The Function of Multiple Pribnow Box on the Aerobic-Anaerobic Switch Control of *aeg-46.5* Gene Expression

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The gene *aeg-46.5*, which is expressed under anaerobic condition, has putative triple −10 regions and four transcription start sites. The mRNA transcription level and its start point change depending on the aerobic/anaerobic growth condition. RNA polymerase and its regulatory proteins must choose which of three −10 region to use. The putative triple 10 region was mutated to make only one of them function with consensus −10 region sequence (TATAAT) and the other two as non-functional region. The results show that the second and third −10 regions are used for the aerobic/anaerobic expression. The third −10 region is responsible for the high aerobic to anaerobic switch ratio. This suggests that only the last two of the putative triple −10 region have functions on *aeg-46.5* gene expression switch control. The phenotype of the mutated promoter was tested in the wild type cell and *narL⁻* cell. The results indicate that the control by NarL is independent from the selection of −10 region. The expression patterns on multi-copy plasmids and on single-copy chromosome were compared. These results show that the aerobic/anaerobic switch control of *aeg-46.5* is through the choice of −10 region. The mechanism of choosing different −10 region remains to be seen.

Keywords : Multiple Pribnow Box, Aerobic-anaerobic switch, aeg-46.5 gene, NarL/NarP regulatory system.

Introduction

Escherichia coli, a facultative anaerobe, can grow in either aerobic or anaerobic environment. *E. coli* breaks varieties of carbon sources and oxidizes them to CO_2 *via* glycolysis and TCA cycle. These processes provide a large flow of electrons into electron-transfer chain. In aerobic condition, electrons that are passed from respiratory chain reduce terminal electron acceptor O_2 to H_2O and the potential energy released during this process gets transduced into proton concentration gradients and electric potential differences across the membranes. This proton motive force is used to drive the synthesis of ATP catalyzed by F_0F_1 protein.[1] In anaerobic condition, *E. coli* gets energy by anaerobic respiration or by fermentation. Nitrate,[2,3] fumarate,[4,5] or DMSO[6] can be used as a terminal electron acceptor in the absence of oxygen. As terminal electron acceptors, they have lower reduction potential than oxygen so that the efficiency of ATP production with these compounds is lower than that with oxygen. Without any terminal electron acceptor ATP can be produced by glycolysis, and the TCA cycle and electron transfer chain are replaced by fermentation.

The anaerobically expressed gene *aeg-46.5* was identified by operon fusion technique using *λplac*Mu53.[7] Under anaerobic condition, *aeg-46.5* was induced by nitrate and repressed by NarL protein.[8] The consensus sequences of Fnr, NarL and NarP regulatory protein binding sites, −35 region and −10 region have been identified.[8] Symmetry region at −64.5 from transcription start point is a Fnr binding site

(TTGAT-NNNA-ATCAA) and the symmetry region at −44.5 (TACYNMT: Y = C or T, M = A or C) is NarL and NarP competitive binding site. At this site, NarL functions as a repressor and NarP functions as an activator.[9] The computer analysis showed that *aeg-46.5* had putative triple −10 regions. The first −10 region has one mismatch base from consensus −10 region, but the second and the third −10 region have two mismatches. The −35 region has 14-, 16-, 19-base spacing from each putative −10 region.[8]

In this study we determined which of putative triple −10 regions functions as −10 region in aerobic and/or anaerobic condition and whether the interaction of RNA polymerase with NarL regulatory protein is related with the selection of putative triple −10 regions.

Materials and Methods

Chemicals and reagents. Bacto trypton, Bacto yeast extract and Bacto agar are from Difco and other salt reagents are from Junsei or Sigma. Antibiotics used for selection are ampicillin (50 μg/mL), tetracycline (15 μg/mL) and chlorotetracyclin hydrochloride (12.5 mg/mL). The 40 μg/mL of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (XG) was used to select colonies of the *lacZ* expression. Cy5™-dATP labeling mix and Cy5™ AutoRead™ Sequencing Kit are from Pharmacia Biotech. Altered Sites II *in vitro* Mutagenesis Systems is from Promega Corp.

