Construction of Deoxyriboaldolase-Overexpressing *Escherichia coli* and Its Application to 2-Deoxyribose 5-Phosphate Synthesis from Glucose and Acetaldehyde for 2’-Deoxyribonucleoside Production

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There will be a need for 2’-deoxyribonucleoside in the near future due to increasing demand in new medical and biotechnology fields. 2’-Deoxyribonucleoside is a building block of promising antisense drugs for cancer therapy. For some recent development antiviral agents, such as azidothymidine for treatment of human immunodeficiency virus infections, 2’-deoxyribonucleoside is a synthesis intermediate. 2’-Deoxyribonucleoside is also a precursor of an indispensable material used for widespread PCR applications. 2’-Deoxyribonucleoside triphosphate. The current 2’-deoxyribonucleoside sources include hydrolyzed herring and salmon sperm DNA, which are not suitable sources for sudden high demand. The difficulty in chemical synthesis of 2’-deoxyribonucleoside lies in the generation of 2-deoxyribose groups. The chemical synthesis of 2-deoxyribose and the subsequent synthesis of 2’-deoxyribonucleoside involve complex protection and deprotection steps (1, 8, 10, 15). It is likely that introduction of biochemical reactions with high selectivity will solve this problem.

In general metabolism, there are two reactions involving 2-deoxyribose groups. One of these reactions is reduction of ribonucleotide to 2’-deoxyribonucleotide during biosynthesis, and the other is cleavage of 2-deoxyribose 5-phosphate (DR5P) to produce glyceraldehyde 3-phosphate and acetaldehyde. If triose phosphates were supplied from glucose through glycolysis, the proportion became more practical. In this study, the possibility of using glucose as a starting material for DR5P production was examined with *K. pneumoniae* B-4-4 and *Escherichia coli* transformants expressing DERA from *K. pneumoniae* B-4-4 and DR5P production by a strain with an active DERA, *Klebsiella pneumoniae* B-4-4, was described (14). This strain produced DR5P from triose phosphates (G3P and dihydroxyacetone phosphate) and acetaldehyde. If triose phosphates were supplied from glucose through glycolysis, the process became more practical. In this study, the possibility of generating DR5P by a strain expressing DERA from *K. pneumoniae* B-4-4 as sources of glycolytic enzymes. DR5P was produced from glucose and acetaldehyde by DERA-overexpressing *E. coli* cells in the presence of ATP.

There are other sources of glyco-

**MATERIALS AND METHODS**

Enzymes and chemicals. Restriction endonucleases and other DNA-modifying enzymes were purchased from TaKaRa Shuzo Co., Ltd. (Kyoto, Japan) and Toyobo Co., Ltd. (Osaka, Japan). Nucleoside phosphorylase was obtained from Toyobo Co., Ltd. (Osaka, Japan).
Sigma (St. Louis, Mo.). All reagents used were commercially available and analytical grade.

**Bacterial strains and culture conditions.** *K. pneumoniae* B-4-4 (=IFO 16579) was used as the source of genomic DNA (14). *E. coli* strain JM109 and the alkaline phosphatase-defective mutant 10B5 (20) were kindly provided by Hideoaki Suzuki, Graduate School of Biostudies, Kyoto University (Kyoto, Japan). For extraction of genomic DNA, *K. pneumoniae* B-4-4 was cultivated in medium containing 0.5% tryptone, 0.5% yeast extract, 0.1% glucose, and 0.1% KH₂PO₄ (pH 7.0) at 28°C for 12 h. For production of DR5P, *K. pneumoniae* B-4-4 was cultivated in a medium (DR medium) containing 0.1% KH₂PO₄, 0.1% K-HPO₄ (0.01% each), 0.2% NH₄Cl, and 0.3% 2-deoxyribose (pH 7.0) at 28°C for 12 h. *E. coli* strains were cultivated at 37°C in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, 1% NaCl; pH 7.2) for 10 h. For cultivation of recombinant *E. coli*, 0.1 mg of ampicillin per ml was added to LB medium. For induction of the gene under control of the tac promoter, 0.2 mM isopropyl-β-D-galactoside (IPTG) was added to LB medium.

