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Fnr, NarL and NarP Regulation and Time Course Expression of *Escherichia coli aeg-46.5* Gene

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Abstract: The anaerobically expressed gene *aeg-46.5*, which had been identified by the operon fusion technique with a hybrid bacteriophage of λ and Mu, λ*plac*Mu53, was studied for its expression pattern and growth. The expression of *aeg-46.5* was studied in the wild-type cell and mutant cells that have mutation (s) in the control gene of anaerobic respiration (*fnr*) and nitrate response (*narL* and *narP*). The β-galactosidase reporter gene showed maximum expression in *narL* host after two hours of aerobic to anaerobic switch in M9-Glc-nitrate medium. Both 40 mM and 100 mM concentrations of nitrate ion in the medium had little effect on expression level. We propose that *aeg-46.5* is subject to multiple regulations of anaerobic activation by Fnr, nitrate activation by NarP and repression mediated by NarL.
Key words: anaerobically expressed gene, *aeg-46.5*, nitrate induction, NarL, NarP.

Escherichia coli is a facultative anaerobic bacterium. In order to grow under various conditions, it has evolved physiological mechanisms to adapt to those conditions. *E. coli* transforms the chemical energy of a variety of carbon sources into ATP. In this process, it uses oxygen as a terminal electron acceptor. If oxygen supply is limited, *E. coli* grows anaerobically using alternative electron acceptors such as nitrate, trimethylamine-*N*-oxide, dimethyl sulfoxide, or fumarate. Without any electron acceptor, it grows by fermentation for ATP production.

The protein profile showed that the levels of at least 125 proteins were influenced by the switch between aerobic and anaerobic conditions (Neidhardt and Smith, 1983). Fnr, the transcriptional activator protein, is required for the induction of anaerobic respiratory enzymes (Guest and Spiro, 1987; Gunsalus and Melville, 1990). Fnr positively regulates nitrate reductase (*nar GHJI*), fumarate reductase (*frdABCD*), and dimethyl sulfoxide reductase (*dmsABC*) (Gunsalus and Jones, 1987; DeMoss and Li, 1988; Gunsalus and Cotter, 1989).

If various alternative electron acceptors are available, *E. coli* uses regulatory systems which control the expression of anaerobic respiratory genes hierarchically according to the reduction potentials of available alternative electron acceptors. For example, two membrane-bound sensor proteins, NarX and NarQ, can independently sense the presence of nitrate ion and transfer this signal to the DNA-binding regulatory protein NarL (DeMoss and Walker, 1993; Gunsalus *et al.*, 1994). The NarL acts as an activator for nitrate reductase (*narGHJI*) (Stewart, 1982) and also acts as a repressor for fumarate reductase (*frdABCD*), dimethyl sulfoxide reductase (*dmsABC*), and pyruvate formate lyase (*pfl*) synthesis in response to the presence of nitrate in the medium (Lin and Iuchi, 1987; Böck and Sawers, 1988; Gunsalus and Kalman, 1990).

A new anaerobically expressed gene, *aeg-46.5*, which is located at min 46.5 on *E. coli* linkage map was identified by operon fusion technique with a hybrid bacteriophage λ and Mu, λ*plac*Mu53 (Weinstock *et al.*, 1985), by Reznikoff and Choe (1991). The *aeg-46.5 gene* is induced anaerobically in the presence of nitrate ion and repressed by NarL (Reznikoff and Choe, 1991). The control region of *aeg-46.5* was identified and sequenced (Reznikoff and Choe, 1993). Through computer analysis, the binding sites of Fnr and NarL and several possible −10 and −35 regions were proposed by Reznikoff and Choe (1993). In order to characterize the regulation of *aeg-46.5*, we report here the time course expression pattern of *aeg-46.5* in the *fnr*, *narL*, *narP* mutant background.

Materials and Methods

Strains

All bacterial strains used in these experiments are derivatives of *E. coli* K-12 and are described in Table 1. The construction of RZ4500 and RZ4546.5 has

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Table 1. *E. coli* strains used in this study

Strains	Genotype	Source or Reference
MG1655	λ⁻ F⁻	Bachmann (1987)
RZ4500	λ F *lacZΔ145*	Reznikoff and Choe (1991)
RZ4546.5	RZ4500 *aeg-46.5*::λ*plac*Mu53	Reznikoff and Choe (1991)
CA46.5F	RZ4546.5 *fnr-501 zcj*::Tn10	This Study
CA46.5L	RZ4546.5 *narL215*::Tn*10*	This Study
CA46.5FL	RZ4546.5 *fnr-501* *narL215*::Tn*10*	This Study
CA46.5P	RZ4546.5 *narP253*::Tn*10d*(Cm)	This Study
CA46.5LP	RZ4546.5 *narL215*::Tn*10* *narP253*::Tn*10*d(Cm)	This Study
RZ8426	MG1655 *fnr-501 zcj*::Tn*10*	Reznikoff and Choe (1991)
RK5278	*narL*::Tn*10*	Stewart (1982)
VJS4325	*narP253*::Tn*10*d(Cm)	Stewart and Revin (1993)