Strains, phages and plasmids. *E. coli* strains RZ4500W, RZ4500L and pRZ4460 were obtained from W. S. Reznikoff. *E. coli* strains ES1301 *mutS*, JM109 and M13K07, R408 helper phage and pALTER-I vector were purchased from Promega Corp. *E. coli* strains ERL41 and W4680 were kind

gifts from David C. LaPorte. Phage P1*vir* was from J. Miller (Table 1).

Plasmid pALTER-I was used to clone the *Eco*RI-*Hin*dIII DNA fragment containing *aeg-46.5*::*lacZ* from pRZ4460 for site-directed mutagenesis. Introduction of *narL215*::Tn*10* was done by P1 transduction.

Primer design. Three 43mer-oligonucleotides were designed with following consideration.

(1) One consensus sequence functional and other two −10 regions nonfunctional

(2) Minimum changes of the total numbers of interstrand hydrogen bonds

(3) Enzyme site (*Bam*HI) for mutagenesis check

(4) No change of space between Pribnow box and transcription start point

(5) Complementarities to the single strand from phagemid

Oligonucleotides were designed by using efficient −10 region base frequency table.[13,14] The −10 region that was made to be active has the consensus "TATAAT" sequence, and nonfunctional −10 regions have most infrequent bases on each base position. Primer used for site-directed mutagenesis is following.

KPr1: 5'-ATGCA TCTTT GACGG ATCC**T ATAAT** TAGTC AGCGG CTATA AAA,

KPr2: ATGCA TCTTT GACGG ATCCG **CTATA AT**GTC AGCGG CTATA AAA,

KPr3: ATGCA TCTTT GACGG ATCCG CGGGT **ATAAT** AGCGG CTATA AAA.

Bold letters are the consensus −10 region sequences and gray boxes are *Bam*HI enzyme sites. All primers have 13-14 matching sequences on both ends.

DNA manipulation and site-directed mutagenesis. Plasmid pCA1 was constructed by cloning 2.2 kb *Eco*RI-*Hin*dIII DNA fragment containing *aeg-46.5*::*lacZ* into pALTER-1 vector. In site-directed mutagenesis, single strand template is more efficient than double stranded template. Singe strand template was prepared from recombinant phagemid with f1 origin in F' cell. R408 helper phage makes the mutant phagemid replicated and packaged as an infectious particle that are secreted into the growth media. This infectious particle was collected to prepare single strand DNA template.

Single stranded pCA1 DNA for mutagenesis was isolated by using R408 helper phage (MOI = 10). Single stranded pCA1 (ss pCA1) was checked by electrophoresis. After being denatured at 75 °C for 10 minutes, ss pCA1 was examined in alkaline agarose gel using electrophoresis buffer of 50 mM NaOH, 1 mM EDTA, pH 8.

Mutagenic oligonucleotides which had phosphorylated 5' end by T4 polynucleotide kinase were annealed to ss DNA template by heating up to 75 °C and cooling down slowly to 45 °C in annealing buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 50 mM NaCl). Ampicillin resistance gene repairing oligonucleotide was used to help the screening of mutagenized plasmids. Polymerization and ligation reaction were done at 37 °C for 90 minutes in synthesis buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM dNTP, 1 mM ATP, 2 mM DTT). JM109 was cotransformed by polymerized dsDNA with R408 helper phage DNA by electroporation with Gene Pulser II Electroporation system (Bio-Rad) at 25 μ F, 1.8 kV/0.1 cm and 200 Ω. Mutagenized phagemid were selected on the plate containing ampicillin and tetracycline.