**DNA manipulation and sequencing.** Total genomic DNA from *K. pneumoniae* B-4-4 was isolated and purified as described previously (16). The protocols used for plasmid isolation, agarose electrophoresis, ligation, and other standard molecular biological techniques have been described previously (18). DNA labeling with alkaline phosphate for Southern blot hybridization was carried out by using the materials and protocols of a chemiluminescence detection kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Sequencing was performed by the dye-deoxy chain termination method by using a CEQ DTCS KIT dye terminator (Beckman Instruments, Fullerton, Calif.) with an automated DNA sequence (CEQ L DNA analysis system; Beckman Coulter, Inc., Fullerton, Calif.). The GENETYX software system (Software Development Co., Tokyo, Japan) was used for computer analysis of nucleotide sequences and deduced amino acid sequences.

**Cloning of the DERA gene.** Oligonucleotide primers were designed based on the nucleotide sequence of the *E. coli* deoC gene (22). Conserved regions were selected for performing a homology analysis of several DERA genes. The primers used for PCR amplification of the partial DERA gene were 5'-GGAGAATTCATGACAGCTCTTCAAGATTTAGTATGATTATGGATAGAGGTTTTGATGAGCGCAGTGGGTAAGCA-3' (sense strand) and 5'-ACCGGTAGAGGTTTTGATGAATTGCTTATGGCATCTTCAGGTCGCTGGCGCTCT-3' (antisense strand), each containing an EcoRI restriction site (underlined). The PCR product was subcloned into the plBlue T vector and extracted by digestion with the EcoRI endonuclease. The DNA fragment of the insert was purified by agarose gel electrophoresis and ligated into the EcoRI site of the pK2K223-3 expression vector (Amersham Pharmacia Bio-tech), yielding plasmid pTS8. DERA expression plasmid pTS8 was then transformed into *E. coli* JM109 (21).

**Preparation and analysis of cell extracts of recombinant *E. coli* strains and *K. pneumoniae* B-4-4.** Recombinant *E. coli* strains were cultivated in 10 ml of LB medium containing ampicillin at 37°C for 12 h, and *K. pneumoniae* B-4-4 was cultivated in 10 ml of DR medium at 28°C for 10 h. The cultivated cells were harvested by centrifugation, suspended in 1 ml of 0.85% NaCl, and then disrupted by sonication with a Powersonic model 50 ultrasonic cell disrupter (Ya- mato Science Co., Ltd., Tokyo, Japan). After centrifugation (10,000 × g, 10 min, 4°C), the resultant supernatants were used for the enzyme assay. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 12.5% polyacrylamide slab gel was performed as described previously (11) with the following standard proteins (Daichi Chemicals Co., Tokyo, Japan): phosphorylase b (molecular weight, 97,400), bovine serum albumin (66,267), aldolase (42,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and lysozyme (14,400).

**DERA activity assay.** DERA activity in cell extracts was monitored by determining DR5P-decomposing activity. DR5P-decomposing activity was analyzed by measuring the decrease in absorbance at 600 nm through the coupled reactions of DERA catalyzing DR5P reduction and alcohol dehydrogenase (Oriental Yeast Co., Ltd., Osaka, Japan), which catalyzes acetaldehyde reduction. A molar extinction coefficient of 6,220 M⁻¹ cm⁻¹ for NADH was used to calculate the specific activity. The 200-μl reaction mixture contained 10 mM Tris-HCl buffer (pH 7.5), 0.5 mM NADH, 7.5 U of alcohol dehydrogenase, 1.0 mM DR5P, and an aliquot of cell extract. The reduction of NADH was monitored at a wavelength of 340 nm with a SPECTRA MAX 190 (Molecular Devices, Sunnyvale, Calif.).