been described previously (Rezinikoff and Choe, 1991). Introduction of *fnr-501 zcj*::Tn*10*, *narL215*::Tn*10*, and *narP253*::Tn*10*d(Cm) was done by P1 transduction (Silhavy *et al.*, 1984). The *fnr-501 zcj*::Tn*10* transduced strain was tested for the *fnr* phenotype by examining their anaerobic growth on M9-glycerol-nitrate agar in an anaerobic jar (BBL Microbiology Systems, Becton Dickinson and Co.). The transductants that did not grow anaerobically on this medium were used. Tn*10* marker which was introduced with *fnr-501* mutation had to be removed for the next genetic manipulation, and the selection of the loss of the Tn*10* marker was done by the method of Bochner *et al.* (1980).

Media and chemicals

LB and M9 minimal media were described previously (Miller, 1992). The concentration of antibiotics used was 40 μg/ml for kanamycin, 15 μg/ml for tetracycline, and 25 μg/ml for chloroamphenicol. Potassium nitrate or sodium nitrite was used as an electron acceptor.

β-Galactosidase assays

β-Galactosidase assays were performed as described by Miller (1992), using chloroform and 0.1% sodium dodecyl sulfate to permeabilize cells. β-galactosidase activities were measured in triplicate. The seed cultures were grown aerobically overnight in 50 ml of M9-glucose medium in Erlenmeyer flask (250 ml). Assay cultures (500 ml) were made in media bottles (1 l) inoculated with 50 ml of seed culture. The assay cultures were incubated aerobically for 2 h and switched to anaerobic conditions with or without the supplement of electron acceptor. Cells were always grown at 37℃.

Results and Discussion

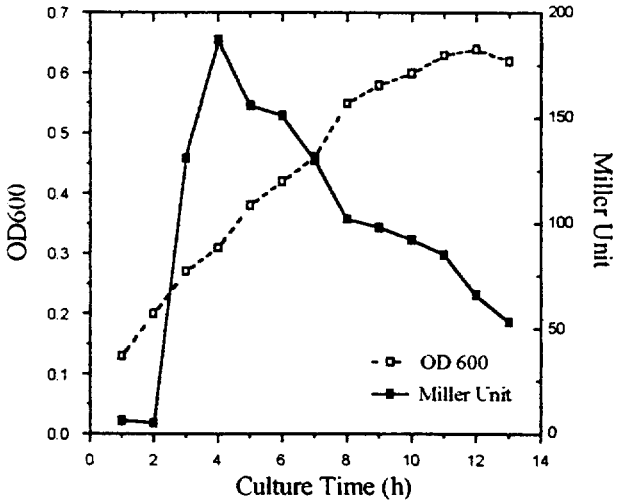

Fig. 1. The expression pattern of *aeg-46.5* and growth curve of its fusion strain with *narL* mutation in 40 mM KNO_3-M9-Glc medium. After two hours in aerobic conditions KNO_3 was added to the medium and switched to anaerobic conditions.

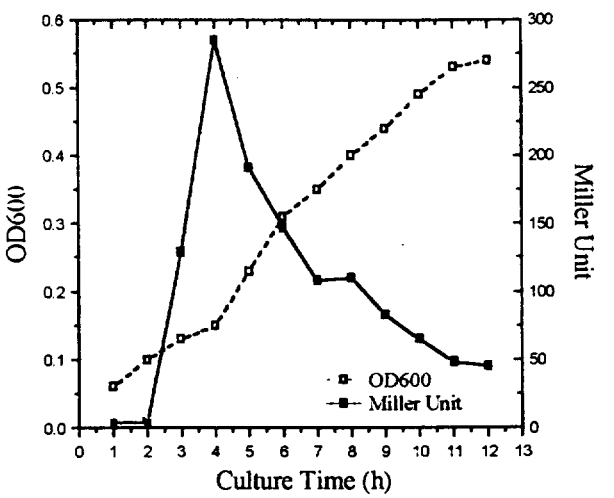

Fig. 2. The expression pattern of *aeg-46.5* and growth curve of its fusion strain with *narL* mutation in 100 mM KNO_3-M9-Glc medium. After two hours in aerobic conditions, KNO_3 was added to the medium and switched to anaerobic conditions.

narL regulation and nitrate induction

NarL, the *narL* product, mediates nitrate activation of the *narGHJI* and *fdnGHI* operons and nitrate repression of *frdABCD* and *dmsABC* operons. The role of *narL* in the regulation of *aeg-46.5* was examined by transducing the *narL215*::Tn*10* allele into RZ4546.5. β-galactosidase activity was measurd in M9-Glc medium in the presence of 40 mM of 100 mM nitrate ion.

