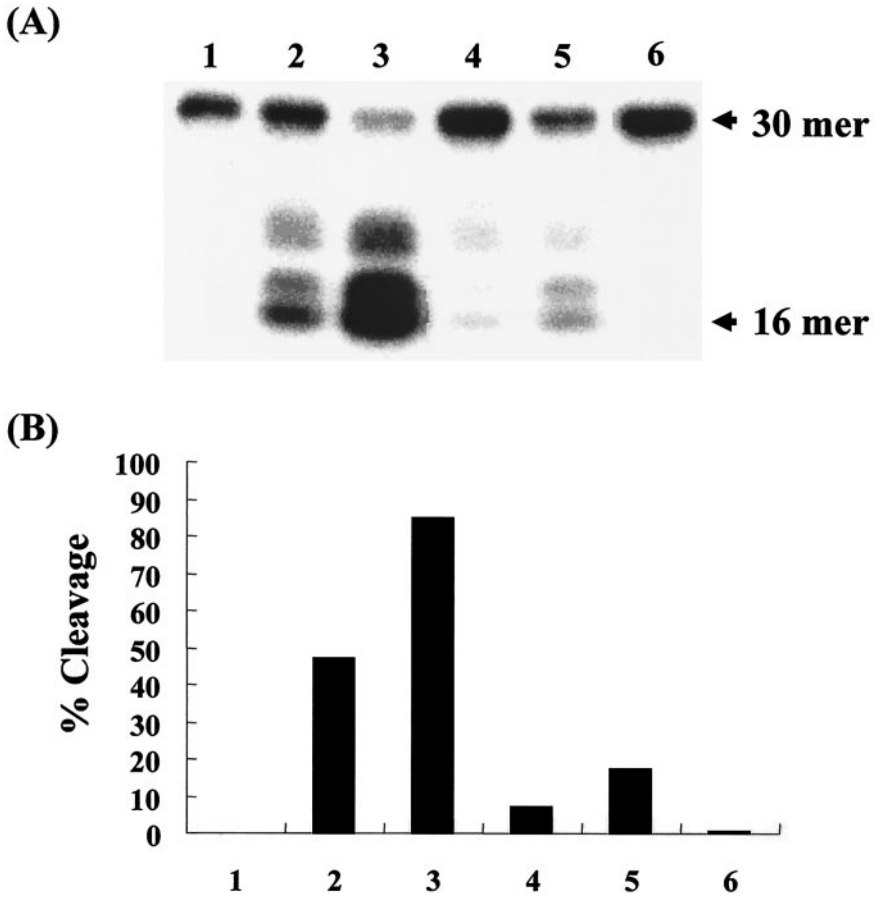

FIG. 2. Assay of uracil-DNA glycosylase (Ung) activity. (A) Ung activity was assayed as described in Materials and Methods. For this assay, cell extracts (lanes 2 and 4, 25 ng; lanes 3 and 5, 50 ng) from BW25113(pCA24ΔGFP) (lanes 2 and 3), BW25113(p41-5ΔGFP) (lanes 4 and 5), and BWung (lane 6, 50 ng) were used. The 30-mer oligonucleotide was cleaved by Ung and NaOH to form a 16-mer product. In lane 1, no cell extract was used. (B) Cleaved DNA products (from panel A) were quantified with BAS 1000 Mac (Fuji film). Percent cleavage was determined by dividing the intensity of the cleaved product (16-mer) by the total intensity, which was defined as the sum of the intensities of the intact substrate and the cleaved products.

