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# Expression Patterns of *Bacillus subtilis* Diacylglycerol Kinase Gene Induced by Physiological Stimuli

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Diacylglycerol kinase (DGK) phosphorylates the second messenger diacylglycerol (DAG) to phosphatidic acid and it may play a role in signal transduction in *Escherichia coli* as well as in eukaryotic cells. In addition, DGK is important for microorganisms to adapt to several physiological stimuli. In *Bacillus subtilis*, the effect of stress on *dgk* transcription was examined by northern hybridization. The high level of *dgk* transcription was induced against high osmolarity, low pH value and low temperature. Transcriptional analysis revealed that the *dgk* gene and *dgk* upstream locus (ORF2, ORF3 and ORF4) were transcribed as a polycistronic mRNA to form an approximately 2.5 kb transcript.

**Key words:** Diacylglycerol kinase, *Bacillus subtilis*, Physiological stimuli

Numerous studies have indicated that inositol phospholipid metabolism plays an important role in the signal transduction of $Ca^{2+}$ mobilizing agents[7,9,16]. According to the current hypothesis, the hydrolysis of phosphatidylinositol 4, 5-bis-phosphate to inositol 1, 4, 5-trisphosphate and sn-1,2-diacylglycerol (DG) triggers signaling to the cell and the products mediate some intracellular hormone actions[1,2,11]. By this hypothesis, inositol 1,4,5-trisphosphate mobilizes $Ca^{2+}$ from intracellular $Ca^{2+}$ stores and DG causes the activation of protein kinase C[5,12], leading to various kinds of cellular responses. However, another proposed idea is that DG is also produced by the cleavage of phosphatidylcholine and phosphatidylethanolamine in response to physiological stimuli. Regardless of the pathway of DG formation, DG plays an essential role in the activation of protein kinase C under physiological condition. Therefore, the attenuation of DG is very important in the regulation of protein kinase C activation. The attenuation of DG is carried out by two pathway, phosphorylation by DG kinase and degradation by DG lipase. The predominant pathway of attenuation is considered to be operated by DG kinase[3]. Thus, DG kinase mainly regulates the activity of protein kinase C through competition for DG [10,14].

Previousely, *dgk* (diacylglycerol kinase) gene of *Bacillus subtilis* was identified and sequenced in our laboratory[8]. *dgk* is 402bp long and its deduced amino acid sequence has high similarity(43.1%) with that of *S. mutans*. It was reported that *dgk* gene of *S. mutans* is important for adaptation of the organism to several environ-mental stress signals[17]. So we studied the effects of pH, osmorality and temperature on the expression of *dgk* gene of *B. subtilis* on transcription level.

## MATERIALS AND METHODS

### Bacterial Strains, Media and Cultural Conditions

The bacterial strains used in this study were *Escherichia coli* JM 109 and XL-1-Blue. Luria-Broth (LB) medium (1.0% Bacto-trypton, 0.5% Bacto-yeast extract, 1.0% NaCl) was used for routine cultures.

The *Bacillus subtilis* strain used in this study was MI113 (*arg, trpC2*) derived from *B. subtilis* 168 (*trpC2*). For the expression study on transcription level, cells were precultured overnight under vigorous agitation at 37°C in LB

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medium. The culture was inoculated into 5 ml of fresh LB medium. Following an incubation for 4 hours at 37°C, the culture was exposed to each conditinal LB medium. The effect of osmolarity (0.05 M, 0.23 M, 0.5 M, 1 M and 1.5 M NaCl), pH value (pH 5, pH 6, pH 7, and pH 8) and temperature (25, 30, 35, 37, and 42°C) were examined with RNAs isolated from the cells exposed to those conditions for 30 min.

**Total RNA Preparation from *B. subtilis***

*B. subtilis* was cultured for 14 hour in 5ml of an appropriate medium, for example, LB medium supplemented with varying concentrations of NaCl, or different pH values, and centrifuged at 7,000 rpm for 5 min at 4°C. The pellet was resuspended in 2 ml suspension solution (10 mM Tris-HCl, 1 mM EDTA, 0.9% NaCl, 0.5 mg/ml lysozyme) treated with diethyl pyrocarbonate (DEPC). 0.24 ml of 10 % SDS was added and saturated phenol was treated for several times. The aqueous phase was re-extracted with phenol/chloroform and the RNA was precipitated overnight at -20°C with 2 volume of ice-cold ethanol. And it was centrifuged at 15,000 rpm for 30 min at 4°C, and then the pellets were washed with 70% (v/v) ethanol and dried. The purified RNA was resuspended in DEPC-treated sterile water and kept at -70°C.