**Reaction conditions for DR5P production from acetaldehyde and G3P.** The recombinant *E. coli* strains were cultivated in 10 ml of LB medium containing ampicillin at 37°C for 12 h, and *K. pneumoniae* B-4-4 was cultivated in 10 ml of DR medium at 28°C for 10 h. The cultivated cells were harvested by centrifugation, washed with 0.85% NaCl, and then suspended in the reaction buffer. The standard 100-μl reaction mixture contained 98.7 mM G3P, 200 mM Tris-HCl buffer (pH 9.0), 200 mM acetaldehyde, and 15% (wt/vol) washed cells in a 1.5-ml Eppendorf tube. The mixtures were incubated at 30°C for 5 h with shaking and then centrifuged (10,000 × g, 10 min). The amounts of DR5P in the resultant supernatants were determined as described below.

**Screening of substrates that were alternatives to G3P for DR5P production.** Reactions were carried out under the standard reaction conditions with G3P and acetaldehyde as the substrates as described above with various compounds (100 mM) involved in the glycolytic pathway and glycerol metabolism instead of G3P. In the reaction with ATP (10 mM), 1% (wt/vol) xylene and 0.4% (wt/vol) polyoxyethylene laurylamine (PL) were added to the reaction mixtures.

**Reproduction conditions for DR5P production from glucose and acetaldehyde in the presence of ATP.** The standard 2-ml reaction mixture contained 200 mM acetaldehyde, 500 mM glucose, 100 mM potassium phosphate buffer (pH 7.0), 15 mM MgSO₄ · 7H₂O, 1% xylene, 0.4% PL, 12.5% (wt/vol) washed wet cells of 10B5/pTS8, and 15 mM ATP in a 15-ml collection tube. The pH of the reaction mixture was adjusted to 7.5 with KOH. The mixtures were incubated at 30°C for 5 h with shaking and then centrifuged (10,000 × g, 10 min). The amounts of DR5P in the resultant supernatants were determined as described below. Optimization of the reaction conditions was carried out essentially under the standard reaction conditions described above by varying the target parameters. Preparative production of DR5P from glucose and acetaldehyde in the presence of ATP was carried out by using a 10-ml reaction mixture containing 200 mM acetaldehyde, 900 mM glucose, 60 mM MgSO₄ · 7H₂O, 1% xylene, 0.4% PL, 12.5% (wt/vol) washed wet cells of 10B5/pTS8, and 100 mM ATP in a 15-ml collection tube at pH 7.5. The mixtures were incubated at 37°C with shaking. Aliquots of each mixture were removed at several times and then centrifuged (10,000 × g, 10 min). The amounts of DR5P in the resultant supernatants were determined as described below.

**Production of 2-deoxyribose nucleoside.** A reaction mixture containing DR5P produced from glucose and acetaldehyde was centrifuged (10,000 × g, 20 min), and the resultant supernatant was used directly for 2-deoxyribonucleoside production. The reaction mixture (total volume, 0.5 ml) contained the 25% (vol/vol) DR5P solution produced as described above (DR5P concentration, 25 mM), 50 mM NaCl, 500 mM glucose, 0.1 mM glucose 1,6-diphosphate, 1 mM MgSO₄ · 7H₂O, 60 mM MnCl₂, 100 mM ATP, 150 mM NH₄Cl-NH₄OH buffer (pH 9.0), 1.0% xylene, 0.4% PL, 0.5 U of nucleoside phosphorylase per ml, and 10% (wt/vol) washed cells of *E. coli* BL21/pTS17 (14). After a 4.5-h reaction at 30°C, the 2-deoxyribonucleoside

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produced was analyzed by high-performance liquid chromatography as described below.

