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Site-Directed Mutation Effect of the Symmetry Region at the mRNA 5'-end of *Escherichia coli aeg-46.5* Gene

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Abstract: The *age-46.5* gene of *Escherichia coli* is induced by nitrate ion and regulated by Fnr, NarL, and NarP during anaerobic growth. *aeg-46.5::lacZ* fusion gene shows its maximum expression in *narL* host after two hours of aerobic to anaerobic switch in M9-Glc-nitrate medium. Fnr and NarP act as positive regulators, and NarL acts as a negative regulator. The control region of the *aeg-46.5* was identified and the binding sites of regulator proteins have been predicted (Reznikoff and Choe (1993)). It has two symmetry regions. One is located at $-52 \sim -37$ bp from the anaerobic mRNA 5'-end, which is the binding site of NarL and NarP. The other is located at $+37 \sim +56$ bp from the 5'-end of mRNA. In this study, the downstream symmetry region from the mRNA 5'-end was investigated by site-directed mutagenesis. The destruction of the symmetry region increases the expression level of *aeg-46.5*. We propose that the symmetry region interferes with the expression of *aeg-46.5* possibly by forming a stem-and-loop structure.
Key words: *aeg-46.5*, anaerobically expressed gene, NarL, site-directed mutagenesis, symmetry region.

Escherichia coli is a facultative anaerobe. It produces chemical energy from a variety of carbon sources and transforms it into ATP. The most efficient way of generating ATP is using oxygen as a terminal electron acceptor. If the oxygen supply is limited, *E. coli* can use a variety of electron acceptors instead of oxygen to generate ATP by an erobic respiration. Without any electron acceptors, it can produce ATP by fermentation. Nitrate, trimethylamine-*N*-oxide, dimethyl sulfoxide and fumarate act as alternative electron acceptors in anaerobic condition.

The switch from aerobic to anaerobic physiology of *E. coli* is controlled by several regulatory systems. The *fnr* is a regulatory gene required for the expression of anaerobic respiratory enzymes (Neidhardt and Smith, 1983; Gunsalus and Melvile, 1990). The Fnr, which is the transcriptional factor encoded by *fnr*, acts as a positive regulator for expression of nitrate reductase (*narGHJI*), fumarate reductase (*frdABCD*), and dimethyl sulfoxide reductase (*dmsABC*) in the absence of oxygen (Gunsalus and Jones, 1987; DeMoss and Li. 1988; Gunsalus and Cotter, 1989). Fnr also acts as a negative regulator for expression of NADH dehydrogenase (*ndh*) required for aerobic respiration (Guest *et al.*, 1989).

Nitrate (NO_3^-) is the most efficient electron acceptor in an anaerobic environment. If nitrate is available, two homologous membrane-bound sensor proteins (NarX and NarQ) monitor the availability of nitrate and transfer this signal to two homologous DNA-binding regulator proteins (NarL and NarP) (DeMoss and Walker, 1993; Gunsalus *et al.*, 1994). The activated NarL and NarP control the expression of target operons. Some of these operons are regulated by the NarL alone (*narGHJI, frdABCD*), but the *nirB* (encoding NADH dependent nitrite reductase) and *nrfA* (encoding formate dependant nitrite reductase) are regulated by both NarL and NarP (Busby *et al.*, 1993; Busby *et al.*, 1994; DeMoss and Walker, 1994).

The *aeg-46.5* was identified by operon fusion technique using λ-placMu53 (Weinstock *et al.*, 1985) by Reznikoff and Choe (1991). It is located at 2315.5 kb in *E. coli* physical map, and λ372 clone of Kohara library (Kohara *et al.*, 1987) contains this gene. In previous studies, it was known that the *aeg-46.5* is subject to multiple regulations of anaerobic activation by *fnr*, nitrate activation by *narP* and repression mediated by *narL*, and the control region of the *aeg-46.5* required for anaerobic expression was identified. Through sequence analysis, the possible -10, -35 regions and Fnr, NarL binding sites were proposed (Reznikoff and Choe, 1993). A 304 bp DNA sequence of the control region of the *aeg-46.5* is shown in Fig. 1.