**Northern Hybridization**

Northern hybridization was performed according to the method of Farrell *et al.* with slight modification[4,13]. Total RNA was denatured by heating for 15 min at 65°C and then incubating for 1 min on ice in the presence of 50% formamide, 2.2 M formaldehyde and 1 × MOPS buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA, pH 8.0). Electrophoresis of total RNA was done in 1.0% agarose/formaldehyde gel in 1 × MOPS running buffer for 4 hours. Nucleic acids were transferred from the gel to a nylon membrane in 10 × SSC by capillary action, and then baked in vacuum for 2 hours at 80°C. Prehybridization and hybridization solutions were composed of 5 × SSPE (3.6 M NaCl, 20 mM EDTA, 0.2 M sodium phosphate, pH 7.7), 50 % formamide, 5 × Denhardt's solution, 100 ug/ml denatured herring sperm DNA, and 0.5% SDS. After prehybridization for 4 hours the hybridization was conducted for 20 hours at 42°C using isotope-labelled probe. The membrane was washed with two times with 1 × SSC/0.1% SDS for 10 min at room temperature and with two times with 0.2 × SSC/0.1% SDS for 30 min at 45°C. For dot blot hybridization, the RNA was denatured at 68°C for 15 min in 1 × SSC containing 50 % formamide and 7% formaldehyde and then cooled on ice. 35 μg, 20 μg and 5 μg of denatured RNA were spotted on nylon membrane. And following steps were the same as in case of northern hybridization. The DNA fragments indicated in Fig. 1 as ORF2 probe and *dgk* probe were labelled with [α-$^{32}$P] dCTP by the random primed DNA labelling kit and used as probes for northern hybridization.

**Preparation of ORF2 Probe**

Two primers (5'-TGGTCAAATCTGTAAAGGACA-3' and 5'-TGATCACAAGCAAAAGTGTTG-3') were used for amplification of the ORF2 region in the *dgk* upstream (Fig. 1). Polymerase chain reaction was carried out with 10 ng of pSO1 [15]. Cycling parameters were programmed as follows : 45 sec at 95°C, 1 min at 48°C and 90 sec at 72°C for 36 cycles.

## RESULTS AND DISCUSSIONS

**Genetic Organization of *B. subtilis dgk* Locus**

The upstream region of *cdd* locus in *B. subtilis* was sequenced[8,15]. For identification of the *cdd* upstream sequence, various restriction fragments of the stretch from the *Eco*RI/*Hind*III and deleted fragment from the *Nru*I/*Hind*III were used. From the resulted sequence we identified *dgk* (diacylglycerol kinase gene) and truncated *phoH* (phosphate starvation-inducible gene) with remaining four ORFs (Fig. 1). In addition, the translated amino acid sequence of the *B.*

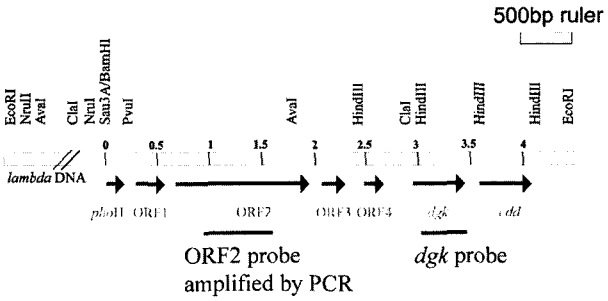


**Fig. 1. Genetic organization of *B. subtilis dgk* locus. A DNA fragment upstream of *Bacillus subtilis cdd* gene was cloned and characterized previously [8, 15]. The *dgk*, *phoH*, *cdd* and four ORFs were indicated by arrows. The indicated *dgk* probe was 0.45 kb restriction fragment. The indicated ORF2 probe was about 0.6 kb DNA fragment amplified by PCR. The accession number is Genbank U29177.**

*sutilis dgk* gene showed 43.1% identity index and 84.3% conservation of amino acid residues to the *Streptococcus mutans dgk* gene.