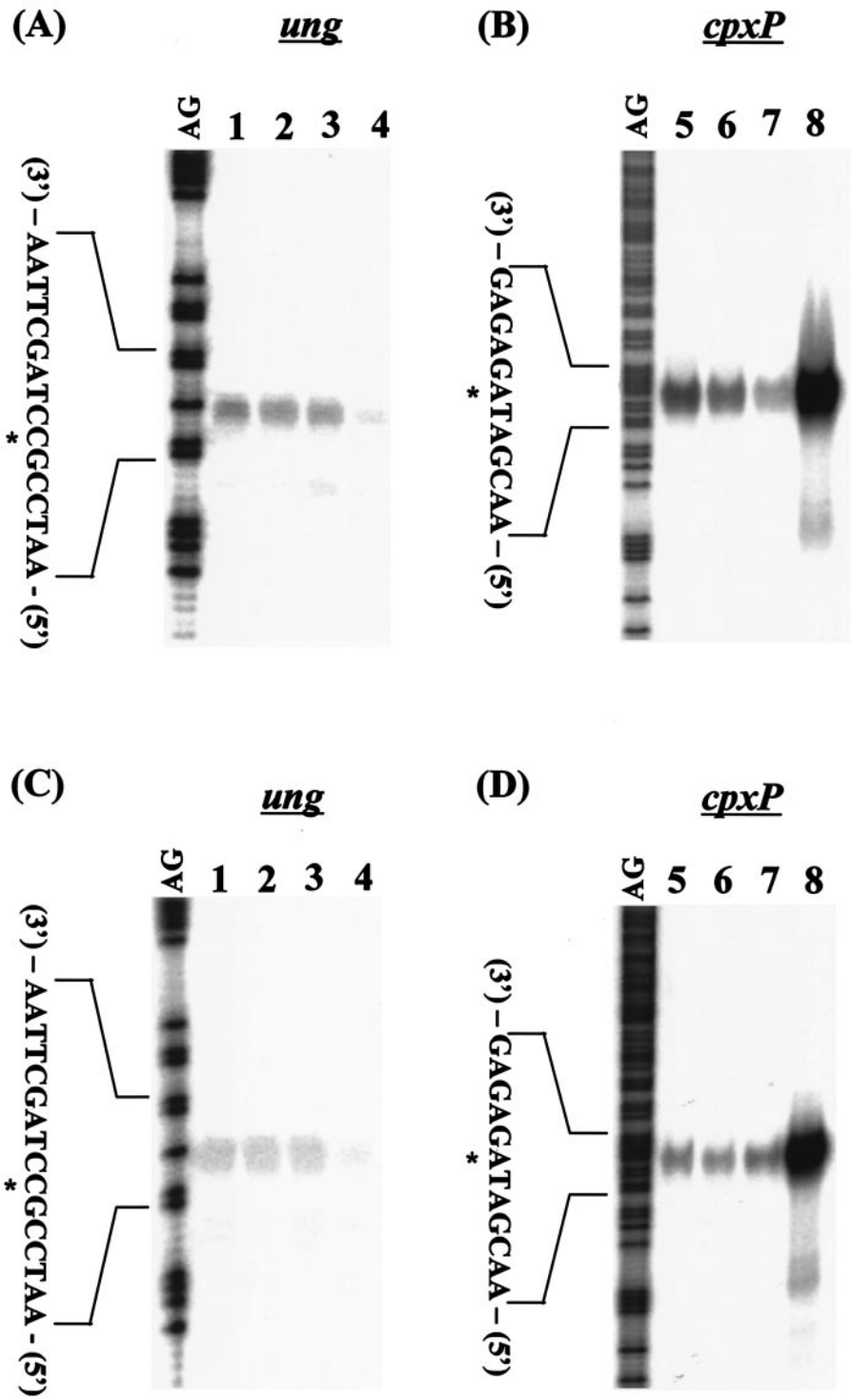

FIG. 3. Effects of overexpressed CpxR or NlpE on *ung* and *cpxP* transcription. BW25113(pCA24ΔGFP) (A and B, lanes 1, 2, 5, and 6) and BW25113(p41-5ΔGFP) (A and B, lanes 3, 4, 7, and 8) were grown to mid-log phase (OD_{600}, 0.6) in the absence (A and B, lanes 1, 3, 5, and 7) or presence (A and B, lanes 2, 4, 6, and 8) of 1 mM IPTG. S1 nuclease assays for *ung* (A) and *cpxP* (B) transcripts were performed as described in Materials and Methods. BW25113(pBAD18) (C and D, lanes 1, 2, 5, and 6) and BW25113(pBADnlpE) (C and D, lanes 3, 4, 7, and 8) were grown to mid-log phase (OD_{600}, 0.6) in the absence (C and D, lanes 1, 3, 5, and 7) or presence (C and D, lanes 2, 4, 6, and 8) of 0.2% arabinose. After that, S1 nuclease assays for *ung* (C) and *cpxP* (D) transcripts were performed. Lane AG represents the Maxam-Gilbert sequence ladder. The transcription start site is marked with an asterisk.

TABLE 2. G · C→A · T mutation frequency

Strain	No. of Lac$^+$ colonies per 10^9 cells[a] (±SD)
CC102	1.7 (±0.021)
CC102ung	5.9 (±0.253)
pCA24ΔGFP/CC102	1.8 (±0.085)
p41-5ΔGFP/CC102	11.2 (±1.060)

[a] Each value represents the average of the data from three independent experiments.

a polyacrylamide gel and then used for S1 nuclease, gel shift, and DNase I footprinting (noncoding strand) assays. The ung-S1R and labeled ung-S1F primers were used to prepare probe B for an S1 nuclease assay (transcription of *yfiD*). Probe C was generated by PCR amplification of the *cpxR* promoter region with the primers cpxRA-S1F (5′-GTTATCGCCTGAACCGACTT-3′) and ^{32}P-labeled cpxRA-S1R (5′-GAAGCCTTCCATCTCGAGCA-3′), and *E. coli* BW25113 genome DNA (100 ng) was used as the template for the Ex *Taq* DNA polymerase. The PCR product with ^{32}P at its terminus was recovered from a polyacrylamide gel and then used for S1 nuclease. The cpxRA-S1R and labeled cpxRA-S1F primers were used to prepare probe D for an S1 nuclease assay (transcription of *cpxP*). The labeled primers were prepared with 10 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol) by T4 polynucleotide kinase (Toyobo).