**Analytical methods.** Qualitative analysis of DRSP was performed by thin-layer chromatography with Kieselgel 60 F254 (Merck, Rahway, N.J.). The developing system consisted of n-butanol, 2-propanol, and H2O at a ratio of 3:1:2 (vol/vol/vol). DRSP was detected with 1% (vol/vol) anisaldehyde and 2% (vol/vol) H2SO4 in acetic acid as a purple spot. Quantitative analysis of DRSP was performed by the chemical colorimetric procedure of the cysteine-sulfate assay described by Stumpf (21). The 2'-deoxyribonucleoside produced was measured by high-performance liquid chromatography at 254 nm with a Cosmosil 5C18-AR column (4.6 by 150 mm; Nacalai Tesque, Kyoto, Japan) by using 100 mM NaClO4 containing 0.1% (vol/vol) H3PO4 at a flow rate of 1.0 ml/min as the eluent; the temperature was room temperature.

**Nucleotide sequence accession number.** The sequence of the DERA gene of *K. pneumoniae* studied here has been deposited in the Center for Information Biology and DNA Data Bank of Japan (DDBJ) under accession number AB999293.

**RESULTS**

**Cloning and sequence analysis of the *K. pneumoniae* DERA gene.** The primers used for cloning of the *K. pneumoniae* DERA gene by PCR were based on the nucleotide sequence of the *E. coli* *deoC* gene (22), as described in Materials and Methods. PCR performed with these primers and genomic DNA of *K. pneumoniae* B-4-4 as the template yielded an amplified band at 0.5 kb. The amplified DNA was then subcloned into the pT7Blue T vector in *E. coli*. The nucleotide sequence of the DNA comprised 483 bp and exhibited 86.3% identity to a partial sequence of the *E. coli* *deoC* gene, indicating that the DNA was a fragment of the *K. pneumoniae* DERA gene.

An approximately 1.5-kb *Pvu*II-digested *K. pneumoniae* genomic DNA fragment was selected as the template for inverse PCR by Southern blot hybridization analysis with probes prepared from the extracted insert of the subcloned plasmid. By using designed primers based on the partial DERA gene sequence and a circularized *Pvu*II digest as the template, an approximately 1.3-kb fragment was amplified by inverse PCR. Then the PCR product was subcloned into the pT7Blue T vector in *E. coli*, and its nucleotide sequence was determined. The sequence comprised 1,258 bp and contained one complete open reading frame that was 780 bp long and encoded 259 amino acid residues (Fig. 1). The predicted molecular weight of the encoded DERA was 27,594.

**Comparison of the deduced amino acid sequence of DERA from *K. pneumoniae* B-4-4 with the amino acid sequences of several other DERAs.** Homology analysis of the deduced amino acid sequence of DERA from *K. pneumoniae* B-4-4 with the amino acid sequences of other known DERAs in a database revealed that the *K. pneumoniae* B-4-4 sequence was quite similar to the sequences of *E. coli* (86% identity; accession no. U14003) (22), *Vibrio cholerae* (79% identity; accession no. AE004305) (7), *Agrobacterium tumefaciens* (51% identity; accession no. AE007954) (6), and *Streptomyces coelicolor* (48% identity; accession no. AL939121) (2). An alignment of these amino acid sequences is shown in Fig. 2.

