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# Protein Engineering of Deoxynucleoside Kinase from *Lactobacillus acidophilus* : Effect of Site-Directed Mutagenesis on Microbial Growth

– Research Note –

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## Abstract

**Deoxynucleoside kinases exist as heterodimeric pairs specific for deoxyadenosine/deoxyguanosine kinase (dAK/dGK) and deoxyadenosine/deoxycytidine kinase (dAK/dCK). The aspartic acid-84 in dGK was mutated to alanine, asparagine and glutamic acid by site-directed mutagenesis. The mutation resulted in a drastic decrease in dGK activity compared to the unmodified cloned enzyme while it increased production of dAK activity. The mutated *dak/dgk* genes, which synthesize tandem deoxyadenosine/deoxyguanosine kinase, were inserted back to the *Lactobacillus acidophilus* and *Lactococcus lactis* by electroporation to determine the effect of site-directed mutation of the enzymes on the microbial growth. However, no significant change was observed in cell growth and lactic acid production between wild type and mutant lactic acid bacteria.**

**Key words:** deoxyguanosine kinase, site-directed mutagenesis, electroporation, *Lactobacillus acidophilus*

## INTRODUCTION

Deoxynucleoside kinases exist naturally in *Lactobacillus acidophilus* R-26 as heterodimeric pairs specific for deoxyadenosine kinase (dAK)/deoxyguanosine kinase (dGK) and dAK/deoxycytidine kinase (dCK) (1). The deoxynucleoside kinases provide a salvage pathway to DNA distinct from *de novo* synthesis of the triphosphate precursors. Because they provide a portal to intracellular incorporation into DNA for a variety of important chemotherapeutic agents, they have been the subject of intensive study (1,2). While thymidine kinase (TK) is readily separable, the other three deoxynucleoside kinase activities of *Lactobacillus acidophilus* are located on separate subunits of two physically similar heterodimeric proteins, deoxyadenosine kinase (dAK)/deoxycytidine kinase (dCK) and dAK/deoxyguanosine kinase (dGK) (3). Each of the deoxynucleoside substrates is phosphorylated at an independent site, all having comparable $K_m$ values and strong feedback inhibition by their homologous deoxynucleoside triphosphates (4).

Tandem *Lactobacillus acidophilus* genes for dAK and dGK (*dak/dgk*) have been cloned and expressed in *Escherichia coli*, using their common upstream promoter and independent Shine/Dalgano sequences (5). Comparison with herpes-viral TKs reveals three conserved regions: glycine- and arginine-rich ATP-binding motifs and a DRS (Asp-Arg-Ser) motif at the putative TK deoxynucleoside site (5-7). The DNA sequences encoding these purine-specific subunits had a 65% identity, but no distinct gene for dCK was found (5). To enable *E. coli* to express a peptide having the native dCK sequence, codon 2 and 3 were deleted from the *dgk* portion of the tandem genes, resulting in expression of a protein having the specificities and regulatory properties of native dAK/dCK (8). It was reported that replacement of amino acids in DRS motif modified the enzyme activity in dGK and dAK in *Lactobacillus acidophilus*. Replacement of Asp-78 of both subunits with Glu, Ala, or Asn reduced dGK and dAK activities to less than 0.2%, whereas replacement of Arg-79 with Lys, either on both subunits in tandem (R79K), or on the dGK subunit only (R79 K:dGK), yielded active but kinetically modified enzymes (9). It would be interesting to replace the amino acid near the DRS motif to understand the role of the amino acid. Since Asp-84 is located near the DRS motif, its function was determined by replacing it to Ala, Glu, and Asn.

*Lactobacillus acidophilus* is a very important industrial microorganism for the production of food and health improvement. The organism is also an inhabitant of the intestinal tract of humans, and so could be utilized as a drug delivery system or for other important purposes. One approach to improve the utility of the bacterium is to increase the productivity of metabolites such as lactic acid by increasing the growth rate of the organism. The change in activities of deoxynucleoside kinases might affect the growth of the microorganism since deoxynucleosides could be utilized as precursors of deoxynucleotides and DNA. This paper describes the effect of a mutation at Asp-84 in deoxyguanosine kinase of *Lactobacillus acidophilus* cloned in *Escherichia coli* on activities of the enzyme and on growth of *Lactobacillus acidophilus and Lactococcus lactis* which were transformed by electroporation of the mutated gene.