RNA isolation and S1 nuclease assay. To prepare total RNA for the S1 nuclease assay, overnight cultures were diluted 100-fold in 100 ml of LB medium grown to an optical density at 600 nm (OD_{600}) of 0.6 or 1.2 at 37°C. Subsequent purification steps were carried out as described previously (19). Ten thousand counts per minute of probe A (2 fmol) was incubated with 100 μg of total RNA in hybridization buffer (80% formamide, 0.4 M NaCl, 20 mM HEPES [pH 6.4]) at 75°C for 10 min, followed by further incubation at 37°C overnight; it was then digested with 50 U of S1 nuclease. The undigested DNA was precipitated by ethanol, dissolved in formamide dye solution (95% formamide, 0.05% bromophenol blue, 0.05% xylenecyanol), and analyzed by electrophoresis on a 6% polyacrylamide gel containing 8 M urea.

Purification of CpxR. His-tagged CpxR for gel shift, DNase I footprinting, and in vitro transcription assays was purified from pKH50-3/BL21(DE3) as described previously (28).

Gel shift assay. Ten thousand counts per minute of probe A (2 fmol) was incubated at 37°C for 10 min with CpxR in 12.5 ml of 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 3 mM Mg acetate, 0.1 mM EDTA, and 0.1 mM dithiothreitol. After addition of the DNA dye solution (40% glycerol, 0.025% bromophenol blue, 0.025% xylenecyanol), the mixture was directly subjected to a 6% polyacrylamide gel electrophoresis.

DNase I footprinting assay. Forty thousand counts per minute of probe A or B (8 fmol) was incubated at 37°C for 10 min with CpxR in 25 μl of 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 3 mM magnesium acetate, 5 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 25 mg of bovine serum albumin/ml. After incubation for 10 min, DNA digestion was initiated by the addition of 5 ng of DNase I (Takara). After digestion for 30 s at 25°C, the reaction was terminated by the addition of 45 μl of DNase I stop solution (20 mM EDTA, 200 mM NaCl, 1% sodium dodecyl sulfate, 250 μg of yeast tRNA/ml). Digested products were precipitated by ethanol, dissolved in formamide dye solution, and analyzed by electrophoresis on a 6% polyacrylamide gel containing 8 M urea.

In vitro transcription assay. A 538-bp EcoRI-BamHI fragment from pRSung, including the *ung* promoter region, or a 205-bp EcoRI fragment from pKB252 (1), including the *lac*UV5 promoter region, was used as the template DNA. Single-round transcription by the reconstituted holoenzymes was carried out as described previously (14). In brief, 0.1 pmol of template was incubated with 0, 1.25, 2.5, 5, and 10 pmol of CpxR for 10 min at 37°C in the presence or absence of 10 mM acetylphosphate in a total volume of 33 μl. Into this reaction mixture, 0.5 pmol of RNA polymerase was added and incubated for 20 min at 37°C to form an open complex. Then a substrate-heparin mixture containing [α-^{32}P]UTP was added and further incubated for 10 min at 37°C. The transcripts were subjected to a 6% polyacrylamide gel containing 8 M urea.

Assay of Ung activity. Cell extract for examining Ung activity was prepared as described previously (16). Here, 5 ml of an overnight culture of *E. coli* cells was inoculated into 200 ml of LB medium (pH 7.5) containing 1 mM of IPTG (isopropyl-β-D-thiogalactopyranoside) and grown at 37°C until reaching an OD_{600} of 1.2. After harvesting by centrifugation, the obtained pellet was washed with 50 ml of H_2O. The cells were again harvested by centrifugation, and the pellet was resuspended in 3 ml of a lysis buffer (50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol). After the cells were sonicated, the cell debris was removed by centrifugation at 15,000 rpm for 20 min at 4°C, and the supernatant was used for assaying Ung activity. The DNA oligonucleotide containing uracil (oligo Lac-461Ruracil, 5′-AGCGCCATGGCCTGACUCATTCC CCAGCGA-3′) was labeled with ^{32}P at the 5′ end by T4 polynucleotide kinase (Toyobo) and hybridized to the unlabeled oligomer, LAC-461F (5′-TCGCTGG GGAATGGGTCAGGCCATGGCGCT-3′), at a molar ratio of 1:10 to form a duplex with a single U · G mismatch. The duplex was separated with a 20% polyacrylamide gel electrophoresis and recovered. It was reacted with cell extract for 30 min at 37°C in a treatment buffer (20 mM Tris-HCl, 10 mM EDTA) and