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

Name	Genotype	Source
RZ450	λ^- F$^-$ *lacZ*Δ145	Reznikoff *et al.*
RZ4500L	RZ4500 *narL215*::Tn*10*	M. Choe *et al.*
CK4	RZ4500 *lacZ*'*aeg46.5*::*lacZ*(first Pribnow Box)	this study
CK5	RZ4500 *lacZ*'*aeg46.5*::*lacZ*(second Pribnow Box)	this study
CK6	RZ4500 *lacZ*'*aeg46.5*::*lacZ*(third Pribnow Box)	this study
CK4L	CK4 *narL215*::Tn*10*	this study
CK5L	CK5 *narL215*::Tn*10*	this study
CK6L	CK6 *narL215*::Tn*10*	this study
ES1301	*lacZ53, mut*S201:Tn5, *thyA36, rha-5, metB1, deoC, IN(rrnDrrnE)*	Promega Corp.
JM109	*endA1, recA1, gyrA96, thi, relA1, hsdR17*(rk$^-$, mk$^+$), *supE44, λ^-, Δ(lac-proAB)*, [*F', traD36, proA$^+$B$^+$, lacI^{q}ZΔM15*]	Promega Corp.
ERL41	Hfr PO1 *lacI' 'bla-kan' 'lacZ, zah-281*::Tn*10 thi⁻ 1 spoT1 supQ80*	D. C. LaPorte
W4680	F$^-$ Δ*lacZ39 rpsL melB4*	D. C. LaPorte
P1*vir*		J. H. Miller
R408	helper phage	Promega Corp.
pRZ4460	pMLB524, 2.2 kb fragment of λ RZ4546.5	Reznikoff *et al.*
pCA1	pALTER-1, 2.2 kb fragment of λ RZ4546.5	this study
pCK1	pCA1, first position functional mutation	this study
pCK2	pCA1, second position functional mutation	this study
pCK3	pCA1, third position functional mutation	this study
pCK4	pRZ4460, first position functional mutation	this study
pCK5	pRZ4460, second position functional mutation	this study
pCK6	pRZ4460, third position function mutation	this study

*Eco*RI-*Hin*dIII mutant fragment was used to replace the wild type fragment of pRZ4460 plasmid.

Converting from multicopy to single-copy chromosomal gene. Recombinant plasmid was transformed into ERL41, which is a derivative of HfrH strain. ERL41 harboring recombinant plasmid was grown to a mid-log phase (OD_{650} = 0.6). Recipient cell, W4680 (OD_{650} = 0.6) was mixed and mated for 45 minutes without shaking. Mated cells were centrifuged and resuspended in LB media with streptomycin, ampicillin and tetracycline. The suspension of exconjugants were incubated at 37 °C for 12 hours.

P1 lysate was prepared by adding P1*vir* (5×10^8 phages/cell) to the exconjugants suspension and the prepared lysate was used to transduce RZ4500. Transductants that were ampicillin sensitive and tetracycline resistant were selected.

It was necessary to remove the Tn*10* insertion before introducing the gene expression regulatory protein mutations that are linked to Tn*10* marker. Bochner (Maloy) plate[10,11] were used to select tetracycline sensitive cells. Selected transductants were cultured for 12 hours without antibiotics and spread on Maloy plate. After 24 hours of incubation, growing cells were selected and checked again for the loss of Tn*10* by replica plating on plates with or without tetracycline. The resulting strains are CK4, CK5, and CK6.

Enzyme assay. β-Galactosidase assays were performed as described previously by Miller,[12] using chloroform and 0.1% SDS to permeabilize cells. Cells were cultured at 37 °C in LB broth overnight and 200 μL of them were resuspended in 5 mL of M9-glucose media. After the cells in M9-glucose media has fully grown, 200 μL of the culture was used to inoculate M9-glucose assay media. Samples for β-galactosidase assay were taken every hour from aerobic culture. Under anaerobic condition samples were taken in every two hours. Anaerobic cells were grown under 95% nitrogen and 5% carbon dioxide atmosphere in rubber stopper sealed vial.