Recently, the whole sequences of *E. coli* centisome

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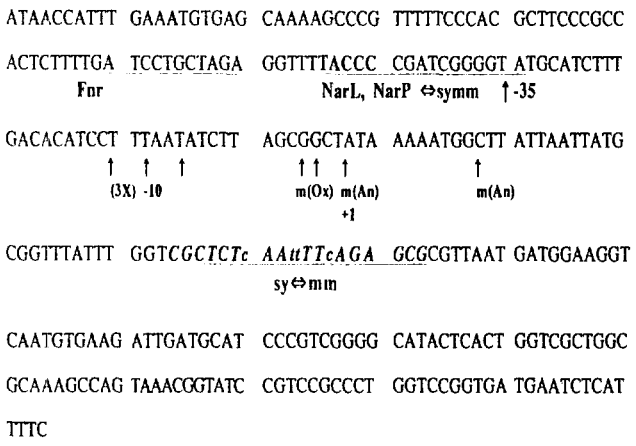

Fig. 1. Upstream DNA sequence of *aeg-46.5*. A 304-bp upstream DNA sequence was determined by Reznikoff and Choe (1993). The binding sites of Fnr, NarL and NarP and putative −10, −35 regions are shown. The 5'-ends of mRNA determined by Reznikoff and Choe (1993) are indicated by ↑ and labeled An (anaerobic) or Ox (aerobic). The symmetry element at the 5'-end mRNA is written by italic with underline and labeled with sy⇔mm.

49 were identified (Richterich *et al.*, 1993). This sequence analysis suggests that the *aeg-46.5* operon encodes proteins resembling the periplasmic nitrate reductase, homologous to NAP protein in *Alcaligenes eutrophus* (Friedrich *et al.*, 1993), and proteins involved in cytochrome c biogenesis in *Bradyrhizobium japonicum* and *Rhodobacter capsulatus* (Hennecke *et al.*, 1995).

In this study, we performed site-directed mutagenesis to elicit the function of the symmetry element downstream from the mRNA 5'-end of the *aeg-46.5* operon *in vivo*. The disruption of the symmetry region increased the expression level of *aeg-46.5* and it indicates that *aeg-46.5* is controlled by this symmetry sequence, which can form a mRNA stem-and-loop structure, in addition to the Fnr, NarL and NarP control on transcriptional level.

Materials and Methods

Strains, plasmids, and phages

All *E. coli* K-12 strains, phage strains and plasmids used in this experiment are described in Table 1. Introduction of *narL215*::Tn*10* allele into RZ4500 was done by P1 transduction (Miller, 1992). Plasmid pRZ4460, which has the control region of the *aeg-46.5*, was used as a template DNA for site-directed mutagenesis.

Media and chemicals

Media used in this experiment was described previously (Miller, 1992). The indicator dye 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XG) was added to final concentration of 40 μg/ml in agar medium. IPTG was

Table 1. *E. coli* strains, phages, and plasmids used in this study

Name	Genotype	Source or Reference
Strains		
RZ4500	λ⁻F⁻ *lacZΔ145*	Reznikoff and Choe (1991)
CA101L	RZ4500 *narL215*::Tn*10*	This study
RK5278	*narL215*::Tn*10*	Stewart (1982)
JM109	*endA1, recA1, gyrA96, thi, relA1, hsdR17*(r_k^-, m_k^+), *supE44*, λ⁻, Δ(*lac-proAB*), [F', *traD36, proA+B+, lacI*^{q}ZΔM15]	Promega Corp.
ES1301 *mutS*	*lacZ53, mutS*201::Tn*5*, *thyA36, rha-5, metB1, deoC*, IN(*rrnD-rrnE*)	Promega Corp.
Phage		
R408	helper phage	Promega Corp.
Plasmids		
pRZ4460	pMLB524, 2.2 kb fragment of λRZ4646.5	Reznikoff and Choe (1993)
pALTER-1	Vector for site-directed mutagenesis	Promega Corp.
pCA1	pALTER-1, 2.2 kb fragment of λRZ4646.5	This study
pCA1L	pCA1, *Nco*I site in +37~+46 of the *aeg-46.5* control region	This study
pCA1R	pCA1, *Nco*I site in +47~+56 of the *aeg-46.5* control region	This study
pCA1T	pCA1, two *Nco*I sites in +37~+56 of the *aeg-46.5* control region	This study
pCA1I	pCA1, inverted +37~+56 sequence of the *aeg-46.5* control region	This study
pCA2L	pRZ4460, *Nco*I site in +37~+46 of the *aeg-46.5* control region	This study
pCA2R	pRZ4460, *Nco*I site in +47~+56 of the *aeg-46.5* control region	This study
pCA2T	pRZ4460, two *Nco*I sites in +37~+56 of the *aeg-46.5* control region	This study
pCA2I	pRZ4460, inverted +37~+56 sequence of the *aeg-46.5* control region	This study

