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Symmetry Region at Beginning of Transcript Inhibits Expression of *Escherichia coli aeg-46.5* Operon

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Abstract The *aeg-46.5* operon of *Escherichia coli* is induced by nitrate and anaerobic conditions. Positive regulators Fnr and NarP, and a negative regulator NarL control the expression of the *aeg-46.5*. It has two symmetry regions [6], one of which is located between +37 and +56 bp from the 5'end of the anaerobic transcription initiation site. In this study, mutagenized symmetry regions were transferred from plasmid to chromosome by homologous recombination to evaluate the mutation as a single copy in the *fnr*, *narL*, *narP*, and *narL-narP* double mutant background. The expressions of the *aeg-46.5* operon with these mutations indicated that the control was not through the possible stem-loop structure. Whether there is a protein that mediates this control remains to be seen. The results from the *narL-narP* double mutant indicated that the anaerobic Fnr induction was independent of NarL repression.

Key words: Anaerobiosis, *aeg-46.5*, symmetry region of mRNA, multicopy and single copy gene dose

Escherichia coli is able to use alternative terminal electron acceptors instead of oxygen for the respiratory electron transfer chain. The uses of oxygen and alternative electron acceptors have been studied in various conditions [20], and it had been known that preferences on electron acceptors are hierarchical depending on the reduction potentials of the acceptor. When the oxygen supply is limited, the expressions of the respiratory genes of *E. coli* are changed by the control of several regulatory systems. Without electron acceptors, *E. coli* can produce ATP by fermentation. The enzymes for anaerobic respiration are synthesized only when the corresponding alternative electron acceptors are present under anaerobic condition. The transcriptional activator protein Fnr mediates the expression control of anaerobic respiration enzymes [9]. The synthesis of most anaerobic respiratory enzymes is also regulated in response to the availability of nitrate, ensuring that the organism makes the most efficient use of a terminal electron acceptor for energy production as nitrate is an electron acceptor with the second highest reduction potential to oxygen [8, 22]. The use of nitrate requires a particular complement of enzymes to be synthesized. Included in this set of enzymes are those with a respiratory pathway comprising a formate dehydrogenase (encoded by the *fdnGHI* operon) and a nitrate reductase (encoded by *narGHIJ*). The presence of nitrate represses the synthesis of other alternative anaerobic respiratory chain components [17]. The synthesis of many anaerobic respiratory enzymes is also controlled by nitrite [7, 9, 10, 13, 17, 23, 24]. Nitrate and nitrite regulation of anaerobic respiratory gene expression is mediated by common regulatory systems [17, 24]. Homologous membrane-bound sensor proteins (NarX and NarQ) monitor the availability of nitrate, nitrite, and regulator proteins (NarL and NarP) [24]. The activated response regulator proteins (NarL and NarP) control the expression of target operons. Some of these target operons are only regulated by NarL protein, whereas others are controlled by both NarL and NarP [11, 14, 24]. A previous study reported that the NarL and NarP proteins recognized common DNA-binding sites in several operon control regions [17]. The *aeg-46.5* (an anaerobically expressed locus at 46.5 min on the *E. coli* linkage map) operon was identified in mutants created by the random chromosomal *lac* operon fusion technique using λ*plac*Mu53 [3-6]. Darwin *et al.* [9] suggested that the NarL protein antagonized the NarP-dependent activation of *aeg-46.5* operon expression. The *aeg-46.5* operon expression was induced by the Fnr protein during anaerobic growth and was further induced by nitrate or nitrite [6, 17].

