Methylglyoxal Is an Intermediate in the Biosynthesis of 6-Deoxy-5-ketofructose-1-phosphate: A Precursor for Aromatic Amino Acid Biosynthesis in *Methanocaldococcus jannaschii*†

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ABSTRACT: A biosynthetic pathway is proposed for creating 6-deoxy-5-ketofructose-1-phosphate (DKFP), a precursor sugar for aromatic amino acid biosynthesis in *Methanocaldococcus jannaschii.* First, two possible routes were investigated to determine if a modified, established biosynthetic pathway could be responsible for generating 6-deoxyhexoses in *M. jannaschii.* Both the nucleoside diphosphate mannose pathway and a pathway involving nucleoside diphosphate derivatives of fructose-1-P, fructose-2-P, or fructose-1,6-bisP were tested and eliminated. The established pathways did not produce the expected intermediates nor did the anticipated enzymes have the predicted enzymatic activities. Because neither anticipated pathway could produce DKFP, *M. jannaschii* glucose-6-P metabolism was studied in detail to establish exactly how glucose-6-P is converted into DKFP. This detailed analysis showed that methylglyoxal and a fructose-1-P- or fructose-1,6-bisP-derived dihydroxyacetone-P fragment are key intermediates in DKFP production. Glucose-6-P readily converts to fructose-6-P, which in turn converts to fructose-1,6-bisP. Fructose-6-P and fructose-1,6-bisP convert into glyceraldehyde-3-P (Ga-P-3), which converts into methylglyoxal by a 2,3-elimination of phosphate. The MJ1585-derived enzyme catalyzes the condensation of methylglyoxal with a dihydroxyacetone-P fragment, which is derived from fructose-1-P and/or fructose-1,6-bisP, generating DKFP. The elimination of phosphate from Ga-P-3 proceeds by both enzymatic and chemical routes in cell extracts, producing sufficient concentrations of methylglyoxal to support the reaction. This work is the first report of methylglyoxal functioning in central metabolism.

The pathway for the archaeal biosynthesis of 3-dehydroquinate (DHQ)[1], the second intermediate in the shikimate pathway leading to the aromatic amino acids, is different from that found in bacteria and eukaryotes. The archaeal pathway begins with the condensation of 6-deoxy-5-ketofructose-1-phosphate (DKFP) and L-aspartate semialdehyde (*1*). DKFP, which derives from glucose-6-P, supplies a hydroxyacetone unit. A transaldolase reaction transfers the intact C_3 hydroxyacetone fragment (carbons 4, 5, and 6) to the L-aspartate semialdehyde, forming 2-amino-3,7-dideoxy-D-*threo*-hept-6-ulosonic acid. This amino sugar then undergoes an NAD-dependent oxidative deamination to produce 3,7-dideoxy-D-*threo*-hept-2,6-diulosonic acid, which cyclizes to DHQ. DKFP's biosynthetic origin is addressed here.

There are several possible routes for generating DKFP. The most obvious biochemical route to DKFP would include a step that involves the loss of water from C-5 and C-6 of an NDP-sugar, as occurs in deoxyhexose biosynthesis. Three well-studied biochemical examples are currently known: the conversion of GDP-D-mannose either to L-fucose (*2*) or to D-rhamnose (*3*, *4*) and the conversion of dTDP-D-glucose to L-rhamnose (*5*). Several genes in the *M. jannaschii* genome could encode for enzymes involved in the formation of 6-deoxysugars and, therefore, possibly DKFP, using modified versions of these pathways (Figure 1).

On the basis of established work, this proposed pathway would begin with the isomerization of glucose-6-P to fructose-6-P, catalyzed by phosphoglucomutase (MJ1605), an established enzyme in *M. jannaschii* (*6*). Fructose-6-P would be isomerized to mannose-6-P by the putative mannose-6-phosphate isomerase (MJ1618). Mannose-6-P would be converted into mannose-1-P, catalyzed by the putative phosphomannomutase (MJ0399 or MJ1100); this reaction would supply the mannose-1-P substrate for the formation of NDP-D-mannose by a putative nucleoside-diphosphate pyrophosphorylase (MJ1101). Loss of water from C-5 and C-6 of the NDP-D-mannose would be catalyzed by an enzyme related to the putative NDP-sugar epimerases/dehydrates (MJ0211 or MJ1055), which are members of the short-chain dehydrogenase/reductase superfamily (*7*), and would generate NDP-5,6-dehydromannose. These enzymes catalyze the elimination of water from C-5 and C-6 of a sugar after an NAD-dependent oxidation at C-4. Subsequent reduction can occur at C-6 or C-4, depending on whether the enzyme is a 4,6-dehydratase or a 4-epimerase (*8*). However, to explain the formation of DKFP, this reaction would have to proceed by the NAD(P)H reduction at C-4 instead of C-6 after water is eliminated. NDP-5,6-dehydro-

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[1] Abbreviations: DHQ, 3-dehydroquinate; DKFP, 6-deoxy-5-ketofructose-1-phosphate; DHAP, dihydroxyacetone-phosphate; TMS, trimethylsilyl; TSP, [2,2,3,3 $^2H_4$] sodium 3-trimethylsilylpropionate.

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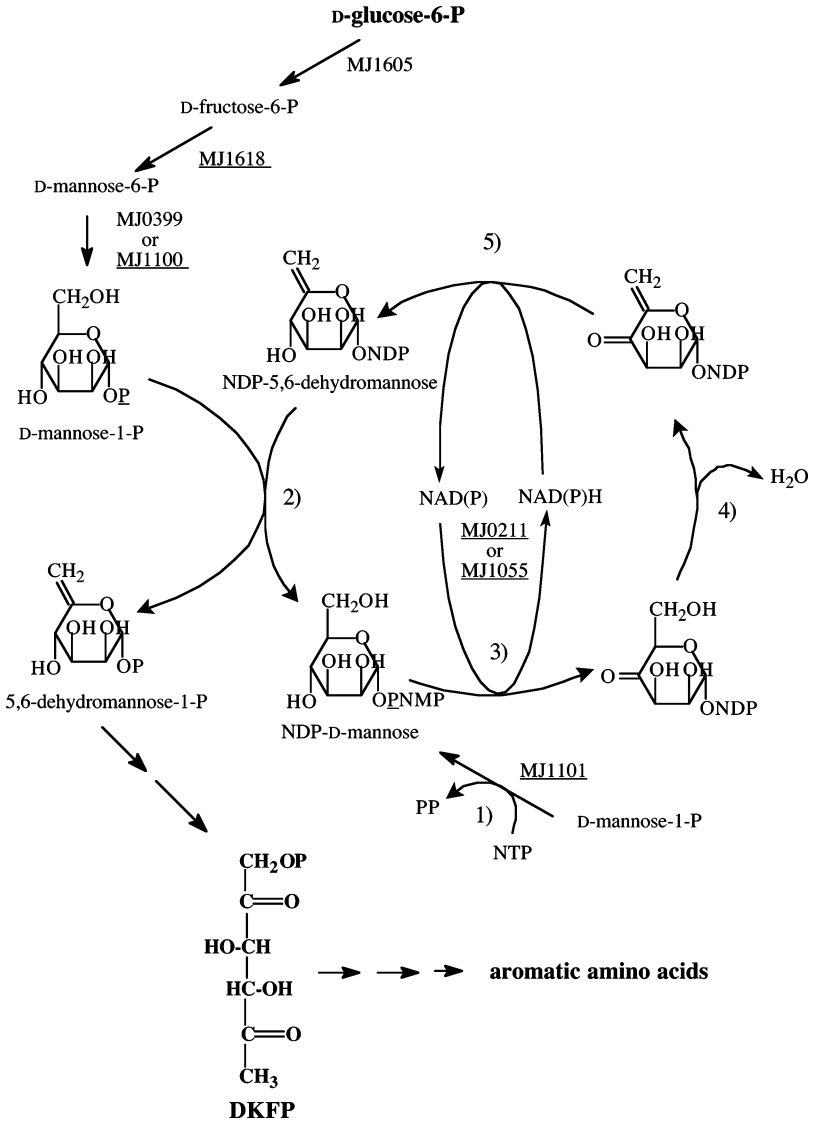

FIGURE 1: Proposed nucleoside diphosphate sugar route to DKFP on the basis of the known formation of L-fucose, D-rhamnose, and L-rhamnose (*3*−*5*). Underlined gene numbers indicate that the protein product of the gene did not catalyze the reaction.

mannose would then undergo a nucleotidyltransferase reaction with D-mannose-1-P to generate 5,6-dehydromannose-1-P. There are two possible routes for converting 5,6-dehydromannose-1-P into DKFP. In one route, the phosphate would have to be removed; the sugar, isomerized; and the phosphate, returned to C-1. This could occur in a single enzyme, as recently proposed for the synthesis of ribulose 1,5-bisphosphate in methanogens (*9*). The second route would involve hydrolysis of the anomeric phosphate ester, isomerization, and readdition of the phosphate to C-1 by a kinase reaction.

Other routes for generating DKFP could use an NDP-fructose linked either at C-1 or C-2 of fructose-1-P, fructose 2,6-bisP, fructose 1,6-bisP, or fructose-2-P (Figure 2). In each case, the formation of NDP-fructose would require the correct fructose-P precursor. Fructose-1-P could be generated from fructose-6-P by a 1,6-phosphomutase, but no example of this conversion has ever been found in archaea (*10*−*12*). Fructose-1-P could also be generated from fructose-1,6-bisP by a specific phosphatase. UDP(2)-fructose has been identified in plants (*13*), and fructose-2,6-bisP is an important regulator of glycogen metabolism (*14*) and is produced from fructose-6-P, catalyzed by fructose-6-P,2-kinase/fructose-2,6-bisphosphatase (*15*). Fructofuranose-2-phosphate is the product of the dephosphorylation of fructose-2,6-bisphosphate (*16*, *17*). In this pathway, the NDP-fructose, after oxidation at C-4, would eliminate water and be re-reduced back to the NDP-5,6-dehydrofructose. If fructose-2-P was the original precursor sugar, a presently unknown mutase would be required to move the phosphate from C-2 to C-1. In any case, ring opening would generate the DKFP. A simpler scheme that does not involve NDP-sugars was proposed earlier by the author, on the basis of results that indicated a possible route to DKFP (*1*). In this scheme, fructose-1,6-bisP, derived from glucose-6-P, is oxidized at C-4 to 4-ketofructose-1,6-bisP. 4-Ketofructose-1,6-bisP then undergoes 5,6-elimination of phosphate to form the enol, which rearranges to the 6-deoxy-triketosugar. Reduction at C-4 would then generate DKFP.