Table 2. The sequence of synthetic mutagenic oligomers

Name	Sequence*	Target site
Oligo-ML (35 mer)	5'-GGTTTATTTGGTCCATGGTCGCTTTCAGAGCGCGT-3'	+37~+46
Oligo-MR (36 mer)	5'-GGTCGCTCTCAATCCATGGTCGCCGTTAATGATGGA-3'	+47~+56
Oligo-MT (45 mer)	5'-GGTTTATTTGGTCCATGGTCGCCCATGGTCGCCGTTAATGATGGA-3'	+37~+56
Oligo-MI (46 mer)	5'-CGGTTTATTTGGTGCGAGACTTTTAACTCTCGCCGTTAATGATGGA-3'	+37~+56

*Underlined sequences indicate mismatched region.

added to final concentration of 0.5 mM. Antibiotics were added to final concentrations of 40 μg/ml for kanamycin, 15 μg/ml for tetracycline, and 100 μg/ml for ampicillin. Potassium nitrate was supplied at a final concentration of 40 mM when the nitrate effect was to be tested.

β-galactosidase assays

β-galactosidase assays were performed as described previously by Miller, using chloroform and 0.1% SDS to permeabilize cells. β-galactosidase activities were measured in triplicate and averaged from three independent experiments.

Design of oligonucleotide for mutagenesis

For site-directed mutagenesis of the symmetry element (+37~+56) of mRNA 5'-end, We designed four mutagenic oligonucleotides. The sequences of the oligonucleotides are shown in Table 2.

Site-directed mutagenesis

For site-directed mutagenesis, we used the Altered Sites® II *in vitro* Mutagenesis Systems (Promega Corp.) and followed the manufacturer's technical manual. Routine DNA manipulations have been described previously (Maniatis *et al.*, 1982). The construction procedure for mutant plasmids is shown in Fig. 2. The pAlter-1 vector contains ampicillin and tetracycline resistance genes. Before the mutagenesis, the ampicillin resistance gene contained a frameshift mutation and it was inactive. During the mutagenesis procedure it is restored by ampicillin repair oligo. The recovery of ampicillin resistance is used as a selection marker for oligonucleotide incorporated plasmid.

The 2.2 kb *Eco*RI-*Hin*dIII fragment of pRZ4460 that contains chromosomal DNA control region of the *aeg-46.5* was isolated and cloned into pAlter-1 polycloning sites. The cells containing recombinant plasmid showed white color, and they were isolated on LB+XG+Tet+IPTG plate. This recombinant plasmid (pCA1) was used for the site-specific mutagenesis in the symmetry element of the *aeg-46.5*. To prepare single-stranded template for the mutagenic polymerization reaction, JM 109 was transformed with pCA1 and infected with helper phage R408.