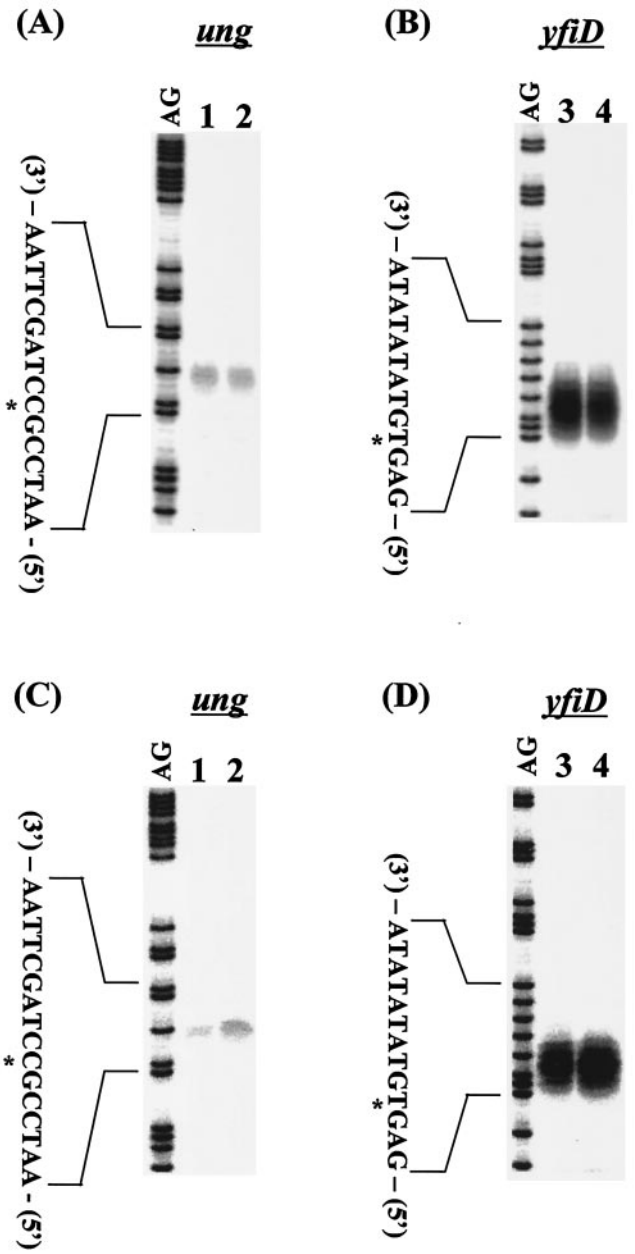

FIG. 4. *ung* and *yfiD* gene expression dependent on growth phase. *E. coli* BW25113 (A and C, lane 1; B and D, lane 3) and BW27559 (Δ*cpxR*) (A and C, lane 2; B and D, lane 4) were grown to mid-log phase (OD_{600} = 0.6) (A, B) or early stationary phase (OD_{600} = 1.2) (C, D). After that, S1 nuclease assays for *ung* (A, C) and *yfiD* (B, D) transcripts were performed. Lane AG represents the Maxam-Gilbert sequence ladder. The transcription start site is marked with an asterisk.