Recently, the whole sequences of *E. coli* centisome 48 was reported [Richterich, P., N. Lakey, K. Robinson, and G. M. Church. 1993. *Genbank accession number U00008*]. This sequence analysis suggested that the *aeg-46.5*-operon-encoded proteins resemble the periplasmic nitrate

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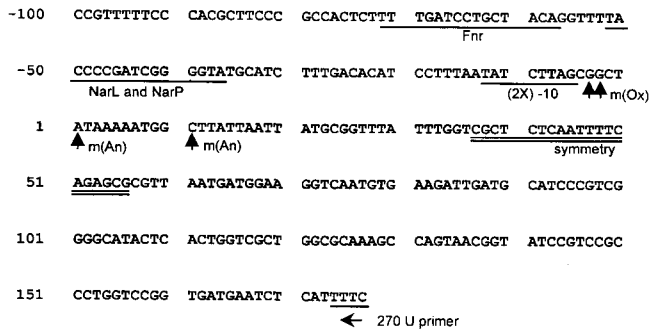

Fig. 1. Upstream DNA sequence of *aeg-46.5*.
Fnr, NarL, and NarP binding sites were identified by Darwin *et al.* [8]. The 5'-ends of mRNA determined by Choe *et al.* [6] are indicated by ↑m and labeled as Ox (aerobic) or An (anaerobic). The NarL, NarP, Fnr binding sites and -10 region are underlined. The symmetry sequence tested in this study is double-underlined and labeled as symmetry. The primers used in mutagenesis are complementary to the sequences double-underlined.

reductase homologous to the NAP protein in *Alcaligenes eutrophus* [20] and also the proteins homologous to those involved in cytochrome c biogenesis in *Bradyrhizobium japonicum, Rhodobacter capsulatus,* and *Paracoccus denitrificans* [12].

The *aeg-46.5* control region has two symmetry regions which are located from -52 to -37, and from +37 to +56, with respect to the anaerobic transcription initiation site [6] as shown in Fig. 1.

The first symmetry region, which is located upstream of the mRNA 5'-end, is an NarL and NarP protein binding site. The Fnr binding site is at the upstream of this NarP and NarL binding site [23]. We had previously changed the sequences of the second symmetry region in the mRNA sequence by site-directed mutagenesis in order to examine the function of this region [2]. In this study, the mutagenized sequences were transferred from plasmids onto chromosome and the effects of the mutations were analyzed. The disruption of the symmetry region changed the expression level of *aeg-46.5*. These results indicate that the expression of *aeg-46.5* is controlled by these symmetry sequences in addition to the Fnr, NarL, and NarP control on the 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. The regulatory gene mutations of *narL215*::Tn*10*, *narP253*::Tn*10*d (Cm), and *fnr-501 zcj*::Tn*10* were introduced into host cells by P1 transduction [21]. Strains ERL41 and W4680 were obtained from David C. Laporte. RK5278 and VJS4325 were obtained from Valley Stewart. RZ4500, RZ8426, and pRZ4460 were obtained from William S. Reznikoff. Plasmid pRZ4460 was used as the template DNA for the site-directed mutagenesis. Four mutant plasmids with mutation at the 5'-end of *aeg-46.5* mRNA, pCA2L, pCA2R, pCA2T, and pCA2I, were constructed in the previous study [2]. pCA2L had the sequence of which the left half was replaced by non-matching sequences, and pCA2R had the sequence of which the right half was replaced by non-matching sequences. In pCA2T, the whole symmetry was replaced by non-symmetrical sequences. pCA2I had the inversed sequence of the wild-type symmetry sequence, which created a different symmetry with the same number of hydrogen bonds between the complementary strands and the same pairing energy as the wild-type symmetry sequence. These mutant plasmid sequences were confirmed using the Cy5™ AutoRead™ Sequencing Kit and ALFexpress DNA Sequencer from Pharmacia Biotech.

Media and Chemicals

The LB and M9 minimal media used in this experiment were described previously [16]. The indicator dye 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) in *N,N*-dimethylformamide was added to a final concentration of 40 μg/ml in agar media. The concentration of antibiotics used was 15 μg/ml for tetracycline, 100 μg/ml for ampicillin, and 100 μg/ml for streptomycin. Potassium nitrate was supplied at a final concentration of 40 mM when the nitrate effect was tested, and the final concentration of sodium nitrite was 5 mM. Cells were always grown at 37°C.