In theory, any of these pathways could produce DKFP, but experiments presented here show that none of them do. After these predicted routes failed to create DKFP experimentally, *M. jannaschii* glucose-6-P metabolism was studied in detail to determine how glucose is ultimately incorporated into DKFP. The work reported here shows that the C-4, C-5, and the C-6 methyl group of DKFP originate from methylglyoxal, which is formed by eliminating phosphate from

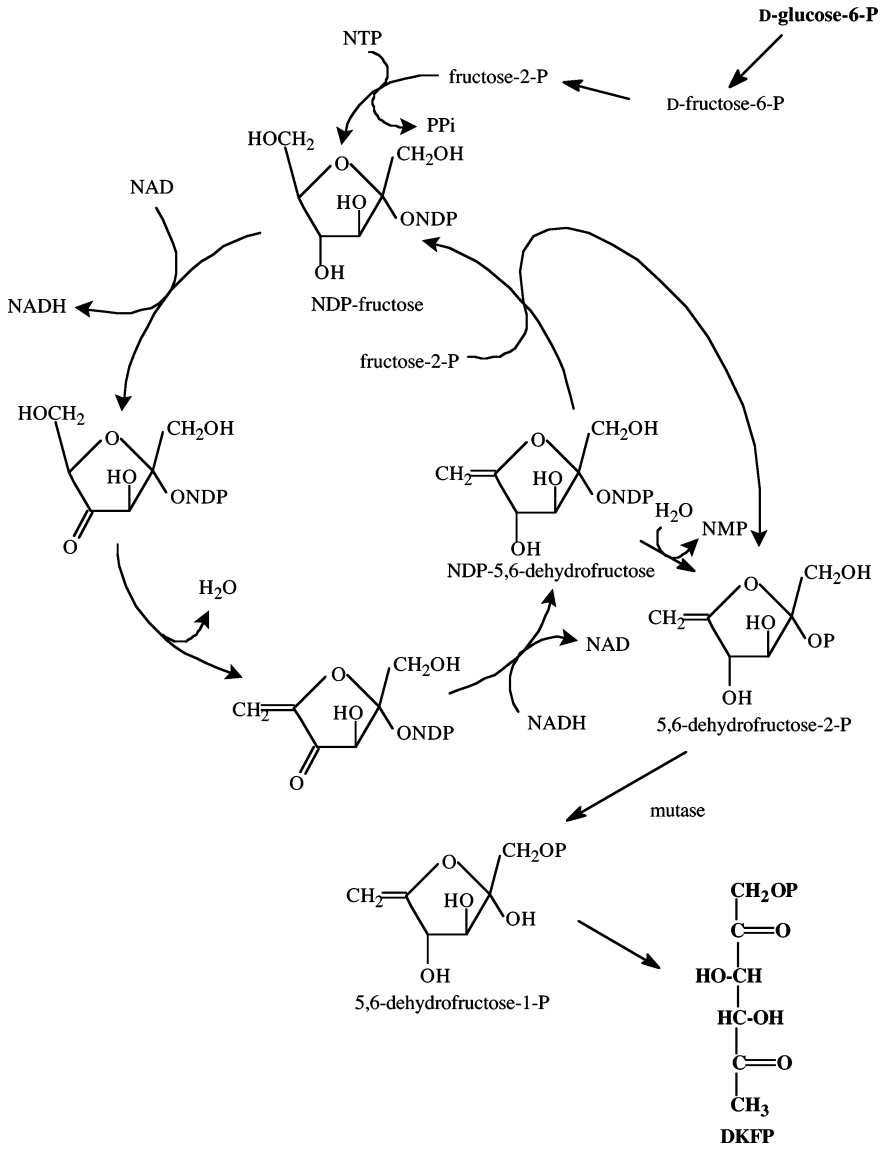

FIGURE 2: Alternate pathway to DKFP formation involving NDP-D-fructose. The pathway shown uses the NDP derivative of fructose-2-P.

glyceraldehyde-3-P (Ga-3-P). Methylglyoxal is a reactive biomolecule that has been identified in many organisms (*18*−*20*) and is metabolized in many different ways (*21*). In cells, it is mostly bound to the side chain arginine, lysine, and cysteine residues of proteins (*22*). Despite its high chemical reactivity, methylglyoxal has recently been proposed as a signal initiator in yeast (*23*) and was once considered to function in the regulation of cell division (*24*). It is readily produced chemically by the elimination of phosphate from Ga-3-P and from DHAP (*25*, *26*). It is also produced enzymatically from several different substrates, including from DHAP catalyzed by methylglyoxal synthase (*27*); from acetoacetate, by myoglobin in the presence of Mn^{2+} and oxygen (*28*); and from lactaldehyde (*29*) or aminopropan-2-ol (*30*).

In the other proposed pathways, an intact hexose is oxidized to generate a carbonyl, which facilitates the elimination of water or phosphate to generate the 5-keto group. When the pathway is based on methylglyoxal, however, an aldolase reaction generates the methyl ketone of DKFP from methylglyoxal. The increased acidity of the carbon-bonded hydrogen next to the aldehyde or ketone facilitates the eliminations. Although the anticipated pathways do not generate DKFP as expected, a novel pathway involving methylglyoxal as a key intermediate does produce DKFP.

EXPERIMENTAL PROCEDURES

Chemicals. All chemicals were obtained from Aldrich/Sigma unless otherwise indicated. Alkaline phosphatase type III from *E. coli* (suspension in 2.5 M $(NH_4)_2SO_4$, 64 units/mg, 2.7 mg/mL) and inorganic pyrophosphate from *E. coli* (1390 units/mg solid) were obtained from Aldrich/Sigma. [U-^{13}C]-D-Glucose-6-phosphate was prepared and purified as previously reported (*1*). DL-Lactaldehyde was prepared by $NaBH_4$ reduction of pyruvic aldehyde dimethyl acetal dissolved in methanol, followed by deprotection with Dowex 50 H^+ (*17*).

Preparation of M. jannaschii Cell Extracts. M. jannaschii cells were grown as previously described (*31*). Cell extracts of *M. jannaschii* were prepared by sonicating 5 g of a frozen cell pellet suspended in 10 mL of TES extraction buffer (50 mM TES/K^+, 10 mM $MgCl_2$, and 10 mM DTT at pH 7.5) under Ar for 5 min at 3 °C. A W-385 sonicator with a

microtip from Heat Systems-Ultrasonics, Inc. was used. The resulting mixture was centrifuged under Ar (27 000*g*, 10 min) and was stored frozen under Ar at −20 °C until used. The protein concentration of the *M. jannaschii* extract was ∼30 mg/mL. Protein concentrations were measured using the BCA total protein assay (Pierce) with bovine serum albumin as a standard.

Cloning, Expression, and Purification of the MJ0211, MJ0399, MJ1055, MJ1100, MJ1101, MJ1585, MJ1605, and MJ1618 Proteins in E. coli. The *M. jannaschii* genes MJ0211 (Swiss Prot accession number Q57664), MJ0399 (Q57842), MJ1100 (Q58500), MJ1101 (Q58501), MJ1055 (Q58455), MJ1585 (Q58980), MJ1605 (Q59000), and MJ1618 (Q59013) were amplified by PCR from genomic DNA using oligonucleoside primers synthesized by Invitrogen as follows: MJ0211-Fwd (5′-GGTCATATGATATTAGTTACTG-3′) and MJ0211-Rev (5′-GCTGGATCCTTATGTCCTATTATTATTTTTC-3′); MJ0399-Fwd (5′-GGTCATATGTTTGGTGATTTG-3′) and MJ0399-Rev (5′-GCTGGATCCTTATAAGGATGCATC-3′); MJ1100-Fwd (5′-GGTCATATGGGAAGATTATTTG-3′) and MJ1100-Rev (5′-GCTGGATCCTTATAAAGCAC-3′); MJ1101-Fwd (5′-GGTCATATGGATGCCATAATATTATG-3′) and MJ1101-Rev (5′-GCTGGATCCCTATTTCAATTTTC-3′); MJ1055-Fwd (5′-GGTCATATGAAATATAAAAATATC-3′) and MJ1055-Rev (5′-GCTGGATCCTTATAATCTTAAAAG-3′); and MJ1585-Fwd (5′-GGTCATATGGGGATTTTTATG-3′) and MJ1585-Rev (5′-GCTGGATCCTTATTTCCTATCTC-3′); MJ1605-Fwd (5′-GGTCATATGCTAAGTTATG-3′) and MJ1605-Rev (5′-CGTGGATCCTTATTGTTTAATC-3′); and MJ1618-Fwd (5′-GGTCATATGATAACCATG-3′) and MJ1618-Rev (5′-GCTGGATCCTCATTCTAATATTTC-3′). PCR was performed as described previously (*23*) using a 45 °C annealing temperature for MJ1055; a 50 °C annealing temperature for MJ0211 and MJ1605; and a 55 °C annealing temperature for MJ0399, MJ1100, MJ1101, and MJ1618. The primers introduced an *Nde*I restriction site at the 5′-end and a *Bam*HI site at the 3′-end of the amplified DNA. The amplified PCR products were purified by QIAQuick spin column (Invitrogen), digested with restriction enzymes *Nde*I and *Bam*HI, and then ligated into the compatible sites in plasmid pT 7-7(USB) by bacteriophage T4 DNA ligase (Invitrogen) to make the recombinant plasmids pMJ0211, pMJ0399, pMJ1100, pMJ1101, pMJ1055, pMJ1585, pMJ1605, and pMJ1585. DNA sequences were verified by dye-terminator sequencing at DVS Sequencing, LLC (Davis, CA) and The Virginia Bioinformatics Institute's DNA Sequencing Facility (Blacksburg, VA). The resulting plasmids were transformed into *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene) cells. Transformed cells were grown in Luria−Bertani medium (200 mL; Difco), supplemented with 100 μg/mL ampicillin at 37 °C with shaking until they reached an absorbance of 1.0 at 600 nm. Recombinant protein production was induced by the addition of lactose to a final concentration of 28 mM. After an additional culture of 2 h with shaking at 37 °C, the cells were harvested by centrifugation (4000*g*, 5 min) and were frozen at −20 °C. Induction of the desired protein was confirmed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) (12% T, 4% C acrylamide, using a Tris/glycine buffer system) analysis of total cellular proteins. This analysis showed the recombinant proteins to be the major cellular protein in each case.