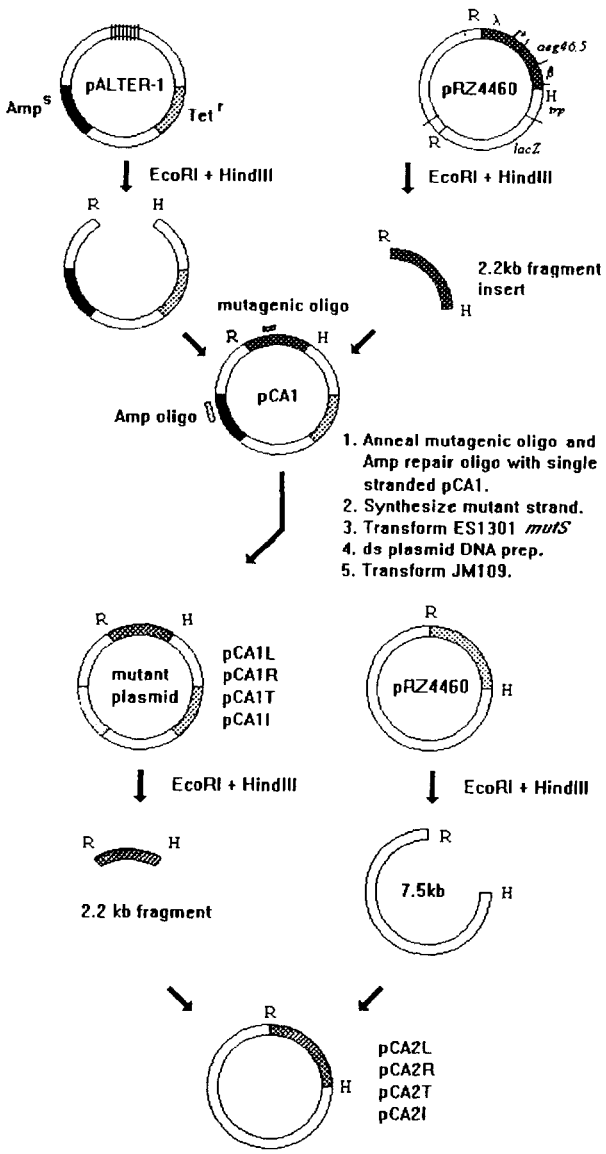

Fig. 2. Construction flow chart of pCA2L, pCA2R, pCA2T, and pCA2I from pALTER-1 and pRZ4460.

The four mutagenic oligo ML, MR, MT, MI were designed. ML destroys the left half of the symmetry, and MR does the right half. MT removes the symmetry in whole. MI replaces the wild-type symmetry sequence with an inverted sequence of the symmetry. It retains the symmetry with the same number of hydrogen bond

between the complementary strands, and has same pairing energy as the wild-type symmetry sequence. They were 5'-phosphorylated with T4 polynucleotide kinase (Promega Corp.) for the ligation and it increased the mutation efficiency. The single-stranded plasmid DNA was annealed with each mutagenic oligo and ampicillin repair oligo. The mutant strand was made with T4 DNA polymerase and T4 DNA ligase (Promega Corp.). The reaction mixtures were used to transform *E. coli* ES1301 *mutS* strain which suppresses *in vivo* mismatch repair. The cells were cultured overnight in LB+Tet+Amp media. The plasmid DNA was extracted through miniprep, and it was used to transform JM109. The transformants were selected on LB+Tet +Amp plates.

A total of 15 colonies were selected for each mutation. The site-specific mutation was confirmed by restriction enzyme digestion analysis. Each confirmed mutant plasmid (pCA1L, pCA1R, pCA1T, pCA1I) was digested with *Eco*RI and *Hin*dIII, and a 2.2 kb fragment was purified. The 2.2 kb fragment was used to replace the *Eco*RI-*Hin*dIII fragment of pRZ4460. The resultant plasmids were named pCA2L, pCA2R, pCA2T, pCA2I, respectively, and used for the β-galactosidase assay.

Results and Discussion

In order to investigate the function of the symmetry element at the mRNA 5'-end of *aeg-46.5*, we constructed four plasmids containing mutations in the symmetry region. The symmetry element consists of 20 base pairs and its sequence is CGCTCTcAAt-tTTcAGAGCG (+37~+56 region). In previous studies, *aeg-46.5* was known to be regulated by Fnr, NarL, and NarP. The Fnr binds at position −71~−58, and NarL or NarP binds at position −52~−37 (Reznikoff and Choe, 1993; Stewart and Darwin, 1995). To investigate the function of the symmetry element for the expression control of *aeg-46.5*, we designed four mutagenic oligonucleotides. The structure of pRZ4460 and the symmetry element sequences of wild-type and mutant plasmids are shown in Fig. 3.