β-Galactosidase Assays

β-Galactosidase activity assays were performed as described previously by Miller [16], using chloroform and 0.1% SDS to permeabilize cells at room temperature. Activities were expressed in arbitrary units, which were determined according to the formula of Miller [16]. The seed cultures were grown aerobically overnight in 5 ml of M9-glucose medium in 15-ml screw cap tubes. Assay cultures were made in new 20 ml M9-glucose media inoculated with seed cultures at a cell density of *E. coli* approximately 0.3 of A_{600}. The assay cultures were incubated aerobically for 2 h and then switched to anaerobic conditions with or without the supplement of an electron acceptor. The anaerobic conditions were made by the following method. The culture was transferred into 15-ml serum stopper capped glass vials, and air was removed by an aspirator. 99.99% nitrogen gas was injected into the culture vials. After three times of repeated deaeration, these vials were incubated in 37°C for 2 h.

Introduction of the Mutational Sequence into the Chromosome of *E. coli*

The method for the introduction of a mutated sequence into the chromosome of *E. coli* is shown in Fig. 2 [18].

Table 1. Strains and plasmids used in this study.

Name	Genotype	Reference or source
Strains		
RZ4500	λ⁻ F⁻ *lacZΔ145*	[5]
CA101L	RZ4500 *narL215*::Tn*10*	[1]
ERL41	Hfr PO *lacI'bla-kan'lacZ, zah-281*::Tn*10 thi-1 relA1 spoT1 supQ80*	[11]
W4680	F⁻ *ΔlacZ39 rpsL melB4*	[11]
RK5278	*narL215*::Tn*10*	[22]
RZ8426	MG1655 *fnr-501 zcj*::Tn*10*	[18]
VJS4325	*narP253*::Tn*10* (Cm)	[22]
CHW	RZ4500 *lacI' bla-aeg-46.5*::*lacZ zah-281*::Tn*10*	this study
CHWL	CHW *Nco*I site in +37– +46 of the *aeg-46.5* control region	this study
CHWR	CHW *Nco*I site in +47– +56 of the *aeg-46.5* control region	this study
CHWT	CHW two *Nco*I sites in +37– +56 of the *aeg-46.5* control region	this study
CHWI	CHW inverted +37– +56 sequence of the *aeg-46.5* control region	this study
CHL	RZ4500 *lacI' bla-aeg-46.5*::*lacZ narL215*::Tn*10*	this study
CHLL	CHL *Nco*I site in +37– +46 of the *aeg-46.5* control region	this study
CHLR	CHL *Nco*I site in +47– +56 of the *aeg-46.5* control region	this study
CHLT	CHL two *Nco*I sites in +37– +56 of the *aeg-46.5* control region	this study
CHLI	CHL inverted +37– +56 sequence of the *aeg-46.5* control region	this study
CHP	CHW *narP253*::Tn*10*d (Cm)	this study
CHPL	CHP *Nco*I site in +37– +46 of the *aeg-46.5* control region	this study
CHPR	CHP *Nco*I site in +47– +56 of the *aeg-46.5* control region	this study
CHPT	CHP two *Nco*I sites in +37– +56 of the *aeg-46.5* control region	this study
CHPI	CHP inverted +37– +56 sequence of the *aeg-46.5* control region	this study
CHF	RZ4500 *lacI' bla-aeg-46.5*::*lacZ fnr-501 zcj*::Tn*10*	this study
CHFL	CHF *Nco*I site in +37– +46 of the *aeg-46.5* control region	this study
CHFR	CHF *Nco*I site in +47– +56 of the *aeg-46.5* control region	this study
CHFT	CHF two *Nco*I sites in +37– +56 of the *aeg-46.5* control region	this study
CHFI	CHF inverted +37– +56 sequence of the *aeg-46.5* control region	this study
CHL2	CHL *narP253*::Tn*10*d (Cm)	this study
CHL2L	CHL2 *Nco*I site in +37– +46 of the *aeg-46.5* control region	this study
CHL2R	CHL2 *Nco*I site in +47– +56 of the *aeg-46.5* control region	this study
CHL2T	CHL2 two *Nco*I sites in +37– +56 of the *aeg-46.5* control region	this study
CHL2I	CHL2 inverted +37– +56 sequence of the *aeg-46.5* control region	this study
Phage		
P1*vir*		[16]
Plasmid		
pRZ4460	pMLB524 2.2 kb fragment of RZ4546.5	[6]
pCA2L	pRZ4460 *Nco*I site in +37– +46 of the *aeg-46.5* control region	[1]
pCA2R	pRZ4460 *Nco*I site in +47– +56 of the *aeg-46.5* control region	[1]
pCA2T	pRZ4460 two *Nco*I sites in +37– +56 of the *aeg-46.5* control region	[1]
pCA2I	pRZ4460 inverted +37– +56 sequence of the *aeg-46.5* control region	[1]