Proteins were released from *E. coli* cell pellets by sonication and were partially purified by heat treatment as previously described (*32*). The MJ0399, MJ1055, MJ1101, and MJ1585 proteins were purified by chromatography on Mono Q (*32*), and the remaining proteins were desalted by Sephadex G-25 chromatography (*32*). SDS−PAGE analysis showed that each enzyme was essentially pure, although some of the samples contained a small amount of the coexpressed chloramphenicol acetyltransferase.

Reduction, Dephosphorylation, and Derivatization of Sugars and Sugar Derivatives. Sugars and sugar derivatives, particularly sugar phosphates, were produced in many of the experiments described here. These sugars and sugar derivatives underwent a largely uniform process before proceeding in the experiment. Sugar and sugar derivative samples were dissolved in ∼100 μL of buffer, and 2−3 mg of sodium borohydride ($NaBH_4$) was added. After 30 min at room temperature, the reaction mixture was acidified to pH 3 by adding 1 M HCl and was evaporated to dryness with a stream of nitrogen gas. The boric acid in the samples was removed by repeatedly suspending the samples in methanol (3 × 0.5 mL) and evaporating the methanol to dryness after each addition with a stream of nitrogen gas. To isolate the sugar phosphates when necessary, the final sample was dissolved in 200 μL of water and adjusted to pH 8−9 by adding 1 M NaOH. The sample was then applied to a small column of DEAE−Sephadex (2 × 5 mm) and washed with 1 mL of water. The sugar phosphates were then eluted with 1 mL of 2 M NH_4HCO_3. NH_4HCO_3 was removed from the sample by evaporating to dryness with a stream of nitrogen gas while the sample was held at 100 °C. For samples in which the sugar-Ps were not isolated, the DEAE−Sephadex isolation step was eliminated.

The phosphate esters in the samples were hydrolyzed by placing them in 150 μL of water; 50 μL of 0.1 M glycine buffer at pH 10.4, with 1 mM $ZnCl_2$ and 1 mM $MgCl_2$; and 1 μL (0.17 units) of the *E. coli* alkaline phosphatase suspension in ammonium sulfate. The sample was incubated for 1−3 h at 37 °C.

Formation of TMS Derivatives. Aqueous solutions containing the polyols and deoxypolyols were dried by evaporation with a stream of nitrogen gas while being held at 100 °C in a water bath. The samples were then reacted with 20 μL of a pyridine, hexamethyldisilazane, and chlorotrimethylsilane mixture (9:3:1 v/v/v) for 2 min at 100 °C prior to GC-MS.

Assay of Mannose Concentration in M. jannaschii Cell Extracts. Mannose, mannose-6-P, and mannose-1-P present in the cell extracts were measured by GC-MS of the mannitol $(TMS)_6$ peak generated after reduction of the samples with $NaBH_4$ and phosphatase treatment as described above. Mannose-6-P was also measured by HPLC as the *O*-(4-nitrobenzyl)hydroxylamine derivative as described below.

Assay of the Phosphoglucose Isomerase Activity of the MJ1605-Derived Enzyme. The MJ1605 enzyme (10 μg), in 50 μL of extraction buffer containing 10 mM glucose-6-phosphate, was incubated for 15 min at 70 °C. Production of the fructose-6-phosphate was assayed as previously described (*33*).

Assay of Mannose-6-P Isomerase (MJ1618). To 100 μL of extraction buffer containing 10 mM mannose-6-P, ∼5 μg of recombinant MJ1618-derived enzyme was added, and the

sample was incubated for 15 min at 70 °C. The sample was reduced with sodium borohydride, was dephosphorylated, and was processed as described below to produce mannitol and glucitol, which were assayed by GC-MS as their $(TMS)_6$ derivatives. Mannitol arises from the reduction of any remaining mannose-6-P substrate as well as from the fructose-6-P product. Glucitol arises from the reduction of the fructose-6-P product. The ratio of the observed area of the glucitol peak to the total of the observed area of the mannitol peak plus the glucitol peak corresponds to the mannose-6-P isomerase activity.

Measuring Phosphoglucose and Phosphomannose Mutase Activity of MJ0399- and MJ1100-Encoded Enzymes. These assays were conducted by incubating 10 mM solutions of either glucose-6-P or mannose-6-P in 50 μL of extraction buffer with the recombinant enzymes (5−10 μg) at 70 °C for 15 min and then measuring the production of either glucose-1-P or mannose-1-P by following the release of phosphate upon acid hydrolysis (*34*).

Assay of Nucleoside-diphosphate Pyrophosphorylase Activity of the MJ1101-Encoded Enzyme. Extraction buffer (55 μL) containing 9.1 mM glucose-1-P, 9.1 mM GTP or UTP, and ∼10 μg of the MJ1101-encoded enzyme was incubated for 15 min at 70 °C. The samples were then incubated in the presence of 5 units of inorganic pyrophosphatase (2 μL of a 2500 units/mL solution) at room temperature for 20 min and were assayed for phosphate (*35*), which is generated from the pyrophosphate produced in the coupling reaction.

Analysis of the NDP-sugar-4,6-dehydratase Activities of the MJ0211- and the MJ1055-Encoded Gene Products. The MJ0211- and MJ1055-derived enzymes were tested for NDP-sugar-4,6-dehydratase activities using a standard colorimetric assay. The formation of a 318-nm-absorbing chromophore upon incubation of the reaction products with 0.1 M NaOH at 37 °C (*36*, *37*) was followed. A GC-MS assay of the 6-deoxyhexitols produced after acid hydrolysis and sodium borohydride reduction of the sugar products was also performed. A mixture containing 8.3 mM NDP-sugar, 1 mM NAD, 1 mM NADP, and ∼80 μg of protein in 120 μL of extraction buffer was incubated for 30 min at 70 °C. After the incubation was complete, 16 μL of 6 M HCl was added, and the precipitated proteins were removed by centrifugation. The resulting separated sample was heated for 1 min at 100 °C to cleave the phosphoglycosidic bond. The sample was then neutralized by adding 16 μL of 6 M NaOH and was reduced by the addition of 1−2 mg of $NaBH_4$. After 30 min at room temperature, the samples were derivatized (TMS) and assayed by GC-MS as described below. Incubations were conducted with UDP-glucose, UDP-mannose, GDP-D-mannose, ADP-glucose, TDP-glucose, UDP-galactose, and UDP-*N*-acetylglucosamine.

To test for the possible exchange of the oxygen of the C-6 hydroxyl group in the sugar portion of the NDP-sugars, the above incubation was also conducted in the same manner but in a buffer containing 50 atom % ^{18}O enriched water.

^{1}H NMR Measurement of Fructose-1-P 1,6-Phosphomutase Activity. ^{1}H NMR (400 MHz, TSP internal standard) of fructose-1-P and fructose-6-P in D_2O containing 10 mM TES buffer at pH 7.2 was measured, and the specific most intense resonances that distinguished the two samples were selected. These were a singlet at 3.86 ppm in the fructose-1-P spectra and a doublet at 3.56 ppm in the fructose-6-P spectra. Then 2 mg of fructose-6-P $(Na)_2$ in 3 mL of water was incubated for 30 min at 70 °C along with 0.1 mg of the activator fructose-1,6-bisP $(Na)_4$ and 50 μL of *M. jannaschii* cell extract. The sample was evaporated to dryness and placed in D_2O, and the ^{1}H NMR spectrum was recorded. The amount of fructose-6-P was not decreased, nor was fructose-1-P produced.

Quantitation, Identification, and Metabolism of Fructose-1-P in M. jannaschii Cell Extracts. To 50 μL portions of *M. jannaschii* cell extracts, 5 μL portions of 0.1 M solutions of glucose-6-P, fructose-1-P, or fructose-1,6-P was added. The samples were incubated for 30 min at 70 °C under argon. A 50 μL portion of cell extract with no additions served as a control. At the end of the incubation, 15 μL of 0.1 M *O*-(4-nitrobenzyl)hydroxylamine and 10 μL of 1 M NaOAc buffer at pH 4.0 were added, and the samples were heated at 100 °C for 20 min. Methanol (100 μL) was then added, and the samples were centrifuged (14 000*g*, 5 min). The clear soluble material was separated from the pellet, evaporated to dryness with a stream of nitrogen gas, and dissolved in 20 μL of methanol. The sample was then purified by preparative TLC using acetonitrile/water/formic acid (88%), 40:10:5 v/v/v, as the developing solvent. The area of the plate containing the *O*-(4-nitrobenzyl)hydroxylamine derivatives, made visible by exposing the TLC plates to UV light, was removed; the derivative eluted with 100 μL of 70% methanol in water. Known samples of sugar phosphates were prepared in the same manner; each of the hexose monophosphate derivatives had an R_f between 0.45 and 0.50, and the fructose-1,6-bisP derivative had an R_f of 0.09. The elution of each of these areas of the plate resulted in one fraction containing all of the hexose-6-P isomer derivatives and another containing the fructose-1,6-bisP derivatives. After evaporation of the solvent, the samples were dissolved in water and adjusted to pH 6.0 for HPLC analysis. The fructose-1-P derivative was confirmed by negative ion LC-ES-MS showing an $M^- = 394.6300$ *m*/*z* using a Finnigan Thermo Quantum Triple Quad LC-MS.