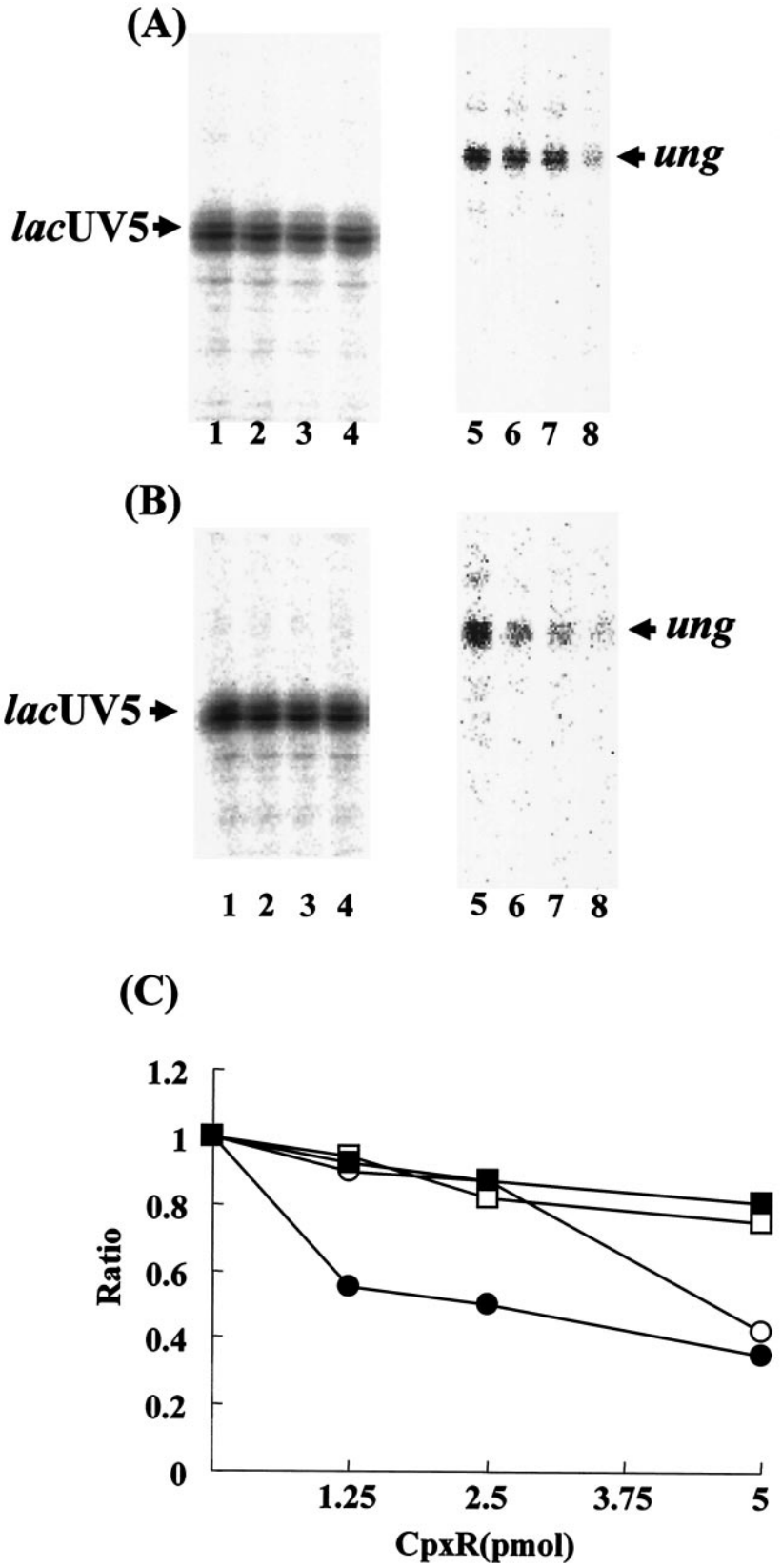

FIG. 5. In vitro transcription assay. Single-round transcription in vitro of 0.1 pmol *ung* DNA template (−452 to +86 of promoter region) (A, lanes 5 to 8; B, lanes 5 to 8) or *lac*UV5 DNA template (A, lanes 1 to 4; B, lanes 1 to 4) was performed in the presence (B) or absence (A) of 10 mM acetylphosphate. The amounts of CpxR were as follows: lanes 1 and 5, 0 pmol; lanes 2 and 6, 1.25 pmol; lanes 3 and 7, 2.5 pmol; lanes 4 and 8, 5 pmol. Electrophoresis was performed with a 6% polyacrylamide sequencing gel. Bold arrows indicate the *ung* and *lac*UV5 transcripts. (C) *ung* (circles) and *lac*UV5 (square) transcripts in the absence (open symbols) and presence (closed symbols) of CpxR were quantified with BAS 1000 Mac (Fuji film). The relative value in shown as a ratio between each transcript and that in the absence of CpxR.

