Evidence for Four Deoxynucleoside Kinase Activities in Extracts of *Lactobacillus leichmannii*

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Extracts of *Lactobacillus leichmannii* (ATCC 7830) catalyze the phosphorylation of the four principal deoxynucleosides. Thymidine, deoxyguanosine, and deoxycytidine kinase activities were found to be optimal with deoxyadenosine triphosphate as the phosphoryl donor, whereas deoxycytidine triphosphate was the optimal donor for deoxyguanosine kinase activity. *L. leichmannii* catalyzes the conversion of deoxycytidine to deoxyuridylic acid, probably by a pathway involving deoxycytidylate deaminase.

Among the bacteria examined, most have been found to be deficient in one or more of the deoxynucleoside kinase activities. For example, deoxycytidine (dC) kinase activity could not be detected in either *Bacillus megaterium* (20), *Salmonella typhimurium* (14), *Diplococcus pneumoniae* (8), or *Escherichia coli* (10). In addition, *E. coli* was also found to lack kinases for deoxyadenosine (dA) and deoxyguanosine (dG) (10), in contrast to mammalian cells which have been reported to phosphorylate the four principal deoxynucleosides, namely deoxythymidine (dT) (4), dA (6, 12), dG (6, 12), and dC (6, 11, 12).

Based on other studies, we examined the possibility that *Lactobacillus leichmannii* would possess all four deoxynucleoside kinase activities. This organism requires vitamin B$_1$ for growth, presumably for the reduction of ribonucleoside triphosphates to the corresponding deoxynucleoside triphosphates (3, 9). The vitamin B$_1$ requirement of this organism can be replaced by a mixture of any one of the common deoxynucleosides plus the other free bases (1, 15). *L. leichmannii* can utilize these substrates to synthesize all four deoxynucleosides with the aid of the enzyme *trans*-N-deoxyribosylase (2, 15). We reasoned that the first step in deoxynucleoside utilization should involve phosphorylation. The experiments reported here show that, in contrast to the previously mentioned bacteria, *L. leichmannii* possesses all four deoxynucleoside kinase activities. While this work was in progress, Durham and Ives (7) reported similar findings with *L. acidophilus*.

**MATERIALS AND METHODS**

Stock cultures of *L. leichmannii* (ATCC 7830) were maintained in 2% agar slabs of Lactobacillus selection medium (Baltimore Biological Laboratory, Cockeysville, Md.). For the preparation of extracts, cells were grown semianerobically in completely filled flasks in Lactobacillus broth AOAC (Difco Laboratories, Inc., Detroit, Mich.) at 37 C. Cells in the late exponential-growth phase (150 Klett units; filter no. 64) were harvested by centrifugation at 0 C, washed twice with cold 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.4, containing 5 mM MgCl$_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol, and suspended in the same buffer at 10,000 Klett units (calculated; equivalent to about 6 x 10$^9$ colony-forming units/ml). Cells were disrupted by two successive passages through a chilled French pressure cell (15,000 lb/in$^2$). The cell lysate was centrifuged at 27,000 x g for 10 min at 0 C, and the resulting supernatant fraction was dialyzed twice at 4 C against 100 volumes of the same buffer mixture used to prepare the cell extract. Samples (0.5 ml) were quick-frozen in acetone-dry ice and stored at −70 C.

