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Characterization of the nar Promoter of *Escherichia coli* to use as an inducible promoter in Wild-type host *Agrobacterium.tumefaciens*

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ABSTRACT

In this study, the nar promoter of E. coli was characterized to see whether the nar promoter cloned onto pBBR122 can be used as an expression promoter of gram negative microbes. For this purpose, a plasmid with lacZ gene expressing β-galactosidase instead of the structural genes of nar operon in a gram negative host strain(*Agrobacterium.tumefaciens*) was used to simplify an assay of induction of the nar promoter. The following effects were investigated to find optimal conditions: methods of inducing the nar promoter, optimal nitrate concentration, maximally inducing the nar promoter, the amount of expressed β-galactosidase and induction ratio(specific β-galactosidase activity after maximal induction/specific β-galactosidase activity before induction). The following results were obtained from the experiments: the growth of *Agrobacterium* with E.coli nar promoter was not much affected by nitrate concentration in the shake-flask; induction of nar promoter was optimal when *Agrobacterium* was grown in the presence of 1% nitrate ion at the beginning of culture and when overnight culture was completely grown in the shake-flask before being transferred to other shake-flask; the amount of β-galactosidase per cell and per medium volume was maximal when *Agrobacterium* was grown under aerobic condition to OD_{600} of 1.7; then the nar promoter was induced under microaerobic and anaerobic condition made by lowering dissolved oxygen level(DO). After 2-3h of induction in the YEP medium selected as a main culture medium, the specific β-galactosidase activity became about 17,000 Miller units in the fermentor cluture.

INTRODUCTION

The induction properties of the nar promoter cloned into pBR322 plasmid are dependant on the nucleotide sequence of the nar promoter, mutations on the chromosome of the host, and host E.coli strains.[1][2][3][4] For example, when the

wild type nar promoter was used, addition of nitrate ion in addition to anaerobic condition was necessary for the maximal induction of the nar promoter.[6] In this case, however, a mutation in the narG gene encoding one subunit of nitrate reductase on a E.coli chromosome(RK5265(RK4353 narG-)) further increased expression level, because lowering of the cellular redox potential by nitrate reductase can supress induction of the nar operon, also resulting in the autoregulation of expression of β-galactosidase from the nar promoter cloned into a plasmid[1)2)] when one of the wildtype nar promoter was tested in a mutant host E.coli(pMW61/RK5265). Induction of the nar promoter was optimal when E.coli was grown in the presence of 1% nitrate at the beginning of culture, the induction ratio was maximal, approximately 300, after approximately 6h of induction, OD_{600} became 3.2 and specific β-galactosidase activity became 36,000 Miller unit, equivalent to 35% of total cellular proteins.[1)]

MATERIALS AND METHODS

The gram negative strains, *Agrobacterium.tumefaciens* LBA 4404 was a wide-type strain. For Plasmids, pNW61 having backbone of pBBR122 to use broad host range vector, a derivative of pMW61 was kindly donated by DeMoss at the University of Texas Medical School in Texas was constructed by fusing the nar promoter containing amino terminus of the first structural gene of the nar operon, narG, with the lac Z gene. The specific β-galactosidase activity, β -galactosidase activity per cell, was measured by the Miller method. Miller units were used to express the specific β-galactosidase activity.

RESULTS & DISCUSSION

To see whether the nar promoter could be used as an oxygen-dependent inducible promoter in *Agrobacterium* with pNW61, the specific β-galactosidase activity was measured during growth of *Agrobacterium* in the absence of nitrate and in the presence of various nitrate concentration.[Fig. 1B)] The growth of E.coli was inhibited but the growth of *Agrobacterium* was not inhibited in presence of nitrate.[Fig. 1A)] To characterize this nar promoter system further, the growth of E.coli was inhibited in presence of nitrate was measured at different OD_{600}.[Fig. 3)] To see the effect of DO level on the expression of nar promoter, Agrobacterium was grown in the presence of 1% nitrate at the high DO level(DO over 80%), and then the nar promoter was induced by reducing the

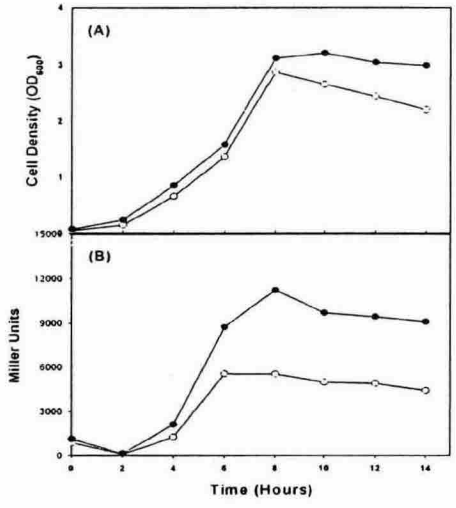

Figure 1. Growth dependence of expession of β-galactosidase. OD_{600}(A) and specific β-galactosidase activity(B). The *Agrobacterium* was grown in the presence of 1% nitrate(●) or in the absence of it(○).