## MATERIALS AND METHOD

### Bacterial strain and growth

*E. coli* XL1-Blue was grown in Luria-Bertani (LB) broth at

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37°C with shaking. *Lactobacillus acidophilus* KCTC3564 was grown MRS broth (Difco Laboratories, Detroit, Mich., USA) without shaking, and *Lactococcus lactis* MG1364 was grown in M17 broth (Difco).

**Enzyme assays**

Deoxynucleoside kinase activities were assayed using DE-81 ion exchange paper by the method of Ives (10) with a minor modification as follows. In the standard assay, the final concentration of each reagent in the reaction mixture (in a 1.5 mL Eppendorf tube) was: ATP, 10 mM; MgCl2, 12 mM; Tris/HCl, pH 8.0, 0.1 M; [$^3$H]dGuo, 0.2 mM (0.5 μCi/assay), respectively. The reaction was started by adding 20 μL of enzyme diluted in 15 mM potassium phosphate buffer, pH 8.0, containing 20% (v/v) glycerol to the warmed reaction mixture tube (final volume, 40 μL). After 30 min of incubation at 20°C, 0.1 mL of formic acid was added to stop the reaction. Aliquots (20 μL) of the mixture were spotted on Whatman DE-81 anion exchange papers for the measurements of radioactivity.

**Site-directed mutagenesis**

The materials and protocols of the Muta-Gene phagemid *in vitro* site-directed mutagenesis kit (version II) from Bio-Rad based on a method described by Kunkel et al. was used (11). Pure oligomers were dried in air, dissolved in 1 mL of 10 mM Tris buffer, pH 8.0, and stored at -20°C until use. The 5' end of each mutagenic oligomer was phosphorylated with polynucleotide kinase to improve the frequencies of mutagenesis. After the polymerization/ligation reaction was continued for 90 min at 37°C, the reaction was stopped by adding Tris-EDTA buffer. Transformants were grown on LB plates containing ampicillin, and each instance of mutagenesis was confirmed by DNA sequencing, using the dideoxynucleotide termination method (12).

**Extraction of enzyme**

*E. coli* XL1-Blue cells containing mutants of the recombinant pBluescript KS (+) clone GTM-K48 were cultured in LB liquid medium, containing 100 μg/mL ampicillin, for expression of the enzyme (5). The cell pellets were washed in icecold cell suspension buffer (0.1 M Tris-HCl, pH 8.0, 25 mM EDTA, 20% glycerol) and used immediately or stored at -20°C for later use. The washed cell pellets were suspended in cell suspension buffer, broken by ultrasonication, and centrifuged at 13,500×g for 1 hr at 4°C.

**Competent cell preparation and electroporation**

Electrocompetent cells from *Lactobacillus acidophilus* and *Lactococcus lactis* were prepared from fresh MRS cultures propagated for 15 hrs in broth containing 1.0% glycine. A 2.0% inoculum was then made to 200 mL of MRS broth containing 1.0% glycine, and the cells propagated to an OD590 of 0.25. The cells were placed on ice for 5 min and centrifuged. The pellet was held on ice and rinsed with distilled water. It was then centrifuged and the supernatant was discarded. The pellet was resuspended with 20 mL of ice cold 0.5 M sucrose containing 10% glycerol. The cell pellet was resuspended in 1.0 ml in cold electroporation buffer and divided into 40 μL aliquots and kept on ice. One aliquot was mixed with 2 μg of plasmid DNA and transfered to a chilled cuvette (0.2 cm electrode gap). After 5 min on ice, the suspension was electroporated at 2.5 kV, 200 ohm and 25 μF with Gene-Pulser™ (Biorad Laboratories, Richmond, CA). It was immediately transfered to 1.0 mL of appropriate broth (MRS for *Lactobacillus acidophilus*, M17 for *Lactococcus lactis*), and incubated at 37°C for 3 hrs prior to plating on the selective medium (MRS agar or M17 agar containing 30 μg/mL ampicillin).