**Construction of DERA-expressing *E. coli* transformants.** For expression of the *K. pneumoniae* DERA in *E. coli*, the DERA gene was ligated into the EcoRI site downstream of the tac promoter of pKK223-3, and the resulting plasmid was designated pTS8. The latter plasmid was transformed into *E. coli* JM109 and the alkaline phosphatase-defective mutant *E. coli* 10B5, and the resulting transformants were JM109/pTS8 and 10B5/pTS8, respectively. These transformants expressed DERA without addition of IPTG to the culture medium, and addition of IPTG was rather ineffective. During sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of cell extracts of JM109/pTS8 and 10B5/pTS8, thick bands corresponding to a molecular weight of about 28,000, which corresponds to the molecular weight of *K. pneumoniae* DERA, were observed. The DERA activities in the cell extracts of JM109/pTS8 and 10B5/pTS8 were 2.75 and 2.48 U/mg of protein, respectively. These activities were about 20-fold greater than those of *E. coli* JM109 (0.11 U/mg of protein) and *E. coli* 10B5 (0.14 U/mg of protein) and threefold greater than that of *K. pneumoniae* B-4-4 (0.77 U/mg of protein). DR5P production from acetaldehyde and G3P was carried out with washed cells of these transformants. In the reaction with JM109/pTS8, the DR5P production from G3P and acetaldehyde by 10B5/pTS8 were 2.75 and 2.48 U/mg of protein, respectively. These activities were about 20-fold greater than those of *E. coli* JM109 (0.11 U/mg of protein) and *E. coli* 10B5 (0.14 U/mg of protein) and threefold greater than that of *K. pneumoniae* B-4-4 (0.77 U/mg of protein). DR5P production from acetaldehyde and G3P was carried out with washed cells of these transformants. In the reaction with JM109/pTS8, the DR5P production from G3P and acetaldehyde by 10B5/pTS8 was compared with the DR5P production by *K. pneumoniae* B-4-4 (Fig. 4). 10B5/pTS8 produced as much DR5P as B-4-4. The 1,258-bp nucleotide sequence containing the DERA gene is shown. The 780-bp open reading frame of the DERA gene is underlined, and the amino acid sequence is given under the nucleotide sequence. An asterisk indicates the stop codon. RBS, ribosome binding site.

**Screening of substrates that are alternatives to G3P for DR5P production.** To determine whether the glycolytic path-
way and glycerol metabolism could provide G3P, various compounds involved in the glycolytic pathway and glycerol metabolism were examined as precursors of G3P in the reactions with washed cells of the *E. coli* transformants and *K. pneumoniae* B-4-4 (Table 1). Dihydroxyacetone phosphate was utilized well for G3P generation by all strains, as found previously in the reactions with *K. pneumoniae* B-4-4 (14). Fructose 1,6-diphosphate resulted in a high level of DR5P production with all strains. Fructose 6-phosphate and glycerophosphate (glycerol 3-phosphate) were used as substrates by JM109/pTS8, and glucose and glucose 6-phosphate were used as substrates by 10B5/pTS8, although the levels of DR5P produced from these compounds were low.

Screening of substrates that are alternatives to G3P for DR5P production in the presence of ATP. Because several phosphorylated compounds were found to be precursors of G3P, screening of substrates that are alternatives to G3P for DR5P production was carried out in the presence of a phosphate (energy) donor, ATP. Xylene and PL were added to the reaction mixtures to enhance the ATP permeability of the cells. As shown in Table 1, glucose, glucose 6-phosphate, and fructose 6-phosphate were used as substrates by all strains in the presence of ATP. Compared to the results obtained with the other strains, 10B5/pTS8 exhibited greater accumulation of DR5P with glucose as the substrate in the presence of ATP.

Optimization of the reaction conditions for DR5P production from glucose and acetaldehyde in the presence of ATP. By using washed cells of *E. coli* 10B5/pTS8 as the catalyst, the

FIG. 2. Comparison of the deduced amino acid sequence of DERA from *K. pneumoniae* B-4-4 with the deduced amino acid sequences of several other DERAs. The deduced amino acid sequences of DERAs from *K. pneumoniae* B-4-4, *E. coli* K-12, *V. cholerae*, *A. tumefaciens*, and *S. coelicolor* are aligned. Asterisks indicate the putative active center residues, Asp-102, Lys-167, and Lys-201.

FIG. 3. Thin-layer chromatography analysis of DR5P production by JM109/pTS8 and 10B5/pTS8. The reactions were carried out with 98.7 mM G3P and 200 mM acetaldehyde as substrates under the conditions described in Materials and Methods. Lane 1, DR5P standard; lane 2, 2-deoxyribose (DR) standard; lane 3, reaction mixture with JM109/pTS8 as the catalyst; lane 4, reaction mixture with 10B5/pTS8 as the catalyst. The arrow indicates the direction of development.