### Effect of Osmotic Stress on *dgk* Transcription

To determine the effect of osmotic stress on *dgk* gene transcription, northern hybridization analysis was carried out. RNA was isolated from cells exposed to LB medium containing 0.05, 0.5, 1 and 1.5 M NaCl concentra-tion for 30 min (Fig. 2A, Fig. 3). In order to compare the level of transcripts at various osmolarity, all lanes on blot contained equal amounts of total RNA (Fig. 3A).

In dot blots, the level of transcript was dependent on osmolarity as expected (Fig. 2A). The level of transcription increased up to 1 M NaCl concentration, but decreased at 1.5 M NaCl concentration. Maximum induction was observed at 1 M NaCl concentration.

The northern hybridization signal showed a single band of approximately 2.5 kb (Fig. 3). The level of transcripts corresponded to those of the dot blots. Also, maximum induction was observed at 1 M NaCl concentration. The results clearly showed that *dgk* gene adaptively responsed to high osmolarity. The size of transcript was determined by comparison of 23S (2.9 kb) and 16S (1.5 kb) rRNA. In addition, these transcripts were too large to account for the *dgk* gene region. Therefore, it was expected that *dgk* gene was transcribed as polycistronic mRNA.

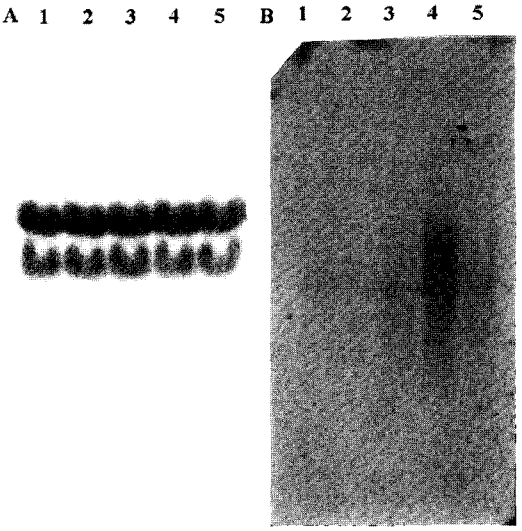


**Fig. 3. Effect of osmotic stress on *dgk* transcription. Tatal RNAs extracted from the *B. subtilis* MI113 treated in LB medium supplemented with various concentrations of NaCl were analyzed with northern hybridization. In each lane the NaCl concentration was indicated as 1, 0.05 M; 2, 0.23 M; 3, 0.5 M; 4, 1 M; 5, 1.5 M.**

A: The patterns of transferred RNAs stained with 0.04% methylene blue on membrane. In each lane the amount of RNA was 25 μg. B: The patterns of northern hybridization analysis. *dgk* gene shown in Fig. 1. was used as a probe.

### Effect of pH on *dgk* Transcription

In order to examine the effect of pH on *dgk* gene expression, total RNA was isolated from cells exposed to pH 5,

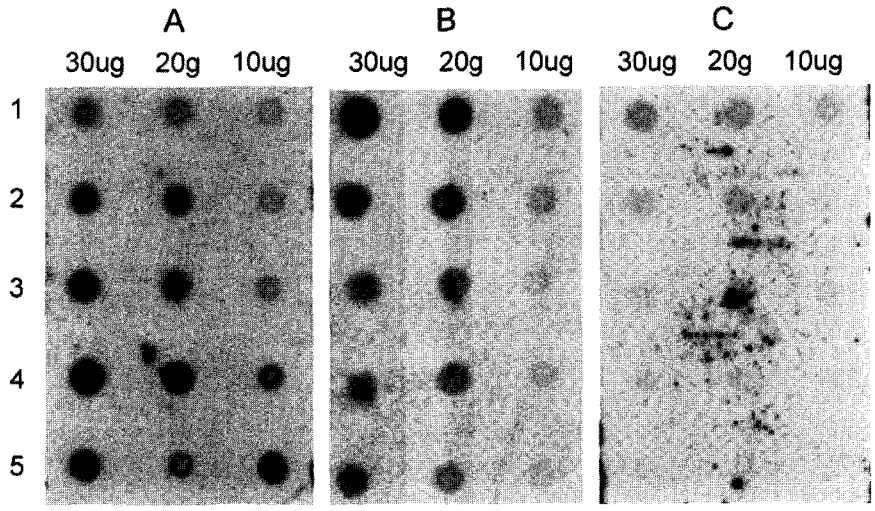


**Fig. 2. RNA dot hybridization. Total RNAs prepared from the *B. subtilis* MI113 treated with various physiological stimuli were spotted on membrane and expression patterns were analyzed with *dgk* probe.**