Results

Cloning of the promoter of *aeg-46.5*. The *aeg-46.5* gene was identified as a transcriptional fusion to λp*lac*Mu53 phage.[8] The phage was induced and *aeg-46.5*::*lacZ* fusion was cloned *in vivo*. pRZ4460 was generated by subcloning *Eco*RI-generated DNA fragment from induced phage containing *aeg-46.5* promoter into pMLB524. The structure of pRZ4460 is shown in Figure 1. The *aeg-46.5* promoter on pRZ4460 was cut with *Eco*RI and *Hin*dIII. DNA fragment containing *aeg-46.5* promoter was transferred to pALTER-I phagemid vector for site-directed mutagenesis with blue/white selection.

Site-directed mutagenesis by polymerization and ligation. Oligonucleotide were purified by extracting with butanol and phosphorylated by using T4 polynucleotide kinase. After the oligonucleotides annealed to single stranded phagemid template, polymerization and ligation was performed. Mixing ratio of oligomers and templates was 1 : 5 : 25 (template : mutation oligomer : ampicillin repairing oligo-
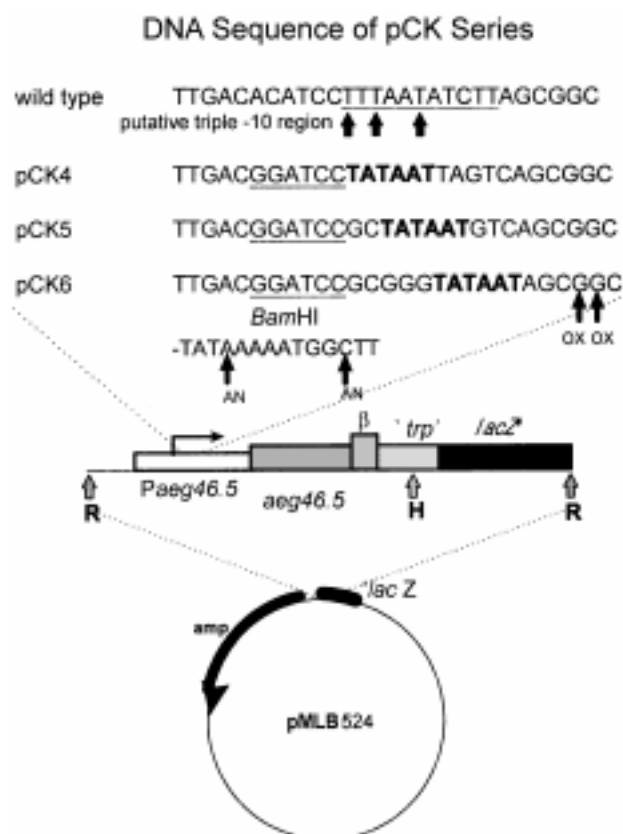
Figure 1. The sequence of triple −10 region of pRZ4460 and mutagenized plasmid pCK4, pCK5, pCK6. pCK4 plasmids are constructed by changing first −10 region to consensus sequence and making other two −10 region nonfunctional; pCK5 by changing second −10 region to consensus sequence and making the others nonfunctional; pCK6 by changing third −10 region to consensus sequence and making the others nonfunctional. Mutagenized recombinant phagemid was partially digested and the fragment containing the promoter region was subcloned into pBR322 derivative pMLB534 vector for β-galactosidase assay. Sequences were examined by automatic sequencer ALF*express*™. The transcription start point was indicated with ox (under aerobic condition), an (under anaerobic condition).

mer). This ratio was optimal for the selection of mutagenized plasmid by ampicillin resistant phenotype.

Cotransformation with R408 helper phage DNA. Polymerized phagemid and R408 helper phage DNA were cotransformed into ES1301 *mutS* competent cell that has lost the host-directed mismatch repair mechanism. Transformed phagemid were replicated and packaged with packaging protein expressed from helper phage in ES1301. This saves time for mutagenesis process by eliminating the steps of competent cell preparation and transformation.