FIG. 4. Time courses of DR5P production by 10B5/pTS8 and *K. pneumoniae* B-4-4. Reactions were carried out as described in Materials and Methods. Symbols: ■, 10B5/pTS8; ▲, *K. pneumoniae* B-4-4.
reaction conditions for DR5P production from glucose and acetaldehyde were examined. ADP, AMP, adenosine, and adenine were used instead of ATP. The amounts of DR5P produced with ADP and AMP were only 46 and 2.3% of the amount produced with ATP, respectively. DR5P was not produced with adenosine and adenine. The effect of the concentration of ATP was examined by using concentrations ranging from 50 to 500 mM. DR5P production increased with increasing ATP concentration up to 100 mM and decreased at concentrations higher than 100 mM.

The effects of the pH of the reaction mixture (in the range from pH 7.0 to 10.5) and temperature (in the range from 18 to 57°C) were examined by using 100 mM ATP. The reaction proceeded well at pH 7.5 and 37°C. The reactions were performed with 100 to 900 mM glucose. The initial rate of DR5P production increased slightly as the glucose concentration increased and was highest with 900 mM glucose. Higher concentrations of glucose might enhance G3P generation by glycolysis of E. coli cells. The effect of acetaldehyde concentration was examined by using concentrations ranging from 100 to 500 mM. DR5P production increased as the acetaldehyde concentration increased up to 200 mM and decreased with acetaldehyde concentrations higher than 200 mM.

The effects of MgSO4·7H2O and potassium phosphate buffer (pH 7.5), which are known to influence reactions involving ATP, were also examined. The effect of the MgSO4·7H2O concentration was examined by using concentrations ranging from 0 to 90 mM. DR5P production increased as the MgSO4·7H2O concentration increased up to 60 mM and decreased with MgSO4·7H2O concentrations higher than 60 mM. The effect of the potassium phosphate buffer (pH 7.0) concentration was examined by using concentrations of 0, 50, and 100 mM. However, the reaction proceeded well without potassium phosphate buffer.

Under the optimal reaction conditions with 100 mM ATP, 900 mM glucose, 200 mM acetaldehyde, and 60 mM MgSO4·7H2O, the reaction was carried out at pH 7.5 and 37°C. As shown in Fig. 5, 100 mM DR5P was produced in a 6.0-h reaction with yields of 100, 10, and 50% for ATP, glucose, and acetaldehyde, respectively.

Production of 2’-deoxyribonucleoside from DR5P produced from glucose and acetaldehyde in the presence of ATP. A reaction mixture containing DR5P produced as described above from glucose and acetaldehyde was centrifuged, and the resultant supernatant was used directly for 2’-deoxyribonucleoside production with PPMase and NPase under the conditions described previously (14). The reaction was carried out with E. coli BL21/pTS17 expressing E. coli PPMase (5) and commercial bacterial purine NPase (Sigma) as catalysts, as described in Materials and Methods. After a 3-h reaction, 10.1 mM 2’-deoxyribonucleoside (0.1 mM 2’-deoxyadenosine and 10.0 mM 2’-deoxyinosine) was produced from 25 mM DR5P produced from glucose and acetaldehyde in the presence of ATP (molar yield for DR5P, 40.4%). 2’-Deoxyinosine was produced via 2’-deoxyadenosine, because E. coli BL21/pTS17 had adenosine deaminase activity. This result indicated that the DR5P solution produced by E. coli 10B5/pTS8 from glucose and acetaldehyde in the presence of ATP can be used directly for 2’-deoxyribonucleoside synthesis without purification.

FIG. 5. Time course of DR5P production by 10B5/pTS8 from glucose and acetaldehyde in the presence of ATP. Reactions were carried out as described in Materials and Methods.