We had previously studied the time course expression of the *aeg-46.5* in the *fnr*, *narL*, and *narP* mutant background(s) using the *aeg-46.5*::*lacZ* fusing strain, RZ 4546.5. From the studies, it was found that *aeg-46.5* showed maximum expression after two hours of aerobic-to-anaerobic switch, and it gave the maximum expression in the *narL* mutant cell and low expressions in the *fnr* and *narP* mutant cells in M9-Glc-nitrate medium. In the present study, the effect of mutations in the symmetry element was tested in the wild-type cell, RZ4500, and the *narL* mutant cell, CA101L, in M9-

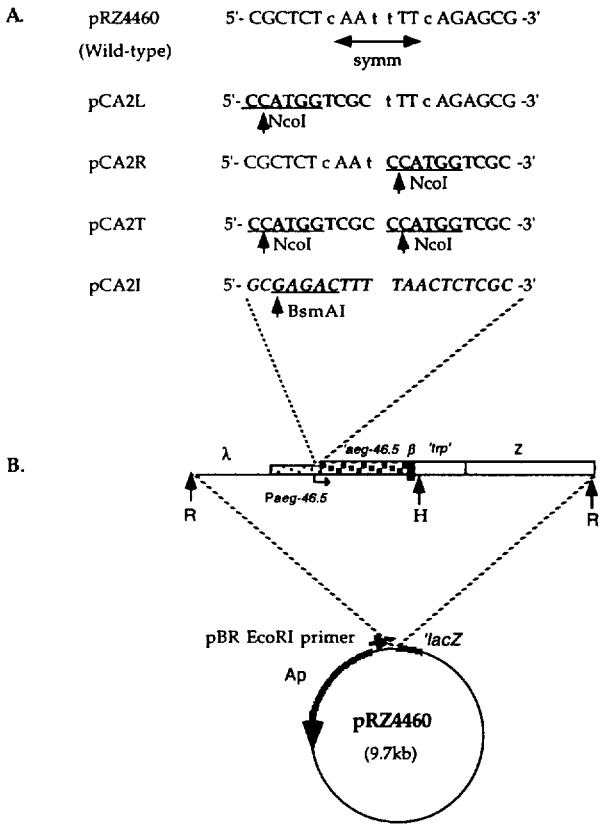

Fig. 3. A. The sequence of the symmetry region at the mRNA 5'-end of *aeg-46.5* in pRZ4460 and mutant plasmids. Four mutant plasmids pCA2L, pCA2R, pCA2T, pCA2I were constructed. pCA2L destroys left half of the symmetry, and pCA2R does the right half. pCA2T removes the symmetry in whole. pCA2I removes the wild-type symmetry sequence with an inverted sequence of the symmetry. It retains the symmetry with the same number of hydrogen bonding between the complementary strands, and has same pairing energy as the wild-type symmetry sequence. B. Structure of pRZ4460. The *Eco*Ri fragment of a specialized transducing phage was cloned in *Eco*Ri-site of pMLB524. The chromosomal DNA was unidirectionally deleted by exonucleaseIII from upstream to get the 2.2 kb *Eco*RI-*Hin*dIII fragment of pRZ 4460. The restriction enzyme sites are indicated by arrows. R: *Eco*RI; H: *Hin*dIII; $P_{aeg\text{-}46.5}$, the promotor sequence of *aeg-46.5*. The drawings are not in scale.

Glc-nitrate medium after two hours of aerobic-to-anaerobic switch.