The produced particles infected JM109 strain that has pili. JM109 cells with mutagenized phagemid were selected on ampicillin and tetracycline plates. Template phagemid that was sensitive to ampicillin became resistant to ampicillin if it was polymerized with ampicillin repairing oligomer.

Transfer onto expression plasmid and sequencing. Plasmid used in site-directed mutagenesis is a high copy number plasmid that is not suitable for phenotype test. For β-

galactosidase assay we subcloned mutated DNA fragment to medium copy number plasmid that can reconstitute functional *lacZ* gene. Mutation was checked by sequencing with pBR322 *Eco*RI site primer (clockwise, 30mer, 5'-CCTAT AA AAA TAGGC GTATC ACGAG GCCCT-3') and 160U oligonucleotide (5'-GATGG AAGG T CAATG TGAAG ATTGA TGCAT CCCGT CGGGG-3').[8]

Converting into a single copy chromosomal gene. As the regulatory proteins for *aeg-46.5* gene expression control can be titered out by the sequence on multi-copy plasmid, mutagenized gene was moved onto chromosome. We made a single-copy chromosomal version of the gene using homologous recombination, conjugation and P1 transduction. Mutagenized plasmid in HfrH strain, ERL41, can integrate into chromosome by homologous recombination. Homologous regions, *bla* and *lacZ*, are located on the each end of *aeg-46.5*::*lacZ*. Plasmid integrates into chromosome and it gets transferred into recipient cell by conjugation. Colonies that have transferred *aeg-46.5*::*lacZ* were selected on ampicillin plates. Subsequent P1 transduction of the gene allows the transfer of the gene without including the plasmids that is produced in the cell by homologous recombinational excision from chromosome (S. H. Lee and M. H. Choe in press). The cells containing integrated *aeg-46.5*::*lacZ* in the chromosome without the plasmids were screened by ampicillin sensitivity, XG blue color and tetracycline résistance.

Phenotype analysis by *β*-galactosidase assay. The effects of mutation altering putative triple −10 region of *aeg-46.5*::*lacZ* on multicopy plasmids are shown in Figure 2.

Changing putative triple −10 region to a sequence that makes only the first −10 region functional with consensus sequence has a minor effect under aerobic condition. Under anaerobic condition, the effect of the mutation results in the decrease of expression levels. These results indicate that the first −10 region has no function under both aerobic and anaerobic condition for the expression of *aeg-46.5*.

Mutations that change the second −10 region or the third −10 region to consensus Pribnow box had a large effect on the expression level under aerobic condition. Each results in an increase of five- to eight-fold in the aerobic expression level. Under anaerobic condition the expression level did not increase significantly, and the anaerobic induction ratios were not as high as that with the wild-type promoter. This result may have been caused by the increased efficiency of −10 region with consensus sequence, which enhances the overall strength of promoter.

To rule out the possibility in which regulatory proteins are titered out, we transferred mutated promoter on plasmid to chromosome and measured the expression levels from single-copy gene dose. The effects of mutation on putative triple −10 regions of *aeg-46.5*::*lacZ* measured on chromosome are shown in Figure 3.