Each plasmid DNA, pRZ4460, pCA2L, pCA2R, pCA2T, and pCA2I, was extracted a using a QIAGEN Plasmid Kit, and used to transform ERL41. Each of the transformed ERL41, harboring plasmid pRZ4460, pCA2L, pCA2R, pCA2T, or pCA2I, and the recipient W4680 were grown to the mid-log phase, A_{600} of approximately 0.6, while shaking at 37°C. 0.1 ml of each culture of the donor and recipient were mixed in a test tube and incubated for 45 min at 37°C without shaking. Five ml LB media was added and the cultures were incubated for 3 h with shaking at 37°C. This medium included ampicillin, tetracycline, and streptomycin to select exconjugants that had inherited a copy of the plasmids integrated into the W4680 chromosome. Following the initial selection, the cells were collected by centrifugation, resuspended in the LB medium, including ampicillin, tetracycline, and streptomycin, and grown

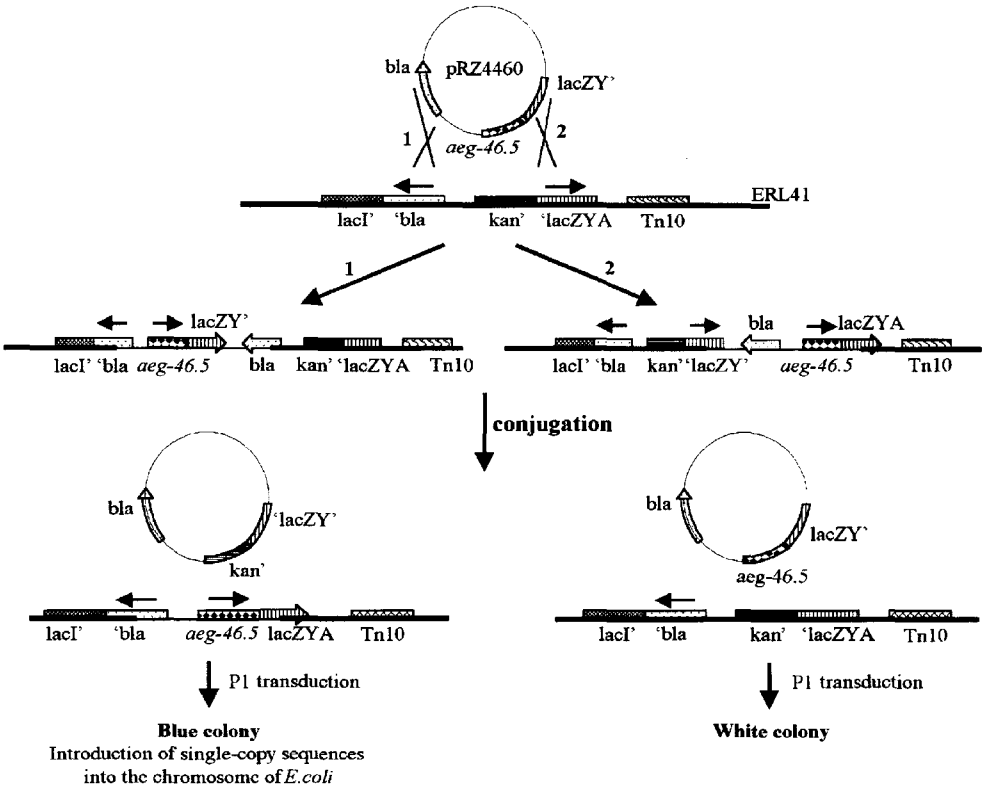

Fig. 2. Method for transfer of mutagenized sequences to the chromosome.
Thick horizontal lines indicate chromosome sequences while plasmid sequences are shown as thin horizontal lines. The directions of transcription are indicated with arrows. Plasmids transferred onto chromosome are pRZ4460, pCA2L, pCA2R, pCA2T, and pCA2I. These are not drawn to scale.

overnight. The recombined sequences on the chromosome were separated from the plasmid by transferring them into a new genetic background, RZ4500, by P1 transduction. The transductants were selected using a drug resistance marker that had been transferred to the chromosome with the mutated sequence. Transductants were tested for ampicillin sensitivity to find out the cells that had not inherited the original plasmid, and selected for *lacZ*$^+$ with X-gal to ensure that they had the *aeg-46.5* promoter fused to *lacZ* on the chromosome. The selection marker Tn*10* exhibited 75% cotransduction frequency with the *aeg-46.5::lacZ*.

RESULTS AND DISCUSSION

In order to investigate the function of the symmetry element at the beginning of the mRNA of *aeg-46.5*, we previously constructed four plasmids containing mutations on the symmetry region [2]. The symmetry region of the mRNA, which is located between positions +37 and +56 from the anaerobic transcription initiation site, consists of 20 base pairs and its sequence is CGCTCTcAAt tTTcAGAGCG [6]. In previous studies, it was shown that Fnr binds at positions -71 to -58, and NarL or NarP binds at positions -52 to -37 in the *aeg-46.5* promoter [6, 8]. To investigate the function of the symmetry element