A: Effect of osmostress. The number was represented as NaCl concentrations by 1, 0.05 M; 2, 0.23 M; 3, 0.5 M; 4, 1 M; 5, 1.5 M. B: Effect of pH. The number was represented as pH values by 1, pH 5; 2, pH 6; 3, pH 6.7; 4, pH 7; 5, pH 8. C: Effect of temperature. The number was represented as temperature gradients by 1, 25°C; 2, 30°C; 3, 35°C; 4, 37°C; 5, 42°C.

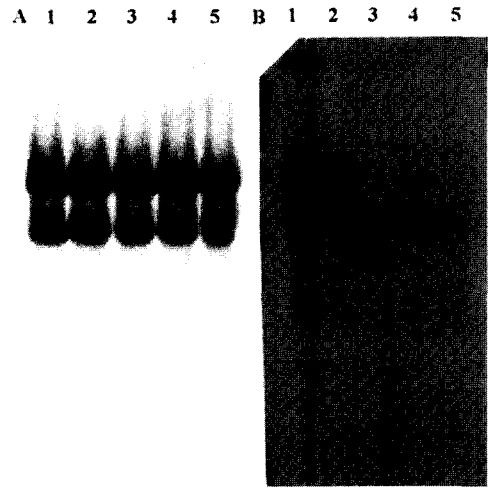


**Fig. 4. Effect of hydrogen ion concentrations on *dgk* transcription. Total RNAs extracted from the *B. subtilis* MI113 treated in LB medium adjusted to various pH values were analyzed with northern hybridization. In each lane the pH values was indicated as 1, pH 5; 2, pH 6; 3, pH 6.7; 4, pH 7; 5, pH 8.**
A: The patterns of transferred RNAs stained with 0.04% methylene blue on membrane. In each lane the amount of RNA was 25 μg. B: The patterns of northern hybridization analysis. *dgk* gene was used as a probe.

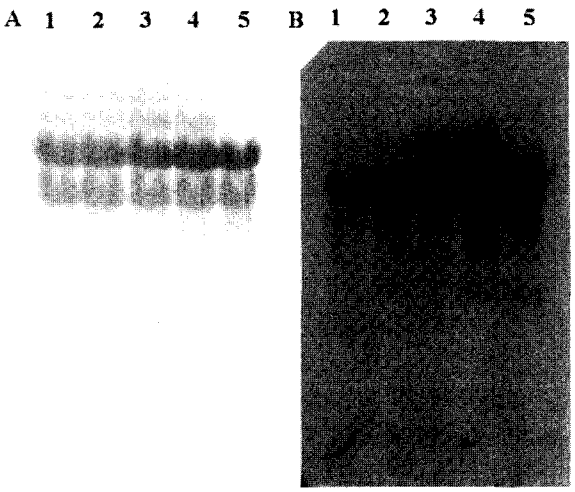


**Fig. 5. Northern hybridization using ORF2-specific probe. Northern hybridization was carried out with total RNAs extracted from the *B. subtilis* MI113 treated with various concentrations of NaCl to know whether the *dgk* gene is expressed as polycistronic mRNA or not.**
A: The patterns of transferred RNAs as in Fig. 3. In each lane the amount of RNA was 25 μg. B: The patterns of northern hybridization analysis with radio-labelled ORF2-specific probe (Fig. 1). The induction method was the same as in Fig. 3. In each lane supplemented NaCl concentrations were indicated as 1, 0.05 M; 2, 0.23 M; 3, 0.5 M; 4, 1 M; 5, 1.5 M.

6, 7, and 8 of LB medium for 30 min (Fig. 2B, Fig. 4).

In the Fig. 2B, high levels of expression were detected when cells were exposed to low pH condition (pH 5). In contrast, low level expression was detected when cells were exposed to neutral condition (pH 7, and 6.7) medium. Maximum induction was observed at acidic condition (pH 5). Yamashita *et al.* also indicated that the *dgk* gene of *S. mutans* was played an important role in adaptation against acid stress[6,17].