The deoxynucleoside kinase assay was a modification of the procedure described by Okazaki and Kornberg (16). Activity was determined at 37 C in 0.10-ml reaction mixtures containing 100 mM Tris (pH 7.5), 6.5 mM MgCl$_2$, 2.5 mM dithiothreitol, 10 mM ribo- or deoxynucleoside triphosphate donor, 3 mM tritiated deoxynucleoside (1 to 7 x 10$^4$ counts per min per μμmol), 30 μg of bovine serum albumin, and 195 μg of bacterial protein. For the assay of dT, dA, and dG kinase activities, samples (5 μliters) were removed as a function of time and deposited on areas of thin-layer cellulose sheets (Brinkmann MN-Polygram Cel 300) containing 25 to 50 nmol each of the appropriate carriers, and the enzyme activity was stopped by the addition of 5 μliters of cold developing.
solvent. For dT and dA kinases, chromatograms were developed by ascending chromatography for 4.5 h with n-butanol-acetone-glacial acetic acid-5% NH₄OH-H₂O (volume ratio, 35:25:15:15:10) (5) as the solvent. For dG kinase, n-butanol-acetone-98% formic acid-5% ammonium formate (volume ratio, 35:25:15:25) (5) was used as the developing solvent. For the assay of dC kinase activity, in which it was also desirable to separate deoxyctydylate monophosphate (dCMP) from deoxyuridine monophosphate (dUMP), two different chromatographic procedures were used. In one, reaction mixtures were spotted onto carrier-containing areas of water-washed sheets of thin-layer PEI-cellulose (Brinkmann Polygram Cell 300 PEI) and developed by ascending chromatography for 2.5 h with 0.12 N formic acid. For better separation between deoxyctydylate diphosphate (dCDP) and deoxyctydylate triphosphate (dCTP), we used thin-layer sheets of diethylaminoethyl (DEAE)-cellulose (Baker-flex), previously washed in 1 M formic acid and then in water, with 0.1 M ammonium formate (adjusted to pH 4.0 with concentrated formic acid) as the developing solvent (7). These chromatographic procedures permitted the separation of deoxyctydyes and their corresponding mono-, di-, and triphosphates from one another. Carrier compounds were located as ultraviolet-absorbing areas which were cut out, and the radioactivity of the phosphorylated reaction products was determined by standard scintillation counting. In all cases, the coincidence between radioactivity and ultraviolet-absorbing area was periodically monitored by cutting out 0.50-cm strips in the direction of solvent flow and counting each strip.

Protein was determined by the method of Lowry et al. (13) on samples precipitated at 0 C with a final concentration of 5% trichloracetic acid by using lysozyme as the protein standard.

Nonradioactive purine and pyrimidine derivatives were obtained from P-L Biochemicals, Inc., Milwaukuee, Wis.; dithiothreitol was from Calbiochem, Los Angeles, Calif.; bovine serum albumin (fraction V) was from Armour Pharmaceutical Co., Kankakee, Ill.; dT-3H (GL) was from International Chemical and Nuclear Corp., Irvine, Calif.; dA-3H (GL) was from Amersham/Searle, Arlington Heights, Ill.; and both dG-3H(GL) and dC-3H(GL) were from New England Nuclear Corp., Boston, Mass.

RESULTS AND DISCUSSION

In all cases, the formation of deoxynucleoside monophosphates was found to be linear for at least 30 min at 37 C, regardless of the phosphoryl donor, and to be linear with respect to L. leichmannii protein concentration for up to about 200 μg of protein per 0.10-ml reaction mixture. A study of the ability of different nucleoside triphosphates to serve as phosphoryl donors showed that deoxyadenosine triphosphate (dATP) is the best donor for dT kinase activity (Table 1). Diaxylated extracts were found to require MgCl₂ for maximal dT kinase activity, with an optimum at about 6 mM Mg²⁺. dCTP is about 88% as effective a donor as dATP for dT kinase activity, whereas deoxycytanosine triphosphate (dGTP) and ATP are about 60% as effective as dATP (Table 1). This is in contrast to the dT kinase activity of B. megaterium (20), which functions optimally with either dATP or dGTP as the phosphoryl donor. The remaining nucleoside triphosphates (Table 1) supported only low levels of activity with GTP > CTP > deoxthymidine triphosphate (dTTP). Although deoxthymidine diaphosphate (dTDP) formation was best with either dATP or dCTP as the phosphoryl donor, in no case was dTTP formation observed.

dA-3H(GL) was also the optimal phosphoryl donor for dG kinase activity (Table 1). ATP was about 70% as effective as dATP, whereas both CTP and dCTP were about 20% as effective. These results differ from those reported for the dG