HPLC Analysis of the O-(4-Nitrobenzyl)hydroxylamine Derivatives. Analyses of *O*-(4-nitrobenzyl)hydroxylamine derivatives were performed on a Shimadzu SCL-6B HPLC using a C-18 reversed-phase column (AXXI Chrom octadecyl silane column, 5 μm, 4.6 mm × 25 cm) eluted with methanol in 25 mM sodium acetate buffer (pH 6.0, 0.02% NaN_3) at a flow rate of 0.5 mL/min. For the elution of the *O*-(4-nitrobenzyl)hydroxylamine hexose-6-P derivatives, an elution gradient from 10% to 70% methanol was used. All *O*-(4-nitrobenzyl)hydroxylamine derivative separations were monitored at 275 nm. Using this gradient, the fructose-1-P derivative eluted as a single peak at 16.4 min; the glucose-6-P derivative eluted as two peaks, the first at 19.2 min and the second at 21.0 min; the mannose-6-P derivative eluted as two peaks, the first at 19.8 min and the second at 21.6 min; the fructose-6-P also eluted as two peaks, the first at 19.7 min and the second at 21.0 min. The ratio of the first to the second peak was ∼0.4 for both the glucose-6-P and mannose-6-P and 0.05 for the fructose-6-P. The fructose-1-6-P derivative eluted as three peaks, the first at 3.3 min, the second at 6.2 min, and the third at 9.6 min each with about equal intensities. Treatment of the samples with phosphatase generated the nonphosphorylated derivatives, which co-chromatographed with known samples by both TLC and

Table 1: Summary of Measured Enzymatic Activities of Gene Products Annotated for an NDP-mannose Pathway

gene product	annotated activity	activity tested	results
MJ0211	nucleoside-diphosphate-sugar epimerase/NDP-sugar-4,6 dehydratase	UDP-glucose 4-epimerase	+
		UDP-acetylglucosamine 4-epimerase	+
		NDP-sugar-4,6 dehydratase	−
MJ0399	phosphomannomutase	phosphoglucomutase	+
		phosphomannomutase	+
MJ1055	nucleoside-diphosphate-sugar epimerase/NDP-sugar-4,6 dehydratase	UDP-glucose 4-epimerase	−
		UDP-acetylglucosamine 4-epimerase	−
		NDP-sugar-4,6 dehydratase	−
MJ1100	phosphomannomutase	phosphoglucomutase	−
		phosphomannomutase	−
MJ1101	Nucleoside-diphosphate-sugar pyrophosphorylase	UTP:glucose-1-P uridylyltransferase	+
		UTP:mannose-1-P uridylyltransferase	−
MJ1605	glucose-6-P isomerase	glucose-6-P isomerase	+
		mannose-6-P isomerase	−
MJ1618	mannose-6-P isomerase	mannose-6-P isomerase	−
		glucose-6-P isomerase	+

HPLC analyses. For the elution of the di-*O*-(4-nitrobenzyl)-hydroxylamine derivative of methylglyoxal, an elution gradient from 70% to 90% methanol was used. The methylglyoxal derivative eluted as two peaks, the first at 29.0 min and the second at 29.5 min; the ratio of the first to the second was 0.95. In all of the above examples in which two peaks were observed, these peaks are assumed to correspond to the syn and anti oxime isomers.

GC-MS Analysis of Hexose-6-P Metabolism in M. jannaschii Cell Extracts. To 100 μL portions of *M. jannaschii* cell extracts in a 0.5 mL plastic centrifuge tube, 10 μL of 0.1 M labeled glucose-6-Ps and 5−10 μL of 0.1 M solutions of the indicated precursors (Table 1) were added, and the samples were incubated for 15 min at 70 °C under argon. After incubation, the extracts were cooled to room temperature, and 2−3 mg of sodium borohydride or sodium borodeuteride was added with mixing. After 30 min at room temperature, 240 μL of methanol was added, and the precipitated proteins were removed by centrifugation (14 000*g*, 10 min). The clear extract was separated, acidified to pH ∼3 by the addition of 1 M HCl, and evaporated to dryness with a stream of nitrogen gas. The samples were then processed as described in the Reduction, Dephosphorylation, and Derivatization of Sugars and Sugar Derivatives section above. The sample was first passed through a Dowex 50W-8X-H^+ column (2 × 10 mm) and then through a Dowex-1X8-200 OH^- column (2 × 10 mm) prior to evaporation of the water and the formation of the TMS derivative.

Glyceraldehyde-3-P Thermal Instability and Enzymatic Breakdown. Solutions of 3.3 mM Ga-3-P (30 μL) in extraction buffer were incubated at 60 °C for 10 min, both with and without the *M. jannaschii* cell extract protein (100 μg). After incubation was finished, the samples were assayed for the released inorganic phosphate (*35*). Methylglyoxal was confirmed as a product of the reaction as described below.

Quantitation and Identification of Methylglyoxal in M. jannaschii Cell Extracts. Two methods were used to assay for the presence of methylglyoxal in *M. jannaschii* cell extracts and incubation mixtures. The first reacted methylglyoxal with 1,2-diaminobenzene to form 2-methylquinoxaline (*18*, *38*). Although this method allowed methylglyoxal to be identified by HPLC and TLC, the method proved to be unreliable because of the large number of interfering, unknown additional peaks observed. In the second method, methylglyoxal was converted into its di-*O*-(4-nitrobenzyl)-hydroxylamine derivative, which, after purification by preparative TLC, was identified by HPLC.

Cell extracts (50 μL) were treated in four different ways before analyzing their methylglyoxal content. These included a nonincubated sample, a sample incubated without precursors, and samples incubated with either 9 mM glucose-6-P or 9 mM fructose-1,6-bisP. After each procedure, the samples were mixed with 40 μL of 1 M NaOAc buffer at pH 4.0 and 20 μL of 0.1 M *O*-(4-nitrobenzyl)hydroxylamine and then heated at 100 °C for 20 min. Methanol (240 μL) was added, and the samples were centrifuged (14 000*g*, 5 min). The clear soluble material was separated from the pellet, evaporated to dryness with a stream of nitrogen gas, dissolved in 20 μL of methanol, and purified by preparative TLC using methylene chloride as the eluting solvent. The area of the plate containing the di-*O*-(4-nitrobenzyl)hydroxylamine derivative of methylglyoxal (R_f = 0.3), made visible by exposing the TLC plates to UV light, was removed; the derivative eluted from the support with 100 μL of methanol. Samples were dissolved in 50% methanol for HPLC analysis as described in the HPLC Analysis of the O-(4-Nitrobenzyl)hydroxylamine Derivatives section above. Quantitation of the derivative was obtained from the volume of the solution; concentrations of the solutions were established using ϵ = 9490 M^{-1} cm^{-1} for *O*-(4-nitrobenzyl)hydroxylamine.

Chemical Synthesis of the Different 6-Deoxyhexitols. The structures and synthetic routes used to prepare the different deoxyhexitols isomers are shown in Figure 3. A mixture of DL-6-deoxyfructose and DL-6-deoxysorbose was prepared by condensing dihydroxyacetone with DL-lactaldehyde in the presence of Amberlite IRA-400 (OH^-) as previously described (*39*). ^1H NMR and ^{13}C NMR analyses of the reaction products showed a mixture of 6-deoxyfructofuranose and