In Fig. 4, about 2.5 kb major transcripts were detected. This result was same with osmostress study. Expression pattern was the same with dot blots (Fig. 2B). These results indicated that *dgk* transcription was induced by low pH value as well as high osmolarity.

### Effect of Temperature on *dgk* Transcription

The effect of temperatures on *dgk* gene expression was studied at various temperatures. RNA was isolated from cells exposed to various temperatures (25, 30, 35, 37, and 42°C) for 30 min and dot blot analysis was carried out (Fig. 2C).

The level of transcription was dependent on temperature. The *dgk* transcription was increased at low temperatures. However, it was reduced at high temperature (42°C). Maximum expression was obtained at 25°C. Therefore, it was supposed that the expression of *dgk* gene was induced at relatively low temperature.

Analysis of transcription levels of the *dgk* gene after stress showed an induction of *dgk*-specific mRNA in response to high osmolarity, low pH, and low temperature stresses.

### *B. subtilis dgk* Gene was Transcribed like a Polycistronic mRNA

In northern hybridization, detected mRNA was 2.5 kb in size (Fig. 3, Fig. 4). It was much lager than the size of *dgk* mRNA deduced from nucleotide sequences[8]. The result indicated that *dgk* gene was transcribed in polycistronic mRNA with upstream or downstream sequences. In order to confirm that the *dgk* gene and the ORF2 in the *dgk* upstream region could be transcribed in the same unit, the probe specific for ORF2 was introduced to northern hybridization experiment (Fig. 1).

The *B. subtilis* mRNA was isolated from the cells exposed to osmostress. The hybridized signals of the *dgk* and ORF2 probe had the same migrating rate slightly below 23S rRNA. The level of RNA transcription was same with northern blot hybridization using *dgk* probe (Fig. 5). The

level of transcript was dependent on osmolarity and maximum induction was observed at 1 M NaCl concentration.

These results confirmed that the *dgk* and ORF2, 3, and 4 genes were transcribed in a single mRNA transcript of approximately 2.5 kb in length. Additionally the transcript was correlated well with the DNA region including ORF2, 3, 4 and *dgk* gene.

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국문초록

# *Bacillus subtilis dgk* (diacylglycerol kinase) 유전자의 생리적 자극에 의한 유도발현

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Diacylglycerol Kinase (DGK)는 *E. coli* 및 진핵세포에서의 신호전달에 관여하며, 또한 미생물에서 생리적 자극에 따라 다른 발현 양상을 보이는 것으로 밝혀져 있다. *Bacillus subtilis*에서 이 유전자에 대한 환경에서의 자극 신호들, 즉 pH 변화, 삼투압의 변화 및 온도의 변화에 따른 발현양상을 연구하였다. 이미 동정된 *dgk* locus의 *Kpn*I-*Hind*III의 0.45 kb의 DNA fragment를 probe로 하여 Dot blot, Northern blot analysis를 통해 발현량을 조사해 본 결과 *dgk* 유전자는 pH 변화, 삼투압의 변화 및 온도의 변화에 대응하여 발현되는 유전자임을 알 수 있었다. 특히 낮은 pH, 고삼투압, 및 저온에서 dgk 유전자의 발현량이 많아짐을 확인 할 수 있었다. Northern hybridization에서 약 2.5kb의 mRNA가 관찰되었다. *dgk* gene의 ORF size는 약 0.4 kb로 관찰된 transcript size와는 일치하지 않았다. 따라서 *Streptococcus mutans*의 *dgk* gene과 마찬가지로 *B. subtilis*의 *dgk* 유전자도 polycistronic mRNA로 발현되는 것을 추정할 수 있었으며, 염색체상의 *dgk* gene에서 상류의 ORF2까지의 크기가 약 2.5 kb로 관찰된 mRNA size와 동일하였다. *dgk* gene 상류의 ORF2영역의 0.6 kb의 DNA fragment를 probe로 하여 northern blot hybridization을 수행한 결과, 2.5 kb의 mRNA가 관찰되었으며 발현되는 형태도 *dgk* probe를 이용한 결과와 동일하였다. 따라서 *dgk* gene은 상류부위의 ORF2 gene과 operon을 형성하여 polycistronic mRNA로 전사되는 것으로 판단된다.

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