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Identification of Lactaldehyde Dehydrogenase in *Methanocaldococcus jannaschii* and Its Involvement in Production of Lactate for F_{420} Biosynthesis

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One of the early steps in the biosynthesis of coenzyme F_{420} in *Methanocaldococcus jannaschii* requires generation of 2-phospho-L-lactate, which is formed by the phosphorylation of L-lactate. Preliminary studies had shown that L-lactate in *M. jannaschii* is not derived from pyruvate, and thus an alternate pathway(s) for its formation was examined. Here we report that L-lactate is formed by the NAD^+-dependent oxidation of L-lactaldehyde by the MJ1411 gene product. The lactaldehyde, in turn, was found to be generated either by the NAD(P)H reduction of methylglyoxal or by the aldol cleavage of fuculose-1-phosphate by fuculose-1-phosphate aldolase, the MJ1418 gene product.

Coenzyme F_{420} is an important cofactor involved in hydride transfer reactions in methanogenic archaea as well as methanotrophic and other bacteria. Although coenzyme F_{420} contains a deazaflavin moiety, it is biochemically analogous to the nicotinamide cofactors. Coenzyme F_{420} is involved in a variety of biochemical transformations, including methanogenesis (12), DNA photorepair (16), and degradation of nitrophenols (15) and nitroimidazofurans (49), and in the biosynthesis of several secondary metabolites (44).

The biosynthetic pathway for the generation of the side chain of coenzyme F_{420} begins with the formation of 2-phospho-L-lactate from L-lactate by a presently unknown kinase. The 2-phospho-L-lactate is then condensed with GTP to form lactyl (2)diphospho-(5′)guanosine (LPPG) and pyrophosphate (21). The second component of F_{420}, Fo, is generated from 4-hydroxyphenylpyruvate and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione by CofGH (20). F_{420}-0 is formed from the condensation of lactyl-phosphate (from LPPG) with Fo by 2-phospho-L-lactate transferase (CofD) (24). F_{420} biosynthesis then proceeds through the addition of two γ-linked glutamate residues by CofE, forming F_{420}-2 (33). Finally, CofF catalyzes the ligation of a final α-linked glutamate residue to the terminal γ-linked glutamate to generate F_{420} (34).

Establishing the biosynthetic origin of L-lactate used in the formation of F_{420} in archaea (23, 24, 33, 34) has been challenging. Although this central metabolite is quite small and simple in structure, a number of experiments in our laboratory were unable to initially identify the metabolic origins of the lactate in *Methanocaldococcus jannaschii*. There are several known biochemical pathways leading to lactate. Previous attempts to establish its origin via reduction of pyruvate proved negative (25). Several recombinant enzymes from *M. jannaschii* were tested for this activity, and none were found to work, despite the fact that one of the enzymes was annotated as a lactate dehydrogenase (25). One of these enzymes, MdhI, encoded by MJ1425, was found to catalyze the formation of lactate but only at very low levels when incubated with high concentrations of pyruvate (22).

Here we report the identification of the pathway for the formation of lactate in *M. jannaschii*. Lactate was found to be formed by the NAD-dependent oxidation of lactaldehyde catalyzed by the MJ1411 gene product, lactaldehyde dehydrogenase. In addition, two pathways for lactaldehyde formation have been identified in *M. jannaschii*. The first route involves the NADPH reduction of methylglyoxal. The second route involves the cleavage of L-fuculose-1-phosphate by an aldolase, encoded by MJ1418, to dihydroxyacetone phosphate and lactaldehyde. A homologous enzyme has been shown to be involved in fucose metabolism in *Escherichia coli* (43).

MATERIALS AND METHODS

Chemicals. DL-[2,3,3,3-$^2H_4$]alanine (98% ^2H) and pyruvate (99% $^{13}C_3$) were obtained from Cambridge Isotope Laboratories. All other chemicals, including L-fucitol, were from Sigma-Aldrich unless otherwise noted.