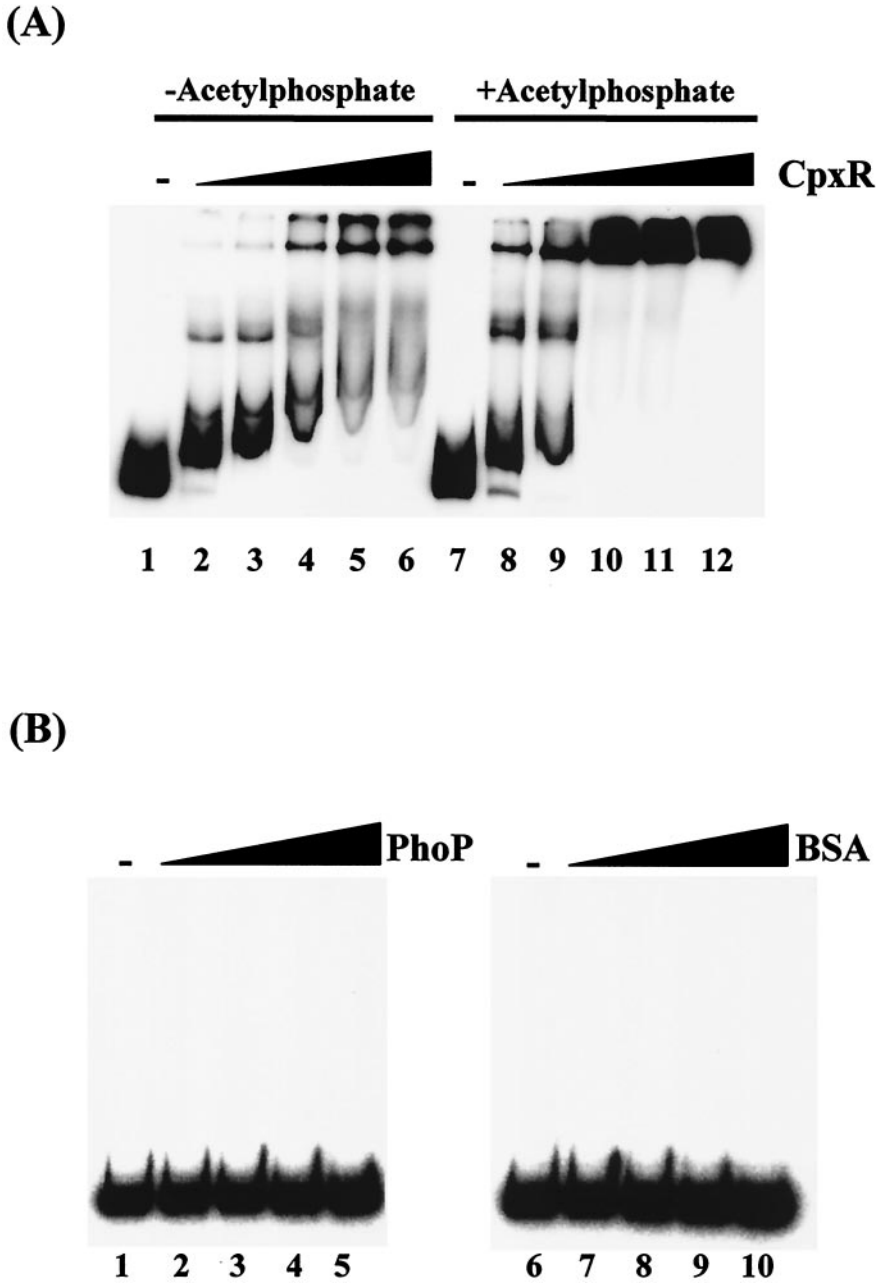

FIG. 6. Gel shift assay. (A) Probe A was incubated at 37°C for 10 min with CpxR (lanes 1 to 6) or CpxR phosphorylated by acetylphosphate at 37°C (lanes 7 to 12). The amounts of CpxR were as follows: lanes 1 and 7, 0 pmol; lanes 2 and 8, 1.25 pmol; lanes 3 and 9, 2.5 pmol; lanes 4 and 10, 5 pmol; lanes 5 and 11, 7.5 pmol; lanes 6 and 12, 10 pmol. (B) Probe A was incubated at 37°C for 10 min with PhoP (lanes 1 to 5) or BSA (lanes 6 to 10) at 37°C. The amounts of PhoP or BSA were as follows: lanes 1 and 6, 0 pmol; lanes 2 and 7, 1.25 pmol; lanes 3 and 8, 2.5 pmol; lanes 4 and 9, 5 pmol; lanes 5 and 10, 10 pmol).

then treated with 0.1 M NaOH to cleave at the apyrimidine site for another 30 min at 37°C. After that, the products were separated in a 20% polyacrylamide containing 8% urea and autoradiographed.

Lac$^+$ mutation assay. To measure the mutation frequency from G · C to A · T, a spontaneous Lac$^+$ mutation assay using *E. coli* CC102 (2) was performed as described previously (17). A single colony was inoculated into 5 ml of LB medium containing 1 mM IPTG and grown with aeration for 16 h. The overnight culture was diluted 10^6-fold with minimal A salt (18). Then, 0.1 ml of the diluted culture was added to 10 ml of minimal A medium containing up to 0.2% glucose, 1 mM $MgSO_4$, 0.0005% thiamine hydrochloride, and tetracycline and/or chloramphenicol. After incubation for 16 h, the culture was harvested by centrifugation. The cell pellet was resuspended in 1 ml of minimal A salt, plated on a minimal A medium (0.2% lactose or 0.2% glucose), and incubated at 37°C. The mutation frequency was then calculated as the ratio of *lac*$^+$ cells to 1×10^9 cells.

RESULTS AND DISCUSSION

Effect of overexpression of CpxR on Ung activity. To determine whether the Cpx system is involved in the regulation of *ung* expression, we examined the Ung activity in crude cell