The results of these assays showed a characteristic regulatory pattern (Fig. 1 and 2). The *aeg-46.5* reached maximum expression in *narL* mutant after two hours of aerobic to anaerobic switch, and the

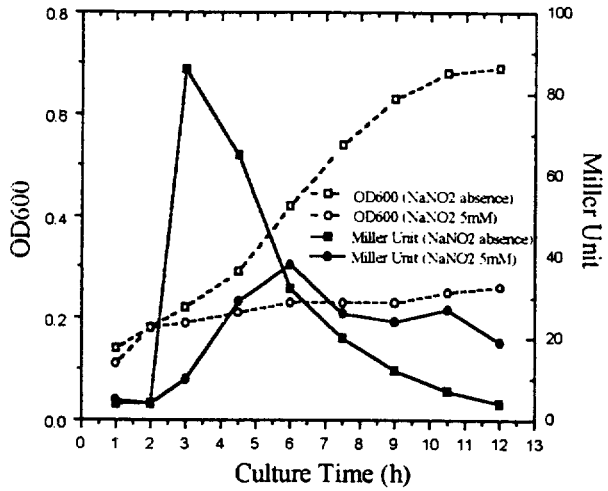

Fig. 3. The expression pattern of *aeg-46.5* and growth curves of its fusion strain with *narL* mutation in 5 mM $NaNO_2$-M9-Glc medium or M9-Glc medium. After two hours culture in aerobic conditions, $NaNO_2$ was added to the medium and switched to anaerobic conditions.

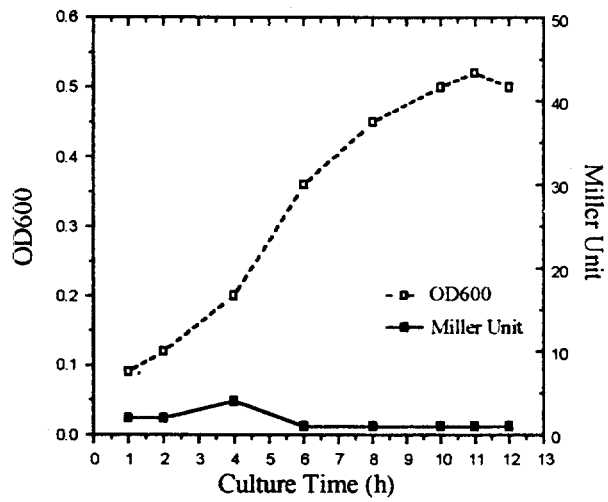

Fig. 4. The expression pattern of *aeg-46.5* and growth curve of its fusion strain with *narP* mutation in 40 mM KNO_3-M9-Glc medium. After two hours in aerobic conditions, KNO_3 was added to the medium and switched to anaerobic conditions.

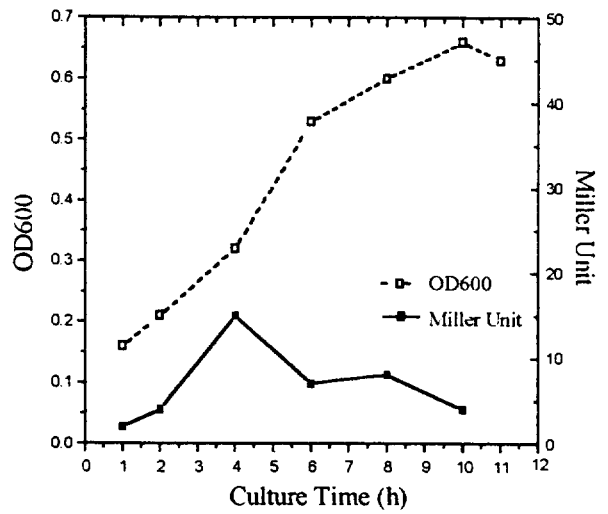

Fig. 5. The expression pattern of *aeg-46.5* and growth curve of its fusion strain with *narL* and *narP* double mutation in 40 mM KNO_3-M9-Glc medium. After two hours in aerobic conditions, KNO_3 was added to the medium and switched to anaerobic conditions.