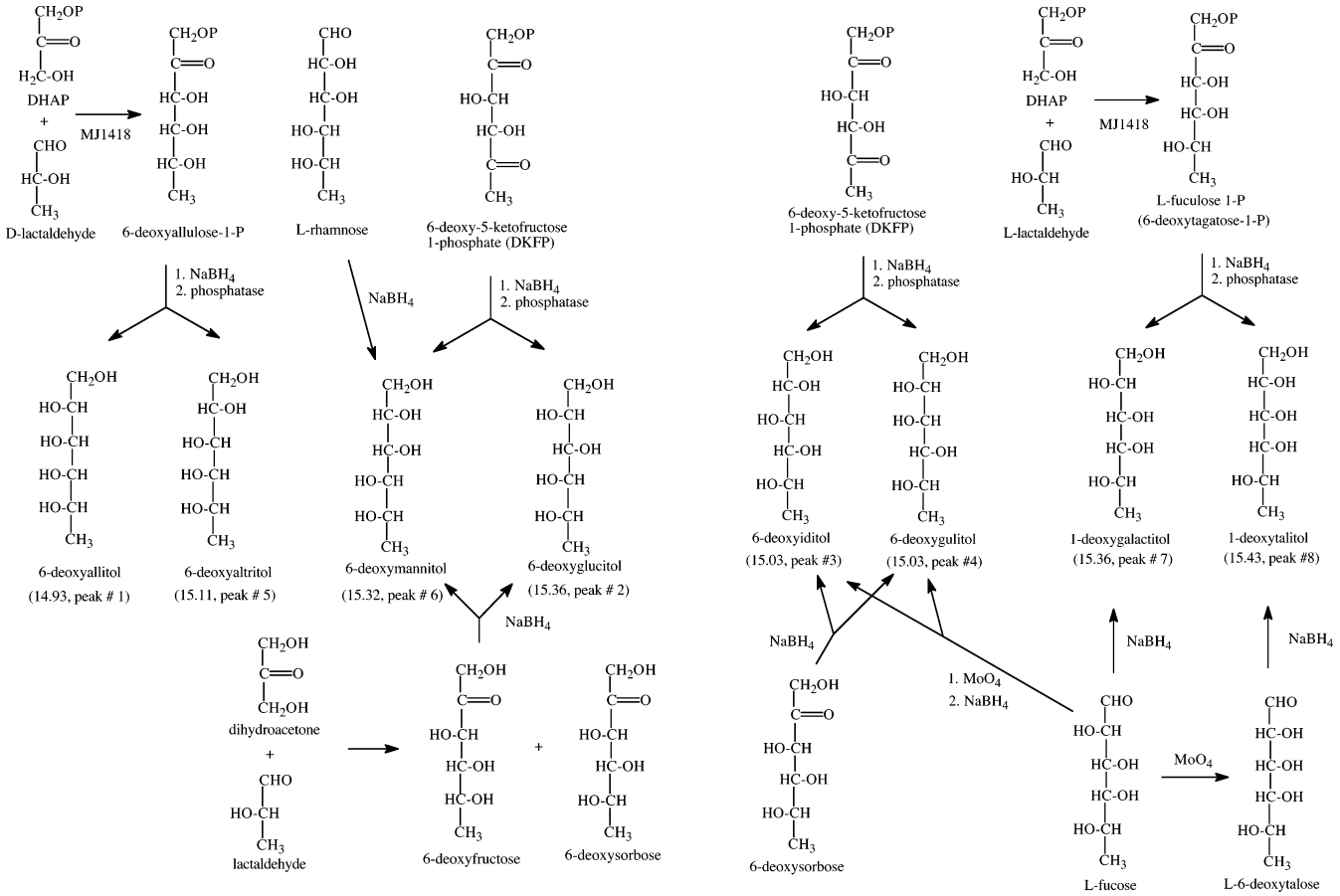

FIGURE 3: Chemical and biochemical syntheses of the 6-deoxyhexitols used in this work. For convenience in comparing the sterochemistries of the different deoxyhexitols, each compound is shown as L-sugars, although some of the reaction products would in fact be D or DL mixtures. The first number in parentheses represents the gas chromatographic elution time in minutes for the $(TMS)_5$ derivatives, and the second represents the order of elution or the peak number.

6-deoxysorbofuranose isomers, which, on the basis of the intensities of their doublet methyl resonances, consisted of 51.4% α-6-deoxyfructofuranose ($J_{6\rightarrow5}$ = 6.19 Hz), 15.8% β-6-deoxyfructofuranose ($J_{6\rightarrow5}$ = 6.19 Hz), 6.3% β-6-deoxysorbofuranose ($J_{6\rightarrow5}$ = 6.42 Hz), and 26.5% α-6-deoxysorbofuranose ($J_{6\rightarrow5}$ = 6.64 Hz). These methyl resonances occurred at 1.329, 1.305, 1.248, and 1.177 ppm, respectively, as previously reported (*39*). The 6-deoxyfructofuranose and 6-deoxysorbofuranose isomers were separated by a preparative TLC using a solvent system of acetonitrile/water/formic acid (88%), 19:2:1 v/v/v. 6-Deoxyfructofuranose had an R_f of 0.53, and 6-deoxysorbofuranose had an R_f of 0.34. 6-Deoxyfructofuranose produced 6-deoxymannitol (peak 6) and 6-deoxyglucitol (peak 2), and 6-deoxysorbofuranose produced 6-deoxyiditol (peak 3) and 6-deoxygulitol (peak 4) upon reduction with $NaBH_4$ (Figure 3). Reduction of DKFP with $NaBH_4$ followed by phosphatase treatment produced 6-deoxymannitol (peak 6), 6-deoxyglucitol (peak 2), 6-deoxyiditol (peak 3), and 6-deoxygulitol (peak 4).

L-Fucose was epimerized at C-2 to L-6-deoxy-D-talose by heating L-fucose for 3 h at 100 °C in the presence of molybdic acid as previously described (*40*, *41*). Longer heating (14 h) resulted in the further epimerization at C-3 producing 6-deoxyiditol and 6-deoxygulitol. A mixture of 6-deoxyallitol, 6-deoxyaltritol, 6-deoxygalactitol, and 6-deoxytalitol was prepared by the aldol condensation of DHAP and DL-lactaldehyde, catalyzed by fuculose-P aldolase (MJ1418), which produced 6-deoxyallulose-1-P and fuculose-1-P (6-deoxytagatose-1-P) (*42*). These ketose-Ps were reduced with $NaBH_4$, and the phosphates were removed with phosphatase. This generated the desired 6-deoxypolyols (Figure 3), with the allitol and altritol isomers arising from 6-deoxyallulose-1-P, and the galactitol and talitol isomers arising from fuculose-1-P.

Reductions were accomplished at room temperature by dissolving a few milligrams of solid $NaBH_4$ or $NaBD_4$ in solutions of the sugars. After reducing for 30−60 min, excess $NaBH_4$ or $NaBD_4$ was destroyed by acidifying the solutions with dilute HCl; borate was removed by evaporation with methanol. The samples were desalted on ion-exchange resins (see below), and the dried samples were converted into their TMS derivatives for GC-MS analyses.

GC-MS Analysis of TMS Derivatives. GC-MS analysis of the sugars in the cell extracts was obtained using a VG-70-70EHF gas chromatography−mass spectrometer operating at 70 eV and equipped with a HP-5 column (0.32 mm by 30 m) programmed from 95 to 280 °C at 10 °C per min. Analyses were conducted on the sugars reduced with either $NaBH_4$ or $NaBD_4$ not only to limit the number of GC peaks generated from each sugar but also to determine the position and number of carbonyls in the original sugars on the basis of the fragmentation of the sugar and number of deuteriums incorporated when $NaBD_4$ was used as the reductant (Figure 3). Under the GC-MS conditions employed, the TMS derivatives of the following compounds had the following retention times (min) and mass spectral data (molecular

weight, base peak, and the most abundant ions with masses over 150 *m*/*z* listed in order of decreasing intensities): erythritol $(TMS)_4$ derivative (9.05) [410, 73, 205, 217, 189, 191, 307]; xylitol $(TMS)_5$ derivative (13.65) [512, 73, 217, 205, 319, 307, 422]; arabitol $(TMS)_5$ derivative (13.98) [512, 73, 217, 205, 319, 307, 422]; ribitol $(TMS)_5$ derivative (14.09) [512, 73, 217, 205, 319, 307, 422]; xylitol $(TMS)_5$ derivative (13.65) [512, 73, 217, 205, 319, 307, 422]; 6-deoxyallitol $(TMS)_5$ derivative (14.93, peak #1) [526, 205, 319, 217, 219, 307]; 6-deoxygluitol $(TMS)_5$ derivative (14.97, peak #2) [526, 319, 205, 219, 217, 277]; 6-deoxyiditol $(TMS)_5$ derivative (15.03, peak #3) [526, 205, 319, 217, 219, 307]; 6-deoxygulitol $(TMS)_5$ derivative (15.03, peak #4) [526, 205, 319, 219, 217, 231]; 6-deoxyaltritol $(TMS)_5$ derivative (15.11, peak #5) [526, 205, 319, 219, 217, 231]; 6-deoxymannitol $(TMS)_5$ derivative (15.32, peak #6) [526, 205, 319, 219, 217, 231]; 6-deoxygalactitol (fucitol) $(TMS)_5$ derivative (15.36, peak #7) [526, 117, 217, 205, 319, 219, 231]; 6-deoxytalitol $(TMS)_5$ derivative (15.43, peak #8) [526, 117, 217, 205, 319, 219, 231]; mannitol $(TMS)_6$ derivative (18.49) [614, 319, 205, 217, 307]; glucitol $(TMS)_6$ derivative (18.65) [614, 319, 205, 217, 307]; galactitol $(TMS)_6$ derivative (18.73) [614, 319, 205, 217, 307]; and talitol $(TMS)_6$ derivative (18.73) [614, 319, 205, 217, 307]. The mass spectra of the deoxyhexitols have been previously reported (*43*) and, as observed here, several of the isomers were not separated by GC.

RESULTS

Testing for the Involvement of a Possible NDP-mannose-Based Pathway to DKFP. An NDP-mannose-based pathway requires the involvement of several enzymes that are annotated to catalyze the indicated reactions in the pathway (Figure 1). These seven proteins were recombinantly expressed and their enzymatic activity was tested (Table 1). The protein derived from the MJ1605 gene readily catalyzes the conversion of glucose-6-P to fructose-6-P. However, no activity was observed for the MJ1618-derived enzyme, annotated as a mannose-6-P isomerase. The product of the MJ0399 gene serves both as a phosphoglucomutase and a phosphomannomutase, but no similar activity was observed for the product of the MJ1100 gene, also annotated as a phosphomannomutase. The MJ1101-derived protein was demonstrated to be a UTP/glucose-1-P uridylyltransferase that forms UDP-glucose from glucose-1-P and UTP but not UDP-mannose from mannose-1-P and UTP. The MJ0211-derived protein is both a UDP-glucose 4-epimerase and a UDP-acetylglucosamine 4-epimerase. Neither the protein from MJ0211 nor that from MJ1055 functioned as a 5,6-dehydratase when tested with UDP-glucose, UDP-mannose, GDP-D-mannose, ADP-glucose, TDP-glucose, UDP-galactose, and UDP-*N*-acetylglucosamine. Only three of the seven recombinantly generated enzymes performed the necessary reaction for the proposed NDP-mannose pathway. These results eliminate an NDP-mannose-based pathway to DKFP.

Elimination of Other Plausible Biochemical Pathways to Deoxyhexoses. Chemically, the most direct route for forming DKFP uses fructose-1-P or fructose-1,6-bisP as the direct precursor. Such a pathway had been previously proposed (*1*). Alternately, a pathway involving an NDP-D-fructose could form DKFP (Figure 2). However, if the proposed precursors, fructose-1-P and fructose-1,6-bisP or NDP-D-fructose, are demonstrated to not be intermediates, then these pathways can be eliminated. As this experiment shows, these three precursors do not lead to DKFP production, thus eliminating these pathways. In each of these proposed cases, the elimination of water or phosphate from C-5 and C-6 of the fructose generates a 5,6-dehydrofructose-containing molecule, which, after ring opening, produces DKFP.