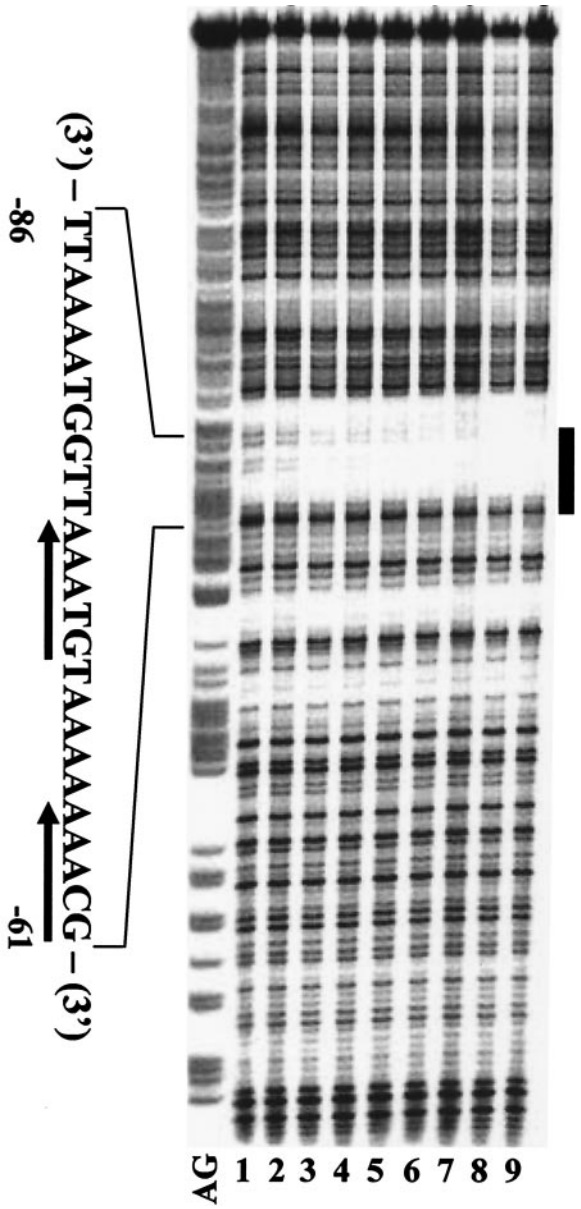

FIG. 7. DNase I footprinting assay. Probe A was incubated with various amounts of the purified CpxR (lane 1, 0 pmol; lane 2, 10 pmol; lane 3, 20 pmol; lane 4, 30 pmol; lane 5, 40 pmol; lane 6, 50 pmol; lane 7, 60 pmol; lane 8, 70 pmol; lane 9, 80 pmol) and subjected to DNase I footprinting assays. Lanes AG represent the Maxam-Gilbert sequence ladder. The black boxes and bold arrows indicate the CpxR binding region and the direct repeat, respectively.

extracts from *E. coli* BW25113(p41-5ΔGFP) overexpressing CpxR. When *E. coli* BW25113(p41-5ΔGFP) was incubated in the presence of IPTG, it was confirmed that the *cpxR* transcript from the *lac* promoter on the plasmid was overexpressed (Fig. 1A, lane 4). To measure Ung activity, we used a model substrate consisting of a synthetic polynucleotide of 30 nucleotides in length containing a single uracil residue at position 17 from the 5′ end. When cell extracts from *E. coli* BW25113 (pCA24ΔGFP) without CpxR expression were used, the labeled polynucleotide (30-mer) containing uracil was cleaved after treatment with 0.1 M NaOH to form a 16-mer oligonucleotide and two other bands as cleaved DNA products (Fig. 2A, lanes 2 and 3). These bands were not detected in the absence or presence of the cell extracts from BWung (Fig. 2A, lanes 1 and 6). When CpxR was induced by addition of IPTG in BW25113(p41-5ΔGFP) (Fig. 2A, lanes 4 and 5), Ung activity decreased to about fivefold lower than that of BW25113 (pCA24ΔGFP) (Fig. 2A, lanes 2 and 3), implying that the Cpx system repressed *ung* expression.

As described previously (9), a G · C-to-A · T transition was stimulated in *E. coli ung* mutants (CC102ung) (Table 2). We investigated the possible effects of CpxR overexpression on the mutator activity of the G · C-to-A · T transition by using a spontaneous Lac^+ mutation assay (17). The results in Table 2 indicate that the G · C-to-A · T transition frequency for strain CC102(p41-5ΔGFP), overexpressing CpxR, was stimulated to about sixfold more than that for CC102(pCA24ΔGFP).

Negative regulation of *ung* gene expression by overexpression of CpxR. To investigate the effect of CpxR overexpression on the transcription of the *ung* gene, we performed an S1 nuclease assay using RNAs prepared from CpxR-expressing BW25113(p41-5ΔGFP) as well as BW25113(pCA24ΔGFP). When CpxR was overexpressed by addition of IPTG (Fig. 1A, lane 4; Fig. 3A, lane 4; Fig. 3B, lane 8), the transcription of *ung* decreased by fivefold (Fig. 3A, lane 4) compared to that of BW25113(pCA24ΔGFP) (Fig. 3A, lane 2) while *cpxP* increased by 10-fold (Fig. 3B, lanes 6 and 8). In addition, we examined whether overproduction of the outer membrane lipoprotein NlpE can repress the *ung* expression (Fig. 3C, lane 4), since overproduction of NlpE activates the Cpx signal transduction pathway (3, 7, 25). When *nlpE* expression was induced by arabinose, the *ung* transcription was repressed by fivefold (Fig. 3C, lane 4) compared to that of BW25113(pBAD18) (Fig. 3C, lane 2), while the transcriptional level of *cpxP* increased by 10-fold (Fig. 3D, lanes 6 and 8). Neither IPTG nor arabinose repressed *ung* and *cpxP* transcriptions in BW25113 (data not shown); therefore, these results suggest that the *ung* expression was negatively regulated by the Cpx pathway.

To confirm this result, we next examined the influence of *cpxRA* deletion on the expression of the *ung* gene. In wild-type BW25113 cells, the level of *ung* mRNA decreased in the stationary phase (Fig. 4C, lane 1), but the level of *yfiD* transcription did not change independently of the growth phase (Fig. 4B and D). The growth phase-coupled reduction in *ung* transcription was not observed in strain BW27559 (Fig. 4A and C, lane 2). These results indicate that CpxR negatively regulates *ung* transcription in the stationary phase and that this repression does not take place in the absence of the Cpx system.

To further confirm the role of CpxR in the transcription of the *ung* gene, we performed in vitro transcription using reconstituted RNA polymerase holoenzyme $E\sigma^{70}$ and truncated DNA fragments (538 bp) containing the promoter region of *ung* in the presence or absence of CpxR. Addition of CpxR caused repression of *ung* transcription, while it did not affect the expression from the *lac*UV5 promoter (Fig. 5A). Such a repression was stimulated in the presence of 10 mM acetylphosphate (Fig. 5B, lanes 5 to 8).