The expression levels of single-copy version were about 20-fold lower than multicopy. However, the expression patterns were still similar to those of multicopy. Wild-type promoter has low expression under aerobic condition and
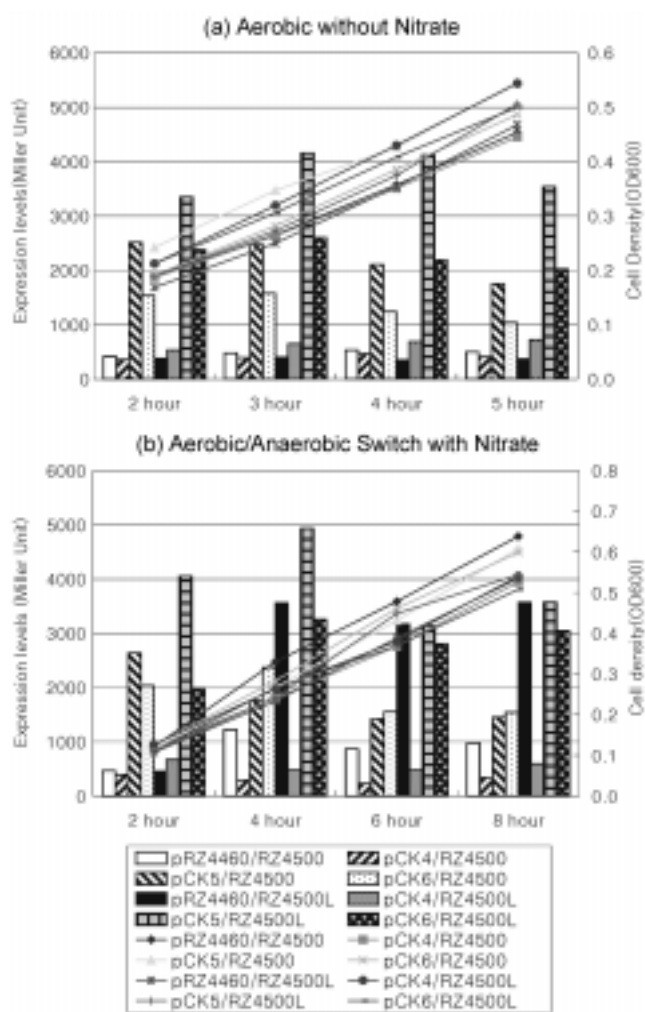
Figure 2. *β*-Galactosidase assays of wild-type plasmid (pRZ4500) and mutant plasmids (pCK4, pCK5, pCK6) in wild-type (RZ4500) and *narL* mutant cell (RZ4500L) in M9-glucose medium under aerobic condition (a) and in M9-glucose-nitrate medium under anaerobic condition (b). After two hours of growth and sampling under aerobic condition the cultures were switched to anaerobic condition with the addition of KNO_3 (b). *β*-Galactosidase activities were measured in triplicate and averaged from three independent experiments.

showed anaerobic induction at two-hours after aerobic/anaerobic switch. CK4 showed notably decreased expression under both aerobic/anaerobic conditions, which appeared to be independent of NarL regulatory protein and aerobic/anaerobic condition. This result indicates that the first −10 region of *aeg-46.5* promoter has no function for the expression of *aeg-46.5*.