Synthesis of DL-lactaldehyde. Pyruvic aldehyde dimethyl acetal (1.18 g, 10 mmol) was dissolved in 1 ml of methanol, and 0.4 g of $NaBH_4$ was added in small portions over 10 min to the stirred solution with ice cooling. The resulting solution was then stirred for 1 h at room temperature and the methanol removed by evaporation with a stream of nitrogen gas. The resulting DL-lactaldehyde dimethyl acetal was dissolved in 4 ml methylene chloride and washed with 2 M aqueous $NaHCO_3$ (2 ml), and the methylene chloride layer was separated and dried over Na_2SO_4 to yield 0.7 g of a colorless oil. Data for ^1H nuclear magnetic resonance (NMR; 400 MHz) ($DCCl_3$) referenced to tetramethylsilane are as follows: δ 1.194 (3H, d, $J_{H\text{-}3\rightarrow H\text{-}2}$ = 6.44 Hz, H-3), 2.313 (1H, d, $J_{H\text{-}1\rightarrow H\text{-}2}$ = 3.32 Hz, H-1), 3.432 (3H, s, OCH_3), 3.461 (3H, s, OCH_3), 3.76 (1H, m, H-2), 4.08 (1H, d, $J_{OH\rightarrow H\text{-}2}$ = 6.25 Hz, OH). The resulting lactaldehyde dimethylacetal (4 ml of a 0.3 M aqueous solution) was deprotected by incubation with 2 ml Dowex 50W-X8 H^+ 200- to 400-mesh resin for 18 h at room temperature (29). A small amount of the resulting solution (10 μl) was diluted into 600 μl D_2O containing 0.436 μmol sodium 3-trimethylsilylpropionate-2,2,3,3-d_4 (TMS) to confirm deprotection of the dimethylacetal and to determine the concentration of lactaldehyde. Data for ^1H NMR (400 MHz) (D_2O) are as follows: δ 1.18 (3H, d, $J_{H\text{-}3\rightarrow H\text{-}2}$ = 6.3 Hz, H-3), 3.67 (1H, dq, $J_{H\text{-}2\rightarrow H\text{-}3}$ = 6.3 Hz, $J_{H\text{-}2\rightarrow H\text{-}1}$ = 6.3 Hz, H-2), ~4.8, obscured by DOH (1H, d, H-1). Substituting $NaBD_4$ in the above synthesis produced [2-^2H]lactaldehyde.

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Synthesis of DL-[2,3,3,3-$^2H_4$]lactate. DL-[2,3,3,3-$^2H_4$]alanine (93 mg, 1 mmol) and 23 mg of sodium nitrite were dissolved in 0.4 ml of water and cooled to 0°C in an ice bath. To this solution three 60-μl portions of 6 M HCl were added at 10-min intervals with stirring. The sample was then stirred for 2 h at room temperature and diluted to 10 ml with water. Isotopic dilution analysis using a known sample of sodium lactate demonstrated the concentration of DL-[2,3,3,3-$^2H_4$]lactate in the sample was 0.0285 M, showing that 28.5% of the alanine had been converted into lactate. This sample was then used for the isotopic dilution analysis of lactate in the incubation mixtures.

Preparation of 6-deoxy-L-talitol and fucitol. 6-Deoxy-L-talitol and fucitol were prepared for use as standards in the gas chromatography-mass spectrometry (GC-MS) identification of fuculose-1-phosphate aldolase reaction products. 6-Deoxy-L-talitol was prepared by the epimerization of L-fucose with molybdic acid following the procedure of Bilik et al. and Defaye and Gadelle (3, 11). Thus, 1 mg of molybdic acid and 50 mg of L-fucose were dissolved in 150 μl of water, and the resulting pH 4.0 solution was heated at 95°C for 3 h. To the resulting blue solution was added 3 to 4 mg $NaBH_4$, and after incubation for 3 h at room temperature the sample was acidified with 1 M HCl and the methanol repeatedly evaporated (three times, 0.5 ml) to remove the borate as the volatile trimethyl borate ester. The resulting sample was desalted by passing the solution first through a column of Dowex 50W-X8 H^+ (2.5 by 5 mm) and then through a column of Dowex 1-X8 OH^- (2.5 by 5 mm). After evaporation of the solvent, the resulting polyols were reacted with TMS reagent prior to GC-MS analysis as described below. GC-MS analysis showed a 9:1 mixture of fucitol and 1-deoxy-L-talitol.

Preparation of *M. jannaschii* cell extracts. A cell extract of *M. jannaschii* was prepared anaerobically by sonication of 4.67 g of frozen cells suspended in 10 ml of TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (50 mM TES/K^+, 10 mM $MgCl_2$, pH 7.5) under argon for 5 min at 3°C. *M. jannaschii* cells were grown as previously described (38). The resulting mixture was centrifuged under argon (27,000 × *g*, 10 min) and stored frozen under argon at −20°C until used. The protein concentration of the *M. jannaschii* extract was 38 mg/ml. Protein concentrations were measured using the bicinchoninic acid total protein assay (Pierce) with bovine serum albumin as a standard.