TABLE 1. Screening of substrates for DR5P production without and with ATPa

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K. pneumoniae B-4-4</th>
<th>JM109/pTS8</th>
<th>10B5/pTS8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without ATP</td>
<td>With ATP</td>
<td>Without ATP</td>
</tr>
<tr>
<td>G3P</td>
<td>68.8</td>
<td>52.1</td>
<td>36.7</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Dihydroxyacetone phosphate</td>
<td>64.5</td>
<td>49.5</td>
<td>53.7</td>
</tr>
<tr>
<td>Dihydroxyacetone</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.9</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>ND</td>
<td>8.5</td>
<td>ND</td>
</tr>
<tr>
<td>Glucose 1,6-diphosphate</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fructose</td>
<td>ND</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>ND</td>
<td>31.4</td>
<td>33.0</td>
</tr>
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<td>Glycerol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Glycerophosphate</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a The reactions were carried out with various glycolysis and glycerol metabolism intermediates (100 mM) without or with ATP (10 mM) under the conditions described in Material and Methods.

b ND, not detected.
tion of DR5P from the complex reaction mixture, which simplifies the total process.

**DISCUSSION**

In the production of 2’-deoxyribonucleoside by the reverse nucleoside degradation reaction, DR5P synthesis catalyzed by DERA is the key step generating the deoxyribose moiety. A DERA useful for this reaction should be acetaldehyde resistant in order to shift the reaction equilibrium in the direction of DR5P synthesis. A DERA with this characteristic from *K. pneumoniae* B-4-4 was cloned and expressed in *E. coli*.

Two DERA-expressing *E. coli* transformants, JM109/pTS8 and 10B5/pTS8, were constructed. 10B5/pTS8 was superior for DR5P production because of a defect in the alkaline phosphatase activity that deocomposes DR5P to 2-deoxyribose. In terms of material utilization, JM109/pTS8 is also interesting. JM109/pTS8 used not only glucose metabolites for G3P generation but also glycerol metabolites. On the other hand, 10B5/pTS8 used only glucose metabolites because of a lack of glycerol-3-phosphate dehydrogenase activity (20). Addition of NAD(P)H with ATP enhanced the glycerol metabolite utilization for G3P generation by JM109/pTS8 (J. Ogawa, T. Sakai, N. Horinouchi, and S. Shimizu, unpublished data).

Use of glucose as the starting material for DR5P production was investigated with 10B5/pTS8. 10B5/pTS8 efficiently produced DR5P from glucose and acetaldehyde in the presence of ATP. This suggests that the levels of activity of the glycolytic enzymes of 10B5/pTS8 are high enough for G3P generation but that the energy required for glucose transformation is insufficient. In addition to ATP, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, ADP, and AMP apparently provided the energy (phosphate) for G3P generation from glucose.

Even without purification, the DR5P produced from glucose and acetaldehyde in the presence of ATP could be used for 2’-deoxyribonucleoside synthesis in the coupled PPMinase and NPass reactions. This indicated that it is possible that 2’-deoxyribonucleoside can be synthesized from the simple materials glucose, acetaldehyde, and a nucleobase (Fig. 6). However, to make the process more practical, energy (ATP) should be supplied in more economical forms and recycled. Although production of DR5P from glucose requires ATP, there are some ATP-providing or -regenerating systems (for example, ATP production from adenine by *Corynebacterium ammoniagenes* [12] or from adenosine by a methylo trophic yeast [17] and ATP regeneration by acetate kinase [9] or by baker’s yeast through alcoholic fermentation [23]) which could be coupled with DR5P synthesis from glucose and acetaldehyde via G3P. Coupling of such energy generation systems is currently under investigation.

2’-Deoxyribonucleoside is in great demand for medical and biotechnology purposes. However, efforts to synthesize these compounds on a large scale have been unsuccessful due to the complex protection and deprotection steps involved in chemical synthesis. The results described above indicate that it may be possible to biochemically synthesize 2’-deoxyribonucleoside from its metabolites through reverse degradation reactions. The innovation in this work was using glucose as the starting material for DR5P synthesis, which is practical for economical large-scale production. The improvement in the energy supply (introduction of ATP-providing or -regenerating systems) for this process provides a powerful tool for producing large quantities of 2’-deoxyribonucleoside. This should make 2’-deoxyribonucleoside more accessible to the research and development community.

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