Analyses of *M. jannaschii* cell extracts showed that they contain 0.28 mM fructose-1-P (see below), indicating that this sugar phosphate could potentially serve as a precursor. The fructose-1-P derivative was confirmed by LC-ES-MS of its *O*-(4-nitrobenzyl)hydroxylamine derivative showing an $M^- = 394.6300$ *m*/*z*, the same as that prepared from a known sample of fructose-1-P. Incubation of cell extracts with fructose-1-P, however, failed to increase the amount of DKFP. Early experiments showed that [U-^{13}C]-glucose-6-P readily labels DKFP, the precursor to DHQ and shikimate, to an extent of >90% with $^{13}C_3$ (*1*). Because fructose-1-P is not derived directly from glucose-6-P (see below), this eliminates fructose-1-P from serving as an intact precursor to DKFP. Likewise, because [U-^{13}C]-glucose-6-P labels the precursor in the absence of added ATP, this indicates that fructose-1,6-bisP is also not the precursor in cell extracts.

HPLC analysis of the hexose-Ps in cell extracts showed concentrations of 0.28 mM fructose-1-P, 0.014 mM fructose-6-P, 0.025 mM glucose 6-P, and <0.0005 mM mannose-6-P. The calculated intracellular concentrations would be ~4−5 times higher than those values found in the cell extracts. Incubating a cell extract sample with 9 mM fructose-1-P reduced the amount of fructose-6-P by 53% and glucose-6-P by 18%, indicating that fructose-1-P was not converted into fructose-6-P or glucose-6-P. The amount of fructose-1-P did not increase after the extracts were incubated with glucose-6-P, fructose-6-P, or glucose-1-P, indicating that no 1 → 6 phosphofructomutase activity was present. ^{1}H NMR analysis of *M. jannaschii* cell extracts incubated with fructose-6-P also fails to show the production of any fructose-1-P. These findings are consistent with other work that shows that such a mutase is not present in the archaea (*11*, *12*, *44*). Also, incubation with fructose-1,6-diP readily forms fructose-1-P, indicating that the levels of ADP and ATP in the extracts are low because no net fructose-1,6-diP is formed. Incubating a cell extract with 9 mM fructose-1,6-diP increased the amount of fructose-6-P 1.9-fold to 0.53 mM; fructose-1-P, 2.9-fold to 0.04 mM; and glucose-6-P, 27-fold to 0.67 mM. This demonstrates that the cell extracts have both 1- and 6-fructose-1,6-diP phosphatase activity as well as glucose-6-P isomerase activity. Incubation of a cell extract with glucose-1-P, glucose-6-P, or fructose-6-P increased the amounts of the glucose-6-P and fructose-6-P detected. In each case, the ratio of the glucose-6-P to fructose-6-P (~1.7) was the same, showing that these two sugar-Ps are in equilibrium after the 15 min incubation. The amount of fructose-1-P in a cell extract dropped 44% after incubation, indicating that it was being metabolized by the cell extract. No mannose-6-P was detected in any analysis.

These results eliminate fructose-1-P and fructose-1,6-bisP as direct precursors to DKFP. Glucose-6-P rapidly labels the shikimate intermediates and is not a direct precursor of fructose-1-P. Therefore, fructose-1-P and fructose-1,6-P cannot be precursors along an intact hexose-driven pathway.

Table 2: Amounts and Incorporation of Label from Sugar-P's into the Sugars

	Concentration of products and reactants detected (μM)								
		deoxyhexitols							
experiment[a]	glycerol	1[b]	(2, 3, and 4)	5	6	7	8	mannitol[c]	glucitol[d]
1. cell extract incubated with no substrates	28	all deoxyhexitols <0.03						0.54	0.54
2. nonincubated cell extract + 1 M HCl	270	<1.8 for isomers 1 to 6				75	42	1.3	8.4
3. cell extract + [U-^{13}C]-glucose 6-P, methylglyoxal	31		40		25			25	180
4. cell extract + fructose 1-P, methylglyoxal	340		11		2.5			270	290
5. MJ1585 fructose-1,6-bisP + methylglyoxal	231		9.1		3.2			590	660
6. MJ1585 fructose-1-P + methylglyoxal	37		2		0.5			350	390
7. cell extract + DL-lactaldehyde+ DHAP	270	100	260[e]	3	-	2	4	16	40

[a] Most of the experiments were conducted using 50 μL of cell extract at 70 °C for 15 min in the presence of 9 mM of the indicated substrates. The experiments with the recombinant MJ1585 enzyme contained ~10 μg of protein. Each sample was analyzed after borohydride reduction and phosphatase cleavage of the phosphate esters. [b] The numbers correspond to the following compounds: #1, 6-deoxyallitol; #2, 6-deoxygulitol; #3, 6-deoxyiditol; #4, 6-deoxyalitol; #5, 6-deoxygulitol; #6, 6-deoxymannitol; #7, 6-deoxygalactitol; and #8, 6-deoxytalitol. The compounds are listed in the order of their elution from the GC column and correspond to their peak numbers. Compounds 2, 3, and 4 did not separate under the conditions used. [c] $NaBH_4$ reduction of the fructose-6-P, fructose-1-P and/or fructose-1,6-bisP present or generated in the extracts produced the mannitol peak. [d] The glucitol peak is derived from both the fructose, glucose, and/or their phosphate esters present in the extract. [e] The absence of compound 6 in the analysis indicates that this single peak only contains compounds 3 and 4.

These results also indicate that the intact C_6 unit of a hexose-6-P is not a likely precursor to DKFP.

Establishing the Pathway to Deoxyhexoses by Incubation of Cell Extracts with Possible Precursors. Once all proposed pathways were eliminated, the details of the pathway from glucose-6-P to DKFP was studied in detail. Gucose-6-P and several proposed intermediates were incubated with cell extracts; the concentrations and extent of labeling of any DKFP-related products were measured by GC-MS. An analysis of the TMS derivatives of the polyols generated by borohydride reduction and enzymatic dephosphorylation of the sugar phosphates present in the incubation mixtures was used to identify the compounds produced in the reactions. By using an internal standard of inositol, which is not produced by the cells, the levels of each compound produced could be determined. The specific final compounds assayed were glycerol, arising from dihydroxyacetone, hydroxypyruvaldehyde, D- or L-glyceraldehyde, and/or their phosphates; 6-deoxyhexitols from the 6-deoxyaldoses, 6-deoxyketoses, 6-deoxydioxohexoses, and/or their phosphates (such as DKFP); and mannitol from the fructose and/or fructose phosphates; and glucitol derived both from glucose and glucose-6-P and from the fructose and/or fructose phosphates. By reducing the sugars with either borohydride or borodeuteride, the number of carbonyl groups in each sugar can be determined by the increase in the nominal mass of the derivative. Changes in the masses of the fragment ions are used to determine the positions where the reduction occurred. By isolating the sugar phosphates after the incubation using DEAE−Sephedex chromatography, it was possible to establish whether the sugars are present as their phosphate ester.

An analysis of a cell extract incubated without added glucose-6-P and then reduced by $NaBD_4$ showed no detectable amount of any of the different 6-deoxyhexitol isomers (Table 2, experiment 1). Because this sample was not purified by the DEAE step, which would have isolated the phosphorylated sugars, this result demonstrates that the 6-deoxyhexitols are not present in detectable amounts in the cell extracts as the free deoxyhexoses, deoxyhexitols, or their phosphate derivatives. Small amounts of glycerol, mannitol, and glucitol were detected, however. The M^+-15 ion at *m*/*z* 293 in the glycerol $(TMS)_3$ derivative had 59% $^2H_0$, 12% $^2H_1$, and 29% $^2H_2$; the glycerol M^+-90 ion at *m*/*z* 218 had 70.5% $^2H_0$, 18% $^2H_1$, and 4.8% $^2H_2$; and the glycerol M^+-103 fragment ion at *m*/*z* 205 had 80% $^2H_0$, 7.2% $^2H_1$, and 13% $^2H_2$. From the distribution of the label in these fragments in the mass spectra of the glycerol $(TMS)_3$ derivative (Figure 4), it is clear that both the C-1/3 and C-2 of the glycerol are labeled with a single deuterium. The overall pattern of labeling showed that 59% of the glycerol was derived from glycerol and/or glycerol-P, 12% was derived from dihydroxyacetone or dihydroxyacetone-P, and 30% was derived from hydroxypyruvaldehyde or hydroxypyruvaldehyde-P. Hydroxypyruvaldehyde-P results from the condensation of DKFP with aspartate semialdehyde in aromatic acid biosynthesis in *M. jannaschii* (*1*). This compound has been previously shown to be generated from glucose in human erythrocytes (*45*). As expected, the isolated mannitol and glucitol both contained one deuterium from the borohydride reduction.

A cell extract without added glucose-6-P was not incubated as the previous sample was but instead was hydrolyzed for 10 min at 100 °C with 1 M HCl before $NaBD_4$ reduction. This sample shows an increase in the amounts of glycerol, 6-deoxygalactitol (peak 7), 6-deoxytalitol (peak 8), mannitol, and glucitol (Table 2, experiment 2). Here, the $(TMS)_3$ derivative of the glycerol had an M^+-15 ion at *m*/*z* 293 with 70.4% $^2H_0$, 20% $^2H_1$, 7.0% $^2H_2$, and 2.6% $^2H_3$. Therefore,