Isotopic dilution analysis of lactate. To 50 μl of *M. jannaschii* cell extract was added 5 μl of a 0.1 M solution of precursor (pyruvate with NADH, L-malate, acrylate, acrylate with coenzyme A [CoA] and ATP, acrylyl-CoA, propionyl-CoA, or phosphoenolpyruvate). The samples were incubated for 30 min at 70°C in aerobic or anaerobic conditions under argon or hydrogen. Incubation mixtures were mixed with a known amount of DL-[2,3,3,3-$^2H_4$]lactate and combined with 1.6 volumes of methanol (80 μl) and centrifuged (14,000 × *g*, 10 min). The methanol layer was separated and evaporated, and the residue was dissolved in 50 μl of water. Any insoluble material was removed by centrifugation (14,000 × *g*, 1 min) prior to passing the sample through a Dowex 50W-X8 H^+ column (2 by 5 mm). The desalted solution was evaporated to dryness, reacted with 20 μl of the TMS reagent to form the $(TMS)_2$ derivative, and subjected to GC-MS analysis as described below. From the measured ratios of the intensities of the $M^+ - 15$ ion at *m/z* 219 for the unlabeled lactate and *m/z* 223 for the DL-[2,3,3,3-$^2H_4$]lactate the amount of lactate present in the samples was accurately calculated.

NAD-dependent oxidation of DL-lactaldehyde by *M. jannaschii* cell extracts. To 50 μl of *M. jannaschii* cell extract contained in a centrifuge tube were added 5 μl of 0.1 M DL-lactaldehyde and 5 μl of 0.1 M NAD. The sample was incubated for 15 min at 70°C under an argon atmosphere. At the conclusion of the incubation 5 μl 0.0285 M DL-[2,3,3,3-$^2H_4$]lactate was added and the lactate produced measured by isotopic dilution analysis of the TMS derivative by GC-MS as described above.

NAD-dependent reduction of methylglyoxal to lactaldehyde in *M. jannaschii* cell extracts. To 50 μl of *M. jannaschii* extract were added 5 μl of 0.1 M methylglyoxal and 5 μl of a solution 0.05 M in both NADH and NADPH. The sample was incubated for 20 min at 70°C and mixed with 40 μl of 1 M sodium acetate buffer, pH 4.0, and 20 μl of 0.1 M *O*-(4-nitrobenzyl)hydroxylamine. The samples were heated at 100°C for 20 min, after which time 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 methanol, and purified by preparative thin-layer chromatography (TLC) using methylene chloride-methyl acetate (1:1, vol/vol) as the eluting solvent. The area of the plate containing the *O*-(4-nitrobenzyl)hydroxylamine derivative (R_f = 0.56), made visible by exposing the TLC plates to UV light, was removed and the derivative eluted from the support with 100 μl methanol. The sample had the expected UV λ_{max} of 275 nm and was quantitated by measuring its absorbance at 275 nm. A known sample of the *O*-(4-nitrobenzyl)hydroxylamine derivative of lactaldehyde has an R_f of 0.56, while the acetol derivative had an R_f of 0.48. Mass-spectral analysis of the TMS derivative of authentic lactaldehyde as well as the sample produced identical mass spectra showing a molecular ion at $M^+ = 281$ *m/z*.

Condensation of lactaldehyde with DHAP. To 100 μl of *M. jannaschii* cell extract were added 10 μl of 0.1 M DL-lactaldehyde and 10 μl of 0.1 M dihydroxyacetone phosphate (DHAP), and the sample was incubated for 15 min at 70°C under argon in a 1.5-ml capped microcentrifuge tube. After the sample was cooled to room temperature, 2 to 3 mg of $NaBH_4$ was dissolved in the sample, which was then incubated for 30 min at room temperature. Following incubation, 240 μl of methanol was added and the sample centrifuged (14,000 × *g*, 10 min) to remove insoluble material. The methanol layer was then acidified with the addition of 10 μl of acetic acid, and the methanol and water were removed by evaporation with a stream of nitrogen gas. The sample was dissolved in methanol (500 μl) and evaporated an additional three times to remove the borate as the volatile trimethyl borate ester. The sample was dissolved in 100 μl of water and 50 μl glycine buffer (0.1 M glycine, pH 10.4, 1 mM zinc acetate, and 1 mM $MgCl_2$). Alkaline phosphatase (0.2 units) was added, and the sample was incubated overnight at 37°C. The resulting sample was desalted by passing the solution first through a column of Dowex 50W-X8 H^+ 200 to 400 mesh (2.5 by 5 mm) and then through a column of Dowex 1-X8 OH^- 200 to 400 mesh (2.5 by 5 mm). After evaporation of the water the resulting polyols were reacted with TMS reagent prior to GC-MS analysis as described below.