Identification of the CpxR binding site. To define the mechanism underlying the repression of *ung* transcription by CpxR, we tested whether CpxR directly interacts with the *ung* pro-

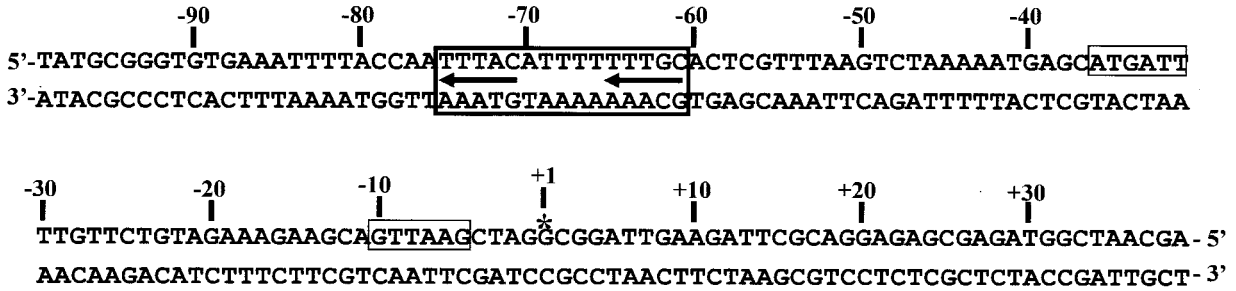

FIG. 8. *ung* promoter and CpxR box. The transcription start site is marked with an asterisk. The nucleotide number represents the distance from the transcription initiation site of the *ung* promoter. Putative recognition sequences for σ^{70} (−10 and −35) are boxed. The arrows indicate the direct repeat in the CpxR box shown previously (6).

moter region. For this purpose, we carried out gel shift assays using the ^{32}P-labeled DNA fragment containing the *ung* promoter region. In the absence of acetylphosphate, a marked shift was observed only at higher concentrations of CpxR (Fig. 6A, lanes 4 to 6). In contrast, a significant gel shift was observed even at the lowest concentration of CpxR when 25 mM acetylphosphate was added (Fig. 6A, lanes 8 to 12). CpxR is a member of the OmpR family of winged helix-turn-helix transcription factors. We investigated the binding ability of another OmpR family protein, PhoP, and that of the bovine serum albumin to the *ung* promoter. No interaction of protein-*ung* promoter DNA was detected (Fig. 6B). These results indicate that the affinity of CpxR to the CpxR box of the *ung* promoter region is increased by phosphorylation.

Next, we tried to identify the specific CpxR binding site on the *ung* promoter region by using a DNase I footprinting assay. CpxR was found to bind to the CpxR box containing the direct repeat between −61 and −86 upstream from the transcriptional start position (Fig. 7). The CpxR box in the *ung* promoter is located 61 bp upstream of the transcription start site (Fig. 8) and corresponds with the site predicted by DeWulf et al. (6). It is well known that the C-terminal domain of the α subunit of RNA polymerase (α-CTD) interacts with an AT-rich sequence (UP element) located upstream of the −35 hexamer (24). The CpxR box in the *ung* promoter also contains an AT-rich sequence corresponding to the UP element. CpxR binding to the *ung* promoter may repress its transcription by interfering with the ability of the α-CTD to bind to the UP element. In *Salmonella enterica*, the transcriptional factor PmrA binding to the 59 bp upstream site in the *pmrD* promoter represses *pmrD* transcription by interfering with the ability of the α-CTD to interact with a putative UP element that overlaps the AT-rich PmrA binding site (15). CpxR seems to repress the *ung* expression in a similar manner as shown for the *pmrD* promoter (15). Taken together, these results indicate that the *ung* gene transcription is directly controlled by phosphorylated CpxR.

In the present study, we demonstrated negative regulation of *ung* expression by overexpression of CpxR (see Fig. 2 and 3). Transcription of the *ung* gene at the stationary phase is about threefold higher in the *cpxRA* deletion strain than in the parental strain (Fig. 4C). DeWulf et al. (6) predicted positive control of the *ung*, *ompC*, *psd*, *mviM*, *aroK*, and *secA* genes by phosphorylated CpxR and negative control of *rpoErseABC* and *aer*. In this paper, we suggest that *ung* is expressed in the exponential growth phase but decreases in the stationary phase concomitantly with the increase in phosphorylated CpxR. This finding indicates that *ung* is a target of negative control by CpxR.

Furthermore, we also found an increase in the mutation level of *E. coli* overexpressing CpxR. The increased mutation might be attributable, at least in part, to the repression of *ung* expression by the increased CpxR. This is because it is not possible to repair DNA uracil residues, which arise as a result of either misincorporation of dUMP by DNA polymerase or deamination of DNA cytosine residues. This finding further implies that the CpxR/CpxA two-component system plays a role in controlling the mutation rate.

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