CK5 did not cause much change in its expression level depending on aerobic/anaerobic condition. Under anaerobic condition the introduction of *narL* mutation into the cell derepressed the expression level about 3- to 5-fold compared to that from wild-type cell, though it is essentially due to the reduced expression level in wild type cell under anaerobic condition. The derepression effect of *narL* mutation in CK5L compared to CK5 under anaerobic condition is smaller than that from wild-type promoter in CHW compared to CHL.
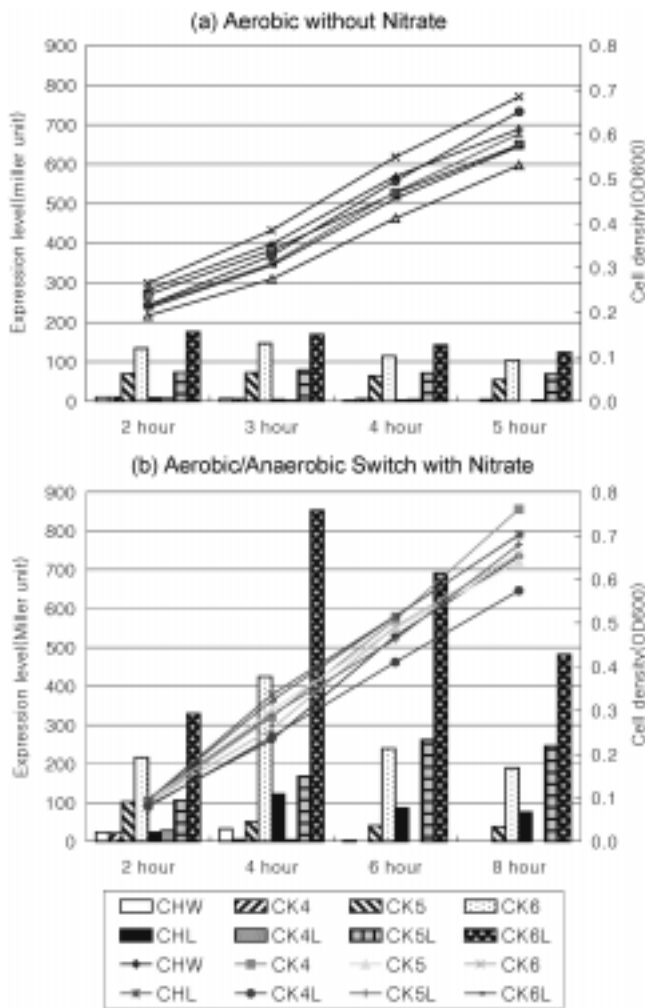
Figure 3. β-Galactosidase assays of wild-type and mutant promoter on chromosome in wild-type and *narL* mutant cells in M9-glucose medium under aerobic condition (a) and in M9-glucose-nitrate medium under anaerobic condition (b). After two hours of the growth and sampling under aerobic condition KNO₃ was added to the medium and switched to anaerobic condition (b). β-Galactosidase activities were measured in triplicates and averaged from three independent experiments.

We could not observe any anaerobic induction in wild type cell though anaerobic CK5L expression shows that the promoter is still under the influence of NarL protein. These result indicate the second -10 region contribute only to a small degree for the aerobic/anaerobic *aeg-46.5* expression.

CK6 showed anaerobic high expression at two hours after aerobic/anaerobic switch. This result correlates with the aerobic/anaerobic expression pattern and the degree of anaerobic induction that were seen with the wild-type promoter. The only apparent difference was a slight decrease of induction ratio.

The switch induction ratios (= anaerobic expression level/ aerobic expression level) are shown in Figure 4.

The induction ratio of mutagenized promoters shows that the second −10 region of a consensus had totally lost its anaerobic induction in the absence of any activity of the third −10 region. The third −10 region mutated to a consen-
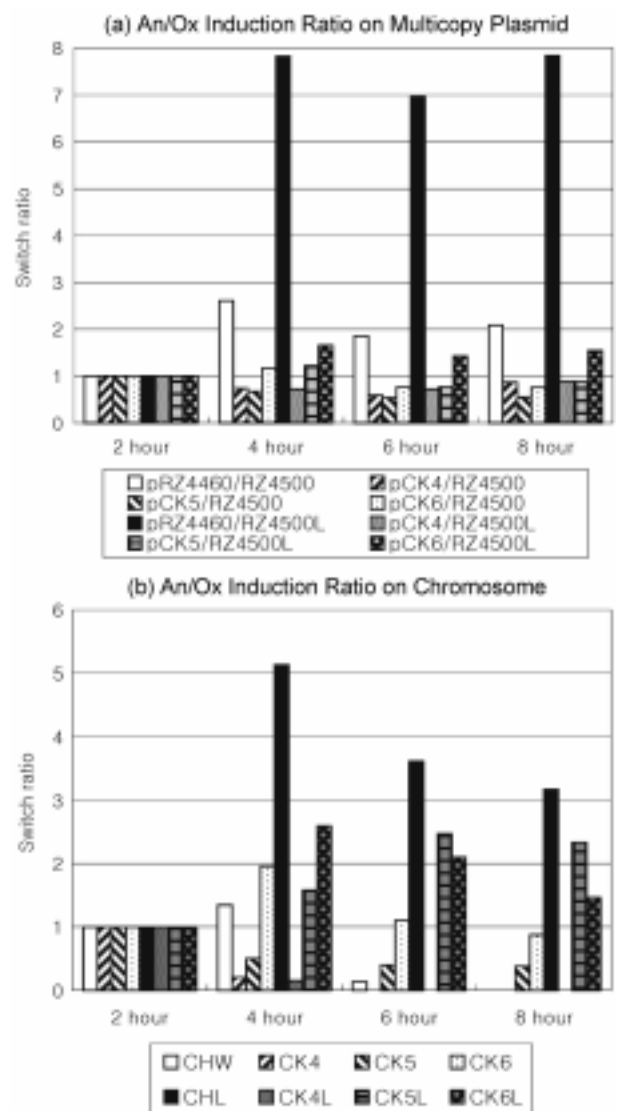
Figure 4. Anaerobic/aerobic induction ratio of multicopy plasmid and single copy chromosomal version. Switch induction ratios of mutant plasmids are less than wild-type plasmid (a). Single copy chromosomal version shows similar pattern as multicopy plasmids but it shows more definite characteristic of each mutation. These results tell that the wild-type −10 region has the most efficient anaerobic induction control.