Formation of TMS derivatives. Samples to be assayed as TMS derivatives were dried by evaporation with a stream of nitrogen gas while being held at 100°C in a water bath. The resulting residue was reacted with 30 μl of a mixture of pyridine, hexamethyldisilazane, and chlorotrimethylsilane (9:3:1, vol/vol/vol) for 2 min at 100°C.

GC-MS confirmation of reaction products. GC-MS spectra of standards and samples were obtained using a VG-70-70EHF gas chromatography-mass spectrometer operating at 70 eV and equipped with a VF-5ms column (0.32 mm by 30 m) programmed from 80°C to 280°C at 8°C per min. Under the GC-MS conditions used the indicated derivatives of the following compounds had the following retention times (parentheses; min) and mass-spectral data (brackets; molecular ions [base peaks are underlined], with the most abundant ions with *m/z* over 100 listed in order of decreasing intensities): 1,2-propanediol (from lactaldehyde) $(TMS)_2$, (2.67) [220, 117, 221, 205, 263]; lactate $(TMS)_2$, (5.17) [234, 117, 147, 219]; glycerol $(TMS)_3$, (6.72) [308, 205, 103, 117, 218, 147]; 1-deoxyallitol $(TMS)_5$ derivative, (14.93) [526, 205, 319, 217, 219, 307]; 6-deoxyaltitol $(TMS)_5$ derivative, (15.11) [526, 205, 319, 219, 217, 231]; 1-deoxygalactitol (fucitol) $(TMS)_5$ derivative, (15.36) [526, 117, 217, 205, 319, 219, 231]; and 6-deoxytalitol $(TMS)_5$ derivative, (15.43) [526, 117, 217, 205, 319, 219, 231]. The fucitol and 6-deoxytalitol peaks were confirmed by coinjection of known standards. The tentative assignments for the 6-deoxyaltitol and 1-deoxyallitol peaks were based on the assumption that the elution order is the same as the corresponding C-2 isomers.

Cloning and expression of the MJ0712, MJ1411, and MJ1418 proteins in *E. coli*. The *M. jannaschii* genes at loci corresponding to Swiss-Prot accession numbers Q58806 for MJ1411 (lactaldehyde dehydrogenase), Q58813 for MJ1418 (fuculose-1-phosphate aldolase), and Q58122 for MJ0712 (glycerol-1-phosphate dehydrogenase) were amplified by PCR from genomic DNA using oligonucleotide primers MJ1411-Fwd (5-GGTCATATGTTCATAGATG-3′), MJ1411-Rev (5′-GCTGGATCCTTATTTTGAGATTATTATTG-3′), MJ1418-Fwd (5′-GGTCATATGGACAAAAAG-3′), MJ1418-Rev (5′-GCTGGATCCCTATTTCTTTAC-3′), MJ0712-Fwd (5′-GGTCATATGATTATAGTCAC-3′), and MJ0712Rev (5′-GCTGGATCCTTAAATAACTCCTG-3′). PCR was performed as described previously using annealing temperatures of 45°C for MJ1411 and 50°C for MJ1418 and MJ0712 (19). The amplified PCR products were purified on a QIAquick spin column (QIAGEN), digested with the appropriate restriction enzymes, and ligated into the compatible sites in plasmid pT7-7(USB). The structure of the resulting plasmids (pMJ1411, pMJ1418, and pMJ0712) was confirmed by sequencing. The resulting plasmids were transformed into *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene). The 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 at 600 nm of 1.0. Recombinant-protein production was induced by addition of lactose to a final concentration of 28 mM. After an additional 2 h of culture, the cells were harvested by centrifugation (4,000 × *g*, 5 min) and frozen at −20°C.