## RESULTS AND DISCUSSION

The DRS motif or its conservatively substituted analog has been implicated as part of the deoxynucleoside site in the HSV TKs (6,7). Therefore, it was attempted to determine the role of Asp-84 located near the motif. Aspartate 84 of dGK subunit in dAK/dGK tandem enzyme was replaced with glutamate, which is conservative replacement except for the longer side chain, with asparagine to eliminate negative charge and with alanine to minimize the volume occupied by the side chain. Three mutagenic oligomers were synthesized and shown in Table 1. The dGK activities in all of Asp-84 mutants were drastically decreased after mutagenesis as shown in Table 2. The remaining dGK activities of D84A (aspartate to alanine), D84N (aspartate to asparagine), and D84E (aspartate to glutamate) mutant enzymes were 0.33, 0.94, and 6.29% activities found in comparable wild type fractions. Therefore Asp-84 seems to be important for deoxyguanosine kinase activity from *Lactobacillus acidophilus*. The mutation of Asp-84 also increased production of dAK activities in tandem dAK/dGK heterodimers. It was also reported that mutation of Asp-78 located in DRS motif also eliminated the dGK activity in *Lactobacillus acidophilus* (9). However, the role of the amino acids for the catalytic mechanism is yet to be determined. The effect of mutation on Asp-84 of dGK on growth of

**Table 1.** Primers used for site-directed mutagenesis of deoxyguanosine kinase

| Mutants | Primer |
|---|---|
| Wild type | 5'GAAAAGAGCATCTTCGTAG3' |
| D84A | 5'GAAAAGAGCAGCTTCGTAG3' |
| D84N | 5'GAAAAGAGCATTTTCGTAG3' |
| D84E | 5'GAAAAGAGCCTCTTCGTAG3' |

Reverse primer

**Table 2.** Production of deoxyguanosine kinase (dGK) and deoxyadenosine kinase (dAK) activities from various mutants

| Mutants | Enzyme activity (unit/mg) | |
|---|---|---|
| | dGK | dAK |
| Wild type | 12.72 | 2.61 |
| D84A | 0.042 | 17.52 |
| D84N | 0.12 | 11.25 |
| D84E | 0.80 | 15.15 |

**Table 3.** Growth of various mutants of *Lactobacillus acidophilus* and *Lactococcus lactis*

| Mutants | *Lactobacillus acidophilus* | | *Lactococcus lactis* |
|---|---|---|---|
| | OD (600 nm) | pH | OD (600 nm) |
| Wild type | 0.71 | 4.27 | 0.51 |
| D84A | 0.61 | 4.47 | 0.50 |
| D84N | 0.72 | 4.30 | 0.53 |
| D84E | 0.65 | 4.32 | 0.49 |

The culture was grown at 37°C for 12 hrs in MRS medium for *Lactobacillus acidophilus* and M17 medium for *Lactococcus lactis*. The inoculum size was 2%.

*Lactobacillus acidophilus* was determined by electroporation of mutant pBSGTM-K48 into *Lactobacillus acidophilus* and *Lactococcus lactis*. *Lactobacillus acidophilus* and *Lactococcus lactis* colonies transformed with mutants of pBSGTM-K48 by electroporation were selected on MRS plates containing 30 μM ampicillin concentration. The transformed *Lactobacillus acidophilus* could grow in MRS medium containing less than 30 μM ampicillin. The growth of lactic acid bacteria transformed with mutated gene with D84A, D84N and D84E was investigated. Since deoxynucleoside kinases are important to synthesize deoxynucleotides, which are necessary for synthesis of DNA, the mutation of deoxynucleoside kinase might affect the growth of the cell. However, no significant change in growth and lactic acid production in the mutant *Lactobacillus acidophilus* was observed as shown in Table 3. The mutant *Lactococcus lactis* also showed no significant change in cell growth. This suggests that the change in purine deoxynucleoside kinase activity in the lactic acid bacteria has little effect on growth of the bacteria, and the cell might possess other mechanisms to synthesize deoxynucleotides enough to provide the necessary deoxynucleotide pool.

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