of the mRNA in the expression control of *aeg-46.5*, four mutagenic oligonucleotides were designed and mutant plasmids were created as previously reported. These mutants had a different *lacZ* expression compared to the pRZ4460 plasmid in wild-type cells and *narL* mutant cells [2]. The fact that pCA2L showed a higher expression level than pCA2T implied a possibility of the existence of a factor that might have specific relationship with the half of symmetry sequences in pCA2L [2].

These multicopy systems can have problems, due to the multiple gene dosage, as follows [18]: (i) The presence of multiple copies of control sequences can result in the titration of key regulatory components that are present in limited amounts. (ii) The physical properties of sequences that normally reside on the chromosome may be significantly different when they are carried by a plasmid. (iii) A plasmid's copy number can vary depending on factors such as growth conditions or the transcriptional activity of the promoters which are carried. (iv) The overproduction of plasmid-encoded products, which usually results from high gene dosage, is often deleterious to the cell. (v) The presence of multicopy plasmids that bear strong promoters can inhibit growth on some carbon

sources. In an effort to circumvent and compare the problems presented by multicopy vectors, the cloned sequences were transferred onto the chromosome by homologous recombination at the *lac* locus.

Nitrate Induction and *narL* Regulation

The effect of a mutation on the expression of the gene was measured by comparing samples taken under aerobic and anaerobic conditions. The samples of anaerobic condition were taken after 2 h of aerobic to anaerobic switch. The phenotypes from the single copy gene on chromosome were observed in the strains CHW and CHL grown in the same medium conditions. CHW was constructed by transferring the mutation on plasmid onto chromosome via homologous recombination [18]. The resulting CHW had Tn*10*-Tet linked with the mutation on its chromosome, and the subsequent introduction of the mutations of gene expression regulatory systems required the removal of the Tn*10*-Tet to make it possible to select the tetracyclin resistant P1 transductants. The tetracyclin sensitive cell from CHW-Tet was constructed using the procedure of Maloy [15]. CHL was made by P1 transduction of the *narL* mutation from RK5278 that was linked to a Tn*10*-Tet into a CHW-Tet^S cell. The β-galactosidase activities were measured and are shown in Table 2 and Fig. 3.