- Table 1. Effect of phosphoryl donor on deoxynucleoside kinase activities

| Donor | dT kinase | dG kinase | dC kinase | dA kinase |
|-------|-----------|-----------|-----------|-----------|
|       | dTMP      | dTMP + dTTP | dGMP      | dGMP + dGTP | dCMP      | dCMP + dCTP | dAMP      | dAMP + dATP |
| ATP   | 0.63      | 0.04      | 1.53      | 0.39      | 0.20      | 0.32      | 0.62      | 0.77      |
| dATP  | 0.97      | 0.16      | 2.48      | 0.25      | 0.55      | 0.52      | 0.97      | 0.13      |
| GTP   | 0.29      | 0.00      | 0.00      | 0.00      | 0.07      | 0.04      | 0.55      | 0.14      |
| dGTP  | 0.67      | 0.00      | 0.00      | 0.00      | 0.00      | 0.00      | 1.94      | 0.78      |
| CTP   | 0.11      | 0.03      | 0.31      | 0.25      | 0.21      | 0.02      | 0.32      | 1.30      |
| dCTP  | 0.84      | 0.16      | 0.30      | 0.30      | 0.04      | 0.02      | 1.57      | 2.00      |
| dTTP  | 0.06      | 0.00      | 0.06      | 0.12      | 0.00      | 0.00      | 0.46      | 0.15      |

* Assays were performed at 37 C as described in Materials and Methods. Each reaction mixture contained 195 μg of bacterial protein. Deoxynucleotide formation was calculated as the average of the values obtained after 10, 20, and 30 min of incubation. For dC kinase, reaction mixtures were chromatographed on PEI-cellulose sheets.
kinase of \textit{B. megaterium} (20), which utilizes dCTP as the optimal phosphoryl donor. Low levels of both deoxyguanosine diphosphate (dGDP) and dGTP were detected.

As with dT and dG kinases, dATP was also found to be the optimal phosphoryl donor for dC kinase activity. ATP and CTP were 52 and 18\%, respectively, as effective as dATP. Based upon DEAE-cellulose chromatography, the formation of both dCDP and dCTP was observed, with a ratio of about 1.6 to 1.0. The conditions that favor the formation of deoxycytidine monophosphate (dCMP) were also found to favor dUMP accumulation (now shown). Because lactobacilli lack the enzyme deoxycytidine deaminase (15, 18), we assume that dUMP formation proceeds via the deamination of dCMP. \textit{L. acidophilus} has been shown to possess an active deoxycytidylyl deaminase (18).

In contrast to the other three kinases, dA kinase functions optimally with dCTP as the phosphoryl donor. dGTP, CTP, and ATP are approximately 76, 45, and 39\% as effective, respectively. The activity observed in the presence of GTP, dTTP, or ATP was less than 20\% of that found with dCTP as the donor. Also, a substantial accumulation of both deoxyadenosine diphosphate (dADP) and dATP was detected in the presence of the most active phosphoryl donors. These results differ from those reported from the dA kinase of \textit{B. megaterium} (20), for which either dGTP or ATP was found to be the optimal donor. The inability of dATP to function as a phosphoryl donor for dA kinase activity is consistent with the finding in \textit{L. acidophilus} (7) that the homologous deoxynucleoside triphosphates are the most effective inhibitors of deoxynucleoside phosphorylation.

Therefore, in contrast to many other bacteria, lactic acid bacteria like \textit{L. leichmannii} and \textit{L. acidophilus} appear to contain kinases for all four deoxynucleosides. Members of the \textit{Enterobacteriaceae} such as \textit{E. coli} and \textit{S. typhimurium} lack at least one, and possibly as many as three, of the deoxynucleosides kinases. The biological significance of the presence or absence of a full complement of these salvage enzymes is at present unclear.

Although we were originally unable to detect dC kinase activity in extracts of \textit{B. megaterium} (20), we subsequently were successful in demonstrating this activity (J.T. Wachsman and D.D. Morgan, unpublished data). \textit{B. megaterium} extracts possess an active dC deaminase activity which, if not inhibited, can effectively mask the action of dC kinase. Therefore, \textit{B. megaterium} resembles some members of the genus \textit{Lactobacillus} in possessing a full complement of deoxynucleoside kinases. dC kinase activity has also recently been found in \textit{B. subtilis} (19).

It is clear from these data that extracts of \textit{L. leichmannii} catalyze the phosphorylation of the four deoxynucleosides required for deoxyribonucleic acid synthesis. The possibility that nucleoside phosphotransferases (17) may also be involved is currently under investigation.

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