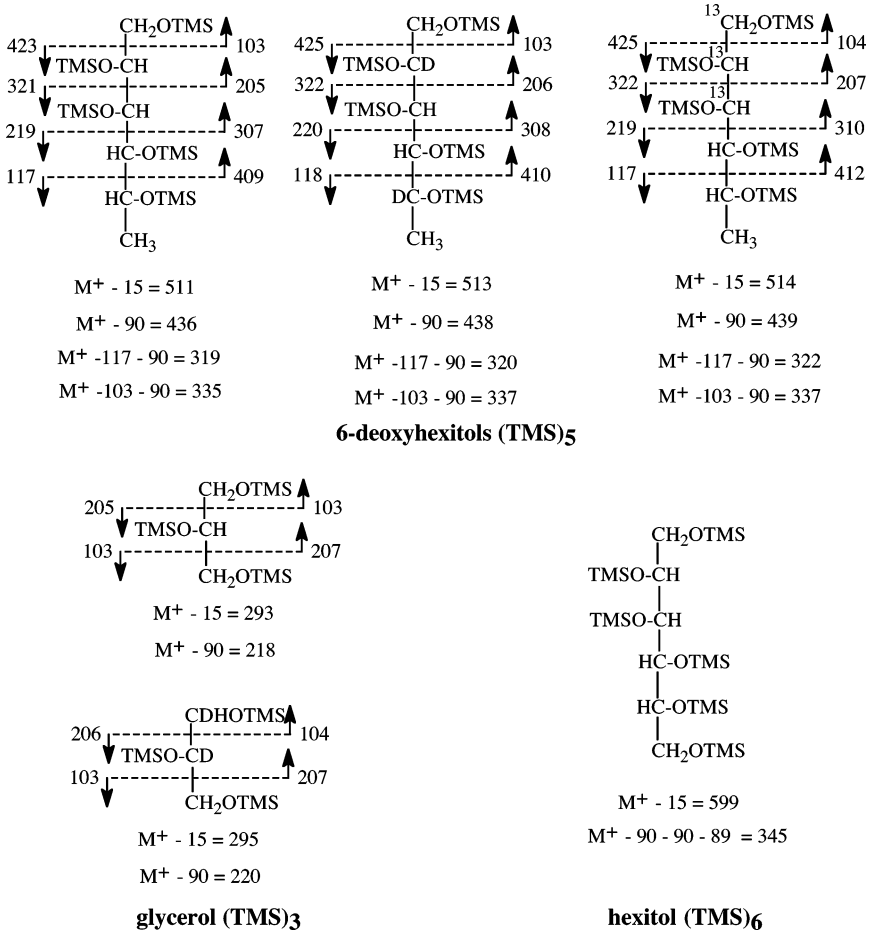

FIGURE 4: Mass spectral fragmentation of the TMS derivatives used in this work. The $M^+ - 15$ refers to the loss of a CH_3 and the $M^+ - 90$ refers to the loss of a TMSOH.

the recovered glycerol is mostly unlabeled. The two 6-deoxyhexitols detected also contain no deuterium, indicating that they, along with most of the glycerol, are present in the cell extract as covalently bound reduced polyols that are released during acid hydrolysis. The sample of the sugars assayed after this acid hydrolysis represents the total sugars present because the DEAE step, which would have specifically isolated the phosphorylated sugars, was omitted. It is highly likely that these 6-deoxyhexitols may be components of the glycan in the flagellins in the S-layer of these cells (*31*, *46*). Rhamnose and fucose occur in the S-layer heteropolysaccharides of *Sulfobacillus asporogenes* (*47*), but no occurrence of 6-deoxyhexitols was found.

Incubation of a cell extract with [U-^{13}C]-glucose-6-P and methylglyoxal (Table 2, experiment 3) readily produced DKFP, which was reduced at C-2 and C-4 by the borohydride to generate 6-deoxyglucitol (compound 2), 6-deoxyiditol (compound 3), 6-deoxygulitol (compound 4), and 6-deoxymannitol (compound 6). The first deoxyhexitol-containing GC peak observed contains 6-deoxyglucitol, 6-deoxyiditol, and 6-deoxygulitol that were not resolved on the GC column, and the second GC peak is 6-deoxymannitol. (This same grouping of peaks is also seen in experiments 4, 5, and 6.) The label distribution in the $M^+ - 90$ ion at *m*/*z* 218 in the glycerol $(TMS)_3$ derivative is 50.8% $^{13}C_0$, 7.3% $^{13}C_1$, 27% $^{13}C_2$, and 15% $^{13}C_3$. Because the labeled glucose-6-P is many times more abundant than the unlabeled glucose-6-P in the cell extract, this result shows not only that both the $^{13}C_2$ and $^{13}C_3$ units of the glucose-6-P were incorporated into trioses but also that the label is substantially diluted after incorporation. The $M^+ - 117 - 90$ ion at *m*/*z* 319 for the 6-deoxyhexitol $(TMS)_5$ derivatives each contained 14.6% $^{13}C_0$, 0.4% $^{13}C_1$, 3.3% $^{13}C_2$, and 81.7% $^{13}C_3$. Fragmentation confirmed that the $^{13}C_3$-label was at C-1, C-2, and C-3 of the 6-deoxyhexitol (Figure 4). The $^{13}C_3$ unit at C-1, C-2, and C-3 arises from the condensation of a DHAP unit (produced via Ga-3-P from [U-^{13}C]-fructose-6-P, which in turn is generated from [U-^{13}C]-glucose-6-P) and unlabeled methylglyoxal to produce DKFP (Figure 5). Most of the DKFP molecules contain a $^{13}C_3$ unit, indicating that the $^{13}C_3$ precursor was not equilibrated with the triose-Ps which were labeled to a lower extent. The label distribution in the $M^+ - 90 - 90 - 89$ ion of the glucitol $(TMS)_6$ derivative at *m*/*z* 345, containing all of the carbons of the sugars, contained 22.1% $^{13}C_0$, 1.0% $^{13}C_1$, 3.0% $^{13}C_2$, 26% $^{13}C_3$, 2.6% $^{13}C_4$, 5.3% $^{13}C_5$, and 39.7% $^{13}C_6$. This labeling pattern indicates that the labeled glucose-6-P has been extensively scrambled via triose-Ps with unlabeled molecules in the cell extract. Cell extracts also generate DKFP when incubated with methylglyoxal and fructose-1-P but to a lesser extent (Table 2, experiment 4). Here, the $M^+ - 15$ ion at *m*/*z* 293 in the glycerol $(TMS)_3$ derivative had 6.1% $^2H_0$, 88.9% $^2H_1$, and 5.0% $^2H_2$, indicating that the glycerol arises from the borohydride reduction of Ga-3-P or DHAP. The $M^+ - 103 - 90$ ion at *m*/*z* 335 for the 6-deoxyhexitol $(TMS)_5$ derivatives (Figure 4) contained 100% $^2H_2$, showing that each is derived from DKFP. Fragmentation confirmed that the deuterium is incorporated at C-2 and C-4 of the 6-deoxyhexitols.

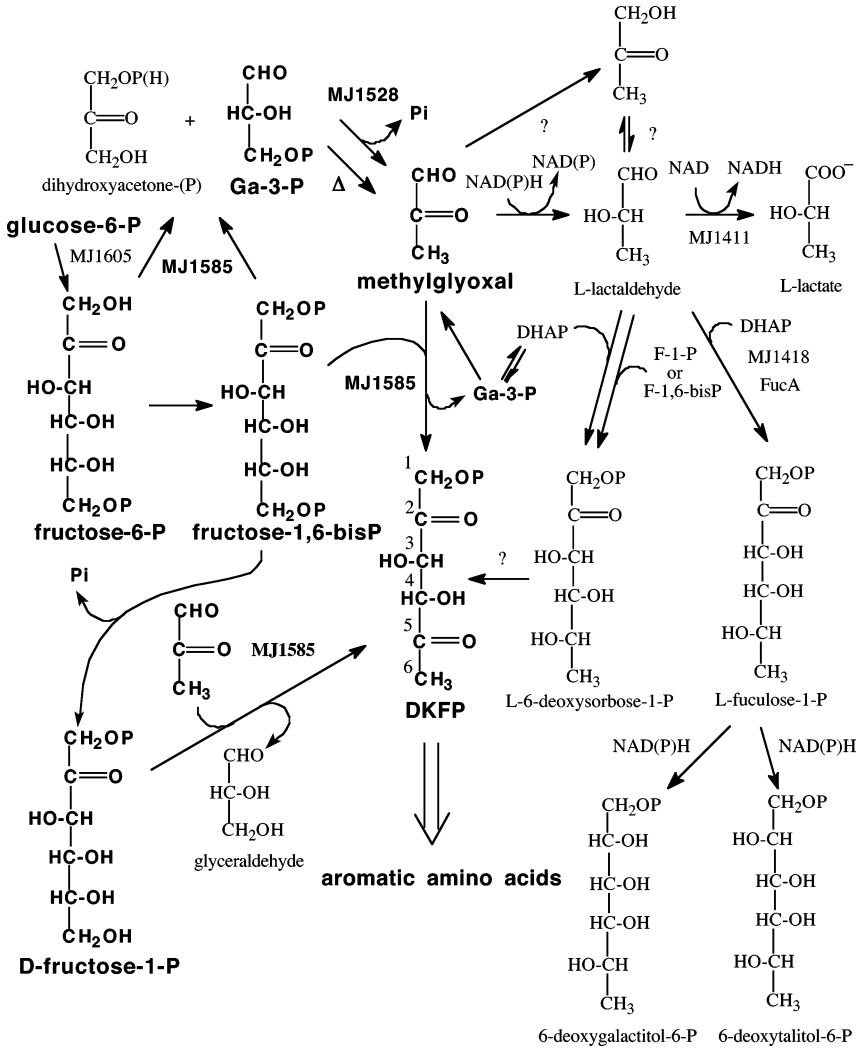

FIGURE 5: Proposed pathways for the formation of methylglyoxal and its conversion into DKFP in *M. jannaschii*. Reactions with bold structures and names represent those established in this work.

This transaldolase reaction between methylglyoxal and a hexose-P is mediated by the product of the MJ1585 gene, an archaeal Class I fructose 1,6-bisP aldolase (*48*). The recombinant enzyme result is confirmed because the gene product catalyzed the reaction of either fructose-1-P or fructose-1,6-bisP with methylglyoxal to produce DKFP (Table 2, experiment 5, 6). In both experiments 5 and 6, each worked up with the $NaBD_4$ reduction, the $M^+ - 103 - 90$ ion at *m*/*z* 335 for the 6-deoxyhexitol $(TMS)_5$ derivatives contains 100% $^2H_2$, and the mannitol and glucitol contain a single deuterium. In the incubation with fructose-1,6-P, the $M^+ - 15$ ion at *m*/*z* 293 in the glycerol $(TMS)_3$ derivative has 3.0% $^2H_0$ and 97% $^2H_1$; in the fructose-1-P incubation, the $M^+ - 15$ ion at *m*/*z* 293 in the glycerol $(TMS)_3$ derivative has 14.5% $^2H_0$ and 85.5% $^2H_1$. These results again show that the glycerol is derived from a triose-P. No deoxyhexoses were observed when fructose-6-P was incubated.