Table 2. β-Galactosidase activity of mutant single copy constructs bearing symmetry region variations (wild-type, L, R, T, I) in wild-type RZ4500 (CHW), *narL* (CHL), *fnr* (CHF), *narP* (CHP), *narL/narP* (CHL2) mutant cells.

Strain	β-Galactosidase activity (miller units)	
	Aerobic	Anaerobic
CHW	5	27
CHWL	4	32
CHWR	4	25
CHWT	4	27
CHWI	4	25
CHL	5	157
CHLL	4	201
CHLR	3	132
CHLT	4	150
CHLI	3	126
CHP	4	4
CHPL	3	3
CHPR	3	3
CHPT	3	3
CHPI	3	3
CHF	5	6
CHFL	3	6
CHFR	3	5
CHFT	3	5
CHFI	3	4
CHL2	4	5
CHL2L	4	5
CHL2R	3	3
CHL2T	3	4
CHL2I	3	3

At the switch from aerobic to anaerobic conditions, potassium nitrate was added to make a final concentration of 40 mM. There was no difference in expression level between wild-type plasmid (pRZ4600) and the mutant plasmids (pCA2L, R, T, I) in the wild-type strain RZ4500 and the narL mutant strain CA101L under aerobic conditions as reported in previous study. In the case of the single copy version, the CHW and CHL series also showed no differences in their expression levels under aerobic conditions (Table 2 and Fig. 3). However, under anaerobic condition, the symmetry variants in wild type cells either in multicopy plasmid or in single copy version showed similar β-galactosidase activities as wild-type sequence except the plasmid pCA2L/RZ4500 [1]. However, in the *narL*$^-$ cells, CHLL displayed the highest expression and CHLI gave the lowest under anaerobic condition. The plasmid versions of the symmetry variation in the *narL* mutant cells, which was reported previously, also showed similar expression patterns although the pCA2R gave a similar level of expression as pCA2L.

CHLT, which had no symmetry and could not form a stem-loop structure, was expected to give the highest expression if the translational control depended solely on symmetry and the formation of a stem-loop structure. However, the fact that the L mutants had a higher expression than the T mutant implies a possibility of the existence of a factor that may have a specific relationship with the symmetry sequence. The β-galactosidase activities of the I mutants, which had the same pairing energy as

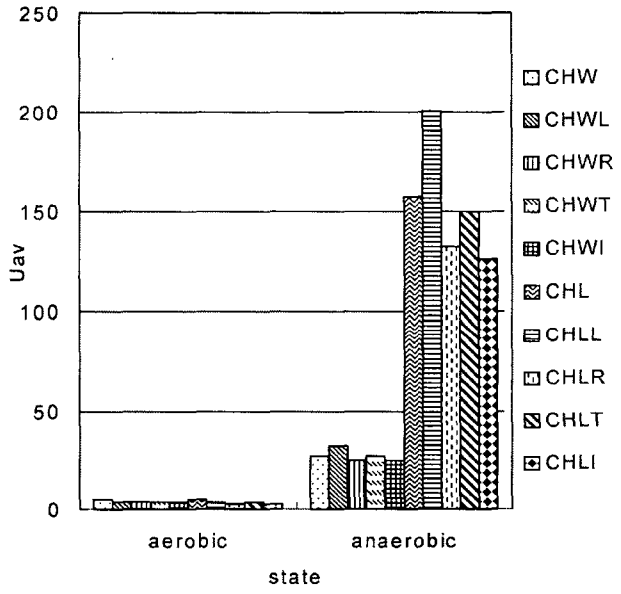

Fig. 3. β-Galactosidase activities from wild-type and L, R, T, I mutant symmetry regions in M9-glucose medium under aerobic condition and in M9-glucose-nitrate medium under anaerobic condition.
After 2 h under the aerobic condition, KNO_3 was added into the medium and switched to an anaerobic condition.

the wild-type, had a lower expression than the wild-type. This also suggests that the symmetry of the sequence may not be the only factor of the function of this region forming a stem-loop structure. The hypothetical additional factor remains to be investigated. If there is no electron acceptor supplemented in the media, *lacZ* is not expressed at all in any cells (data not shown). The single copy chromosomal versions of the mutations clearly showed the effect of the mutations rather than the multicopy plasmid that gave a high expression of the reporter gene from all mutations.