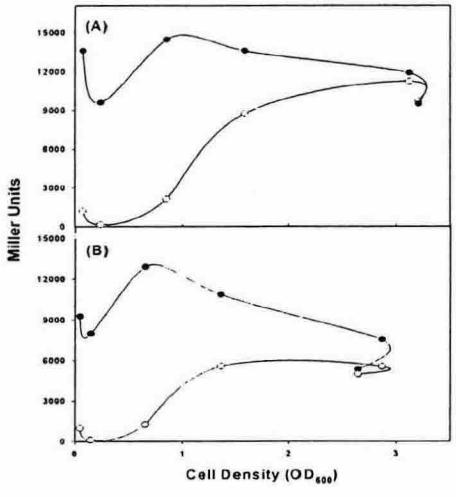

Figure 2. The *Agrobacterium* in the flasks in the presence of 1% nitrate(A) or in the absence of nitrate(B). The specific β-galactosidase activity was measured without induction(○) or after 2 h of induction(●).

DO level. Agrobacterium was grown to OD600 of 1.0, at high DO level. Then, after DO level was dropped to 1-2% *Agrobacterium* was grown continuously. The specific β-galactosidase activity increased sharply and continuously after induction up to OD_{600}= 2.60, where the activity became 16,900 Miller units. As the specific β-galactosidase activity was approximately 200 just before induction, induction ratio was about 85.[Fig. 3] Previously we showed that supply of nitrogen gas instead of air to make DO Level 0% was not good for the maximal induction because E.coli did not grow well[2] but *Agrobacterium* was greatly not affected, so DO level was regulated to 1-2% by manufacturing air and rpm after DO level was made nearly 0% by supplying nitrogen gas. After the specific β-galactosidase activities were obtained for different OD_{600} at the induction time, they were plotted against OD_{600}.[Fig. 4] the maximal specific β-galactosidase activity occurred at OD_{600} = 1.5 at the induction time. Usually the final OD_{600}, where the maximal specific β-galactosidase activity occurred for each induction experiment, was about 2.9-3.4 after 2-4 h of induction. Therefore, the maximal specific β-galactosidase activity still occurs at induction absorbance(OD_{600}) of 1.5(final OD_{600} = 3.11), where the activity was

approximately 17,200 Miller units.

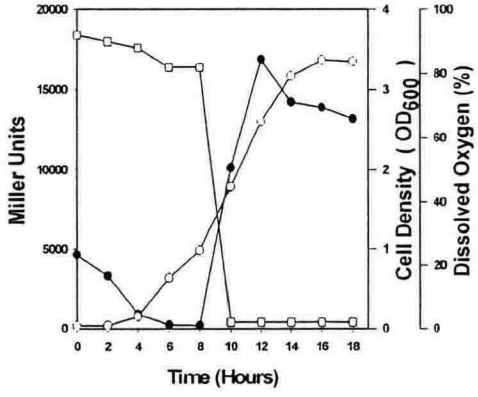

Figure 3. Effect of dissolved oxygen(DO) levels on the expression of the nar promoter under the microaerobic conditions. The specific β-galactosidase activity(●) and OD_{600}(○) were also measured.

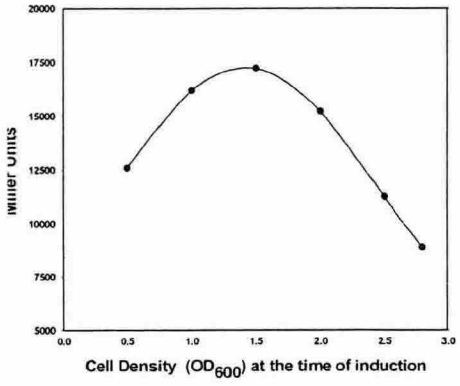

Figure 4. Effect of the growth phase of *Agrobacterium* on the expression of β -galactosidase.

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