sus sequence maintained anaerobic induction but the aerobic-anaerobic switch ratio is less than that of wild-type promoter. Therefore it can be proposed that the third −10 region is essential for *aeg-46.5* anaerobic induction and the second −10 region assists the anaerobic induction and the NarL control on the promoter.

Discussion

aeg-46.5 operon was an anaerobically expressed gene located in the centisome 49 (47 min). *aeg-46.5* operon has 15 genes. The first seven genes were predicted to express periplasmic nitrate reductase and the last eight genes were predicted to encode proteins homologous to protein cyto-

chrome assembly.[15] These 15 genes are expressed by *aeg-46.5* promoter, which is regulated by Fnr, NarL and NarP. NarL protein binding site is positioned at −35 region. This binding site overlaps with the RNA polymerase binding site, suggesting the possibility that NarL protein interacts with RNA polymerase and make it recognize one of putative triple −10 region depending on the aerobic/anaerobic respiration condition. But it is found that NarL protein has no role in selecting −10 region from this experiment.

The results from this study on the change of *aeg-46.5* promoter sequence into consensus sequence imply the following. The first −10 region has no function as Pribnow box and the third −10 region has anaerobic induction function, but the change to consensus sequence decreases the aerobic-anaerobic switch induction ratio in *narL* mutant. On the other hand the second −10 region has no function to aerobic/anaerobic induction but maintains the expression levels under both of aerobic/anaerobic condition. This suggests that last two −10 region of putative triple −10 regions compose *aeg-46.5* promoter required for the full aerobic/anaerobic gene expression control. The function of each −10 region could be clarified by testing the mutated promoters that have only one wild-type −10 region sequence functional and the others nonfunctional.

The gene expression regulatory phenotype *aeg-46.5* is abnormal compared to other genes controlled by NarL protein. *aeg-46.5* is induced by nitrate and repressed by NarL that was activated by nitrate. Regulatory protein NarP has the same binding site in *aeg-46.5* promoter as NarL. This implies a possibility that triple −10 regions can be related with NarP regulatory protein. This will be clarified by the test with mutated −10 regions in the *narP⁻* mutation cell in the future study.

aeg46.5 has four transcription start points. The first two are used for aerobic expression and the last two are for anaerobic expression. This observation is consistent with the results of this experiment in which the second −10 region is used for the basal level expression under aerobic condition,

and the third −10 region is used under anaerobic condition that has the normal distance to transcription start point. Primer extension experiment with mRNA from each mutagenized −10 region is on progress to show the relationship between −10 regions and transcription start point.

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