Recently, it was reported that the primary inducer for NarP-dependent *aeg-46.5* operon expression is nitrite rather than nitrate [8, 17], after measuring the effect of the nitrite ion at a final concentration of 5 mM sodium nitrite. Induction by nitrite was not observed to detectable levels after the switch to an anaerobic state in the strains of this study (data not shown), even when the cells with the multicopy plasmid were used [2]. This discrepancy could be due to a difference of the genotype of the host strain and/or the growth condition. The single copy genes in the strains of this study were located at the 8 min locus of the chromosome and the chromosomal configuration may not be suitable for activator protein, NarP, binding. This problem remains to be seen.

fnr and *narP* Regulation

CHF strains, which do not have the FNR protein but include the NarL and NarP proteins, were constructed by introducing the *fnr* mutation from RZ8426 which had *fnr-501* linked to *zcj*::Tn*10* into CHW-Tet^S through P1 transduction [5] for a single copy test. These CHF cells did not show any expression of *lacZ* under anaerobic conditions (Table 2). Stewart *et al.* [17] identified the *narP* gene, which encodes a response regulator that is 44% identical to the NarL protein. The NarP protein activates the *aeg-46.5* operon while NarL represses it [17]. Experiments were conducted with a *narP* null mutation to determine its regulation. A CHP strain was constructed by introducing the *narP* mutation from VJS4325 that had *narP253*::Tn*10*d (Cm) into CHW through P1 transduction. In the derivatives of this strain that had no NarP activator proteins but included NarL repressors, β-galactosidase activities disappeared (Table 2). This confirms that both Fnr and NarP are required for *aeg-46.5* operon expression.

The effect of the double mutation of the regulatory elements, NarL and NarP, on *aeg-46.5* was tested. In the *narL* and *narP* double mutant strain, CHL2, the *aeg-46.5* expression had been abolished (Table 2). This indicates that the NarL repression has effect only in the presence of NarP activator and the Fnr control is independent of NarL repression. The *fnr* and *narL* double mutant case has already been reported [1] as having no expression.

The regulation of *aeg-46.5* operon expression can be summarized as follows [9]. In an anaerobic environment, the Fnr protein is active and binds to the 64.5 site to induce *aeg-46.5* operon expression. The presence of nitrate results in activation of the NarP and NarL proteins. The NarL and NarP proteins compete for the 44.5 binding site; the binding of NarP further induces the expression of the *aeg-46.5* operon, whereas the binding of NarL has no significant effect on the basal level of anaerobic Fnr-dependent expression. The regulatory sequence of the *aeg-46.5* is an unusual promoter in the sense of the assembly of the binding sites for Fnr, NarL, or NarP proteins compared to other promoters that are regulated by the same regulatory proteins [9, 17]. In the cases of *narGHJI*, *fdnGHI*, and *frdABCD* operons, NarP did not activate these operons, while the role of NarL was an activator, which is in contrast to *aeg-46.5* [17]. That means the *aeg-46.5* operon could have another mechanism for the control of NarL and NarP effects in the presence of nitrate [9, 17].

The *aeg-46.5* operon has a Fnr protein binding site centered at -64.5, and a NarL or NarP binding site at the -44.5 region. The bindings of these proteins are important for *aeg-46.5* operon expression and the +37 to +56 symmetry sequence is also playing a role in the control of the expression of the *aeg-46.5* operon. Whether the inhibition is via a protein binding to this symmetry sequence still needs to be investigated.

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