The β-galactosidase activities were measured and are shown in Fig. 4. In aerobic conditions, there is no difference of expression level between the wild-type plasmid and the mutant plasmids in RZ4500 and CA101L host cells. At the switch from aerobic to anaerobic conditions, potassium nitrate was added to a final concentration of 40 mM. In RZ4500 host cells where the *aeg-46.5* gene is repressed by NarL protein binding, wild-type and mutant plasmids except the pCA2L showed similar β-galactosidase activities. However, in CA101L host cells where NarL repressor is absent, the mutant

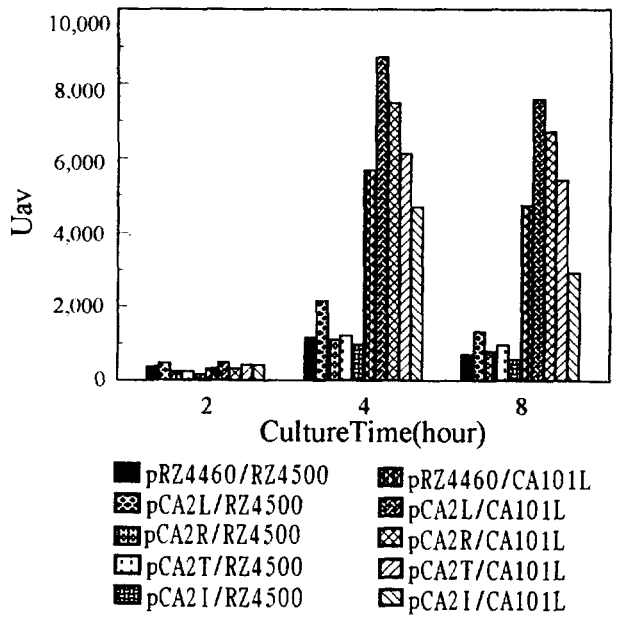

Fig. 4. β-galactosidase assays of wild-type and mutant plasmids in wild-type (RZ4500) and *narL* mutant cells (CA101L) in M9-Glc medium. β-galactosidase activities were measured in triplicate and averaged from three independent experiments. The error of activity measurements was less than± 30%.

plasmids (pCA2L, pCA2R, and pCA2T) that contained mutated symmetry elements showed higher expression than wild-type pRZ4460 and pCA2I. pCA2I retains the symmetry of the sequence which is an inverted sequence of the wild-type and has the same pairing energy as wild-type. The β-galactosidase activities of pCA2I were lower than pRZ4460, and this suggests the symmetry of the sequence may not be the only function of this region. When cells reach stationary phase the difference of expression level between pRZ4460 and pCA2I became larger than the difference on log phase. These results show that the symmetry element which can form stem-and-loop structure affects the expression of *aeg-46.5* and its interference gets stronger as the cell grows from the log phase to stationary phase.

In our previous study, we observed expression of *aeg-46.5* decreased in stationary phase in *narL* mutant cells. This might involve the formation of stem-and-loop structure of the symmetry element at the mRNA 5'-end and its interactions with other factors that accumulate in stationary phase.

The expression level among mutant plasmids pCA2L, pCA2R, and pCA2T shows that pCA2T is expressed most inefficiently. pCA2T has no symmetry and can't form a stem-and-loop structure, and it was expected to give the highest level of expression if the translational level control depends solely on symmetry. The fact that pCA2L has a higher expression level than pCA2T implies the possibility of a factor that might have specific relationship with the sequence that pCA2L retains. The identify of this additional factor remains to be determined.

The regulatory sequence of *aeg-46.5* has a peculiarity in its shape. Many anaerobically expressed operons (*narG, fdnG, nirB,* and *nrfA*) of *E. coli* are controlled by Fnr, NarL or NarP proteins. However, the *aeg-46.5* operon has a different array of binding sites for these regulatory proteins compared to other operons. The Fnr protein (consensus sequence, TTGAT-N_4-ATCAA) binds at −40~−50 region and the NarL protein and NarP protein binding sites are located upstream of this Fnr binding site in other operons. However, *aeg-46.5* has an Fnr binding site at −64.5, and an NarL or NarP binding site at −44.5. For these unique features, we are investigating the binding of the regulator proteins to these sites and regulatory effects of the symmetry element *in vivo*.

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