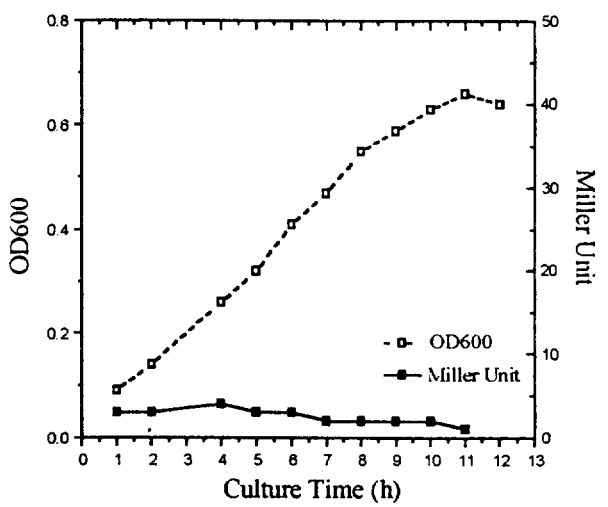

Fig. 6. The expression pattern of *aeg-46.5* and growth curve of its fusion strain with *fnr* and *narL* double mutation in 40 mM KNO_3-M9-Glc medium. After two hours in aerobic conditions, KNO_3 was added to the medium and switched to anaerobic conditions.

expression level decreased slowly after the maximum level. The 40 mM and 100 mM concentration of the nitrate ion in the medium gave little differences on expression level. This result leads us to believe that if the minimum level of nitrate ion required for *aeg-46.5* induction is obtained, the *aeg-46.5* shows its characteristic expression pattern and the excess nitrate ion doesn't increase the maximum level nor expand the expression interval of *aeg-46.5*.

The effect of nitrite ion on the expression of *aeg-46.5* was measured (Fig. 3). The anaerobic growth of *narL* mutant strain in nitrite medium was poorer than in nitrite free medium. The anaerobic expression of *aeg-46.5* was not elevated at all by the presence of nitrite ion in the medium. On early anaerobic phase β-galactosidase units in nitrite medium were lower than that in nitrite free medium.

narP regulation

Recently, NarP, which is homologous to NarL regulatory protein, was identified by Stewart and Rabin (1993) using *aeg-46.5*::*lacZ* fusion gene as a probe system. We introduced *narP253*::Tn*10*d(Cm) allele into RZ4546.5 by P1 transduction and studied the effect of *narP* mutation on the expression of *aeg-46.5* (Fig.

4). Interestingly, there was no expression of *aeg-46.5* during all the time course examined. This suggests that the expression of the *aeg-46.5* absolutely requires NarP as a positive regulator.

The effects of double mutation

We examined the effects of double mutation of regulatory systems on *aeg-46.5* expression. In *narL* and *narP* double mutant, the expression of *aeg-46.5* had been abolished (Fig. 5). This observation leads us to believe that the NarP is an essential regulatory protein needed for *aeg-46.5* expression even in the case of the absence of NarL repression. In the *fnr* and *narL* double mutant, no expression was observed (Fig. 6). These data also show that *aeg-46.5* needs Fnr protein for its anaerobic expression in the absence of NarL repression.

We studied the time course expression pattern of *aeg-46.5* using operon fusion strains which had an *aeg-46.5*::*lacZ* fusion. In *narL* mutation background, the *aeg-46.5* was expressed higher in early-log phase than late-log phase in anaerobic conditions. These data lead us to assume that the products of *aeg-46.5* may be required in early phase of anaerobiosis of *E. coli* for the use of nitrate as an electron acceptor. We monitored the anaerobic growth of wild-type strain (RZ4500), fusion strain (RZ4546.5), and *narL* mutant strain with fusion (CA46.5L) in M9-glucose-nitrate medium. There were no differences observed in their growth rates (data not shown).

Recently, Stewart and Rabin (1993) reported that *aeg-46.5* is also induced by nitrite ion. They identified the second nitrate response regulator NarP, which is 44% identical to NarL, and proposed that the primary signal for *aeg-46.5* induction is nitrite rather than nitrate. But, in our experiments, the cell growth was very poor and the expression was not induced with nitrite ion in the medium. We presume this difference is due to the differences of host strain and media conditions used for the anaerobic culture of the cell.

In *narL* and *narP* double mutants, where the repressor NarL is not made, the expression level of *aeg-46.5* is below 15 units under anaerobic conditions. This suggests that NarP acts as an essential positive regulator for the expression of *aeg-46.5*. In *fnr* and *narL* double mutant, although the NarP has its full activation function and the NarL is absent, there is no expression of the gene. From these results, we conclude that anaerobic expression of *aeg-46.5* needs both of Fnr and NarP activation, and without the activation, the removal of NarL repression alone does not induce *aeg-46.5* expression. The *aeg-46.5* gene is subject to multiple regulations of anaerobic activation by Fnr, nitrate activation by NarP, and repression mediated by NarL.

The transcriptional unit of *aeg-46.5* and the function of its products are currently unknown. We are planning further experiments to investigate the interaction of the Fnr, NarP and NarL proteins with the *aeg-46.5* control region, the mRNA length of *aeg-46.5* and function of its products.

The construction of a merodiploid cell where the *aeg-46.5* products are active will help us to study the function of *aeg-46.5* in detail.

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