The observed stereochemistry of the aldolase reaction is consistent with MJ1585 also functioning as the fructose 1,6-bisP aldolase (EC 4.1.2.13) used in glycolysis. The MJ1585 aldolase did not catalyze the reaction of DL-lactaldehyde with fructose-1-P, fructose-1,6-P, or DHAP to form 6-deoxyallulose-1-P or fuculose-1-P. This reaction is catalyzed by the MJ1418 gene product (*44*). Clearly, the MJ1585-derived aldolase must have a multifaceted function in the archaea. This gene product is involved in aromatic amino acid biosynthesis; the specific activity of this enzyme in *Methanococcus maripaludis* grown on aryl acids (which suppresses the production of the aromatic amino acids via the shikimate pathway) drops 10-fold, confirming the enzyme's involvement (Porat and Whitman, unpublished results).

Cell extracts readily catalyze the condensation of DHAP and DL-lactaldehyde to produce 6-deoxysorbose-1-P, which was detected as a single peak containing 6-deoxyiditol and the 6-deoxygulitol peak (Table 2, experiment 7). In addition, much smaller amounts of 6-deoxyallulose-1-P and 6-deoxyfuculose-1-P were produced (Figure 3), which were detected by the generation of 6-deoxyallitol (compound 1), 6-deoxyaltritol (compound 5), 6-deoxygalactitol (compound 7), and 6-deoxytalitol (compound 8). All of these compounds contain one deuterium at C-2, introduced during the $NaBD_4$ reduction. This is confirmed because the 117 *m*/*z* fragment ion of the deoxyhexitol $(TMS)_5$ derivative contains no deuterium, and the *m*/*z* 319 fragment ion contains one deuterium (Figure 4). The $M^+ - 15$ ion at *m*/*z* 293 in the glycerol $(TMS)_3$ derivative has 21% $^2H_0$, 70% $^2H_1$, and 9% $^2H_2$, and the *m*/*z* 205 fragment ion shows that 78% of the deuterium is on a terminal hydroxymethyl and 22% is at C-2, demonstrating the equilibration of DHAP with Ga-3-P.

The condensation of DHAP and DL-lactaldehyde to produce 6-deoxyallulose-1-P and 6-deoxyfuculose-1-P is catalyzed by the MJ1418 gene product (*42*) and is not related to the formation of DKFP. The production of 6-deoxyallulose-1-P is probably not normal because the cells are not expected to have D-lactaldehyde. Also, because 6-deoxyiditol and 6-deoxyglulitol (combined peaks 3 and 4) represent the largest amount of deoxyhexitols observed in experiment 7, these isomers must result from 6-deoxysorbose-1-P formed by the condensation DHAP and L-lactaldehyde catalyzed by the MJ1585 enzyme. The MJ1585 enzyme would be expected to produce the *threo* isomer at the position of condensation.

The above labeling data indicate that the deoxysugars could arise from methylglyoxal and either fructose-6-P or fructose-1,6-bisP. To be consistent with the aromatic incorporation data (*1*), methylglyoxal would have to be generated as an intact unit from carbons 4, 5, and 6 of either of these molecules. This is consistent with the cleavage of fructose-6-P to Ga-3-P and dihydroxyacetone and fructose-1,6-bisP to Ga-3-P and DHAP. The cleavage is followed by the elimination of phosphate from Ga-3-P to form methylglyoxal. This elimination of phosphate is catalyzed by triose-P isomerase (*26*). If this process is indeed occurring, then the cells should contain methylglyoxal, and its level should increase when the cells extracts are incubated with added glucose-6-P, fructose-6-P, or fructose-1,6-P.

Analysis of Methylglyoxal in Cell Extracts. Incubating cell extracts with different precursors to methylglyoxal can point to the compound's source. Likely precursors will increase the amount of methylglyoxal present in the cell extracts. Analysis of cell extracts shows that they contained 0.07 mM methylglyoxal. Mass spectral analysis (DI-EI) of the derivative, a sample isolated from the cell extracts or prepared from a known sample of methylglyoxal, shows the same mass spectra with an $M^+ = 372$ *m/z*. The derivatized samples also have the same expected UV $\lambda_{max} = 275$ nm and have the same retention time and peak patterns upon HPLC analysis. Incubation of a cell extract with no added precursors increased the level of methylglyoxal to 0.19 mM. Including 10 mM glucose-6-P in the incubation mixture increased the level of methylglyoxal to 0.80 mM, whereas incubation with 10 mM fructose-1,6-bisP increased the level of methylglyoxal to 2.10 mM. After heating a 3.3 mM solution of Ga-3-P in the TES extraction buffer at pH 7.5 at 60 °C for 10 min, 29% of its phosphate was released. Including the cell extract at a concentration of 1.5 mg of protein/mL resulted in the release of 40% of the phosphate. The difference between the two values corresponded to a specific activity of 0.55 nmol/min/mg protein.

DISCUSSION

DKFP can be generated by the condensation of methylglyoxal with a dihydroxyacetone-P fragment that originates from either fructose-1-P or fructose-1,6-bisP in *M. jannaschii* cell extracts. This condensation reaction is catalyzed by the gene product of the MJ1585 gene, a Class I aldolase that also catalyzes the aldolase cleavage of fructose-1,6-bisP. The presence of methylglyoxal in the cell extracts was confirmed and, as previously shown, is expected to be generated in part from Ga-P-3 catalyzed by the triose-3-P isomerase (MJ1528). The concentration of methylglyoxal found in the cell is sufficient to account for the observed biosynthesis of the aromatic amino acids and lactate for F_{420} biosynthesis. This condensation reaction represents the first example of methylglyoxal serving as a primary metabolic precursor. Alternate pathways for the generation of DKFP involving the dehydration of an NDP-hexose were eliminated on the basis of the direct testing of recombinantly produced proteins. Most of these enzymes did not catalyze the expected reactions, opening up the important question of exactly what these enzymes do in *M. jannaschii* metabolism.

The methylglyoxal used for the production of DKFP is derived from both the thermal decomposition of a triose-P and from at least one specific enzymatic reaction. Ga-3-P is exceedingly unstable when heated at 60 °C in neutral buffer, producing methylglyoxal and phosphate. This agrees with the previously reported instability of Ga-3-P samples heated at 39 °C (*25*, *26*). No gene is annotated in the genome of *M. jannaschii* as a methylglyoxal synthase (EC 4.2.3.3) (*27*), indicating that DHAP, derived from Ga-3-P, is not likely the source. This is further confirmed because the methyl group of the methylglyoxal produced in *M. jannaschii* is derived from C-6 of glucose-6-P, whereas the methylglyoxal produced from glucose-6-P via DHAP and Ga-3-P is derived from C-1 (*27*). The route to methylglyoxal from acetoacetate (*28*) is eliminated because no oxygen is present during the growth of *M. jannaschii*. In addition, lactaldehyde is derived from methylglyoxal, and the cells contain no free aminopropan-2-ol (White, R. H., unpublished). Therefore, methylglyoxal may arise enzymatically and exclusively from Ga-3-P via the elimination of phosphate catalyzed by triose-P isomerase (*26*). One could also consider that on the basis of mechanistic grounds, another enzyme could function to catalyze this reaction, such as one of the two enolases known to be present in the *M. jannaschii* genome. Because *M. jannaschii* does not have any of the known enzymatic systems to remove methylglyoxal (*49*), it must capture and use the thermally and enzymatically generated methylglyoxal product to make aromatic amino acids, 6-deoxypolyols, and L-lactate.

The scheme shown in Figure 5 represents the pathways for the metabolism of DKFP and methylglyoxal in *M. jannaschii*. Reactions with bold structures are directly related to DKFP biosynthesis. First, glucose-6-P converts into fructose-6-P, catalyzed by phosphoglucose isomerase (MJ1605). In the presence of ATP, the fructose-6-P is converted into fructose-1,6-P, which, through the action of a presently unknown phosphatase, produces fructose-1-P. A portion of the fructose-6-P or fructose-1,6-P is then cleaved to Ga-3-P; this reaction is catalyzed by the MJ1585 gene product. The resulting Ga-3-P is then converted either nonenzymatically (*25*, *50*) or by triose-P isomerase (MJ1528) (*26*) to methylglyoxal. Methylglyoxal would then react either via a transaldolase reaction with fructose-1-P or fructose-1,6-bisP to form DKFP or would be reduced to L-lactaldehyde. The L-lactaldehyde can be oxidized to lactate by the product of the MJ1411 gene, can be condensed with DHAP to form 6-deoxysorbose-1-P or fuculose-1-P, or can react via a transaldolase reaction with fructose-1-P to form 6-deoxysorbose-1-P. Whether the 6-deoxysorbose-1-P is oxidized to DKFP is currently unknown, but fuculose-1-P could be the source of the 1-deoxygalactitol and 1-deoxy-

talitol or their 6-phosphate esters found in the cells after acid hydrolysis.

These reactions can substitute, in the archaea, for the two-enzyme glyoxalase system used for the removal of toxic methylglyoxal in other organisms (*22*). This would explain the absence of this enzyme system in the archaea, which do not have the genes glyoxalase I and II, methylglyoxal reductase, and methylglyoxal dehydrogenase. The absence of glyoxalase I and II is also consistent with the absence of gluthionine in the archaea. The question now is why the cells do not use the methylglyoxal synthase enzyme to generate the methylglyoxal from DHAP. This could be because methylglyoxal is produced in such a large amount in thermophiles, that the real problem these cells have to address is how to remove it instead of how to make it more easily. Using methylglyoxal as an intermediate to DKFP and, ultimately, the aromatic amino acids could be the archaea's solution to the over abundance of methylglyoxal and the lack of any other means of eliminating the compound.

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