Purification of recombinant protein. *E. coli* cells (~0.5 g wet weight) expressing recombinant glycerol-1-phosphate dehydrogenase, lactaldehyde dehydrogenase, or fuculose-1-phosphate aldolase were resuspended in 4 ml extraction buffer (50 mM TES, pH 7.0, 10 mM $MgCl_2$, 20 mM dithiothreitol) and lysed by sonication, and the insoluble proteins were removed by centrifugation

(14,000 × *g*, 10 min). The soluble cell extract obtained was then incubated at 80°C for 10 min to precipitate *E. coli* proteins, which were removed by centrifugation (14,000 × *g*, 10 min). The resulting heat-soluble lactaldehyde dehydrogenase and fuculose-1-phosphate aldolase solutions were further purified by anion-exchange chromatography on a MonoQ HR column (1 by 8 cm; Amersham Bioscience) with a linear gradient from 0 to 1 M NaCl in 25 mM Tris, pH 7.5, over 55 ml at 1 ml/min. Lactaldehyde dehydrogenase and fuculose-1-phosphate aldolase eluted as single peaks centered at 0.35 M and 0.3 M NaCl, respectively. Both MJ1411- and MJ1418-derived proteins were >95% pure as judged by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with Coomassie blue staining. Protein concentrations were determined using the Bradford protein assay (4).

Assay for lactaldehyde dehydrogenase. Lactaldehyde dehydrogenase assays were performed at room temperature in 1 ml of 100 mM TES, pH 7.5, buffer containing 2.5 mM NAD and 1 mM aldehyde substrate and were initiated with the addition of 9 μg lactaldehyde dehydrogenase. Increase in absorbance at 340 nm, corresponding to the formation of NADH, was measured on a Shimadzu UV-1601 UV-visible spectrophotometer over 10 minutes. For kinetic assays, substrate aldehyde concentration was varied from 0.13 mM to 3 mM with 1 μg lactaldehyde dehydrogenase. Kinetic parameters were estimated from the slope and intercepts of the Lineweaver-Burk plot using Microsoft Excel software.

Confirmation and quantitation of lactaldehyde dehydrogenase reaction product. Lactaldehyde dehydrogenase (9 μg) was incubated with 10 mM DL-lactaldehyde and 10 mM NAD in 100 μl of 100 mM TES for 1 h. At the conclusion of the incubation 25 μl of 0.0285 M DL-[2,3,3,3-$^2H_4$]lactate was added and the lactate measured by isotopic dilution analysis GC-MS of the $(TMS)_2$ derivative as described above.

Assay for L-fuculose-1-phosphate aldolase. Recombinant fuculose-1-phosphate aldolase (0.2 μg; MJ1418) was incubated with 5 mM DHAP and 5 mM aldehyde in 30 mM TES, pH 8.0, in a final volume of 500 μl for 30 min at 70°C. Following incubation 100 μl of the reaction mixture was combined with 2 μl 0.1 M NADH and 1 unit α-glycerophosphate dehydrogenase (Sigma G6751) in a final volume of 1 ml 25 mM TES, pH 7.4, and the decrease in absorbance at 340 nm, corresponding to the DHAP-dependent consumption of NADH, was measured. GC-MS confirmation of L-fuculose-1-phosphate aldolase reaction products was performed by TMS derivation of the reduced, dephosphorylated products as described above. Alternate substrates tested in addition to lactaldehyde were propionaldehyde, glyceraldehyde, glycolaldehyde, crotonaldehyde, acetaldehyde, acrolein, pyruvaldehyde, formaldehyde, and methylglyoxal.

Assay for glycerol-1-phosphate dehydrogenase. Recombinant glycerol-1-phosphate dehydrogenase (MJ0712; partially purified heat-soluble protein) was incubated with 0.5 mM DHAP, 0.5 mM NADH, and 60 mM KCl in 60 mM TES, pH 8.0, in a final volume of 1 ml. The rate of NADH consumption was monitored at 340 nm following the addition of enzyme. Alternate substrates tested include methylglyoxal and dihydroxyacetone.

Temperature stability of lactaldehyde dehydrogenase. Heat stability of lactaldehyde dehydrogenase was determined by incubating the enzyme in 100 mM TES, pH 7.5, at 25, 80, 90, and 100°C for 10 min. Following heat treatment, the enzyme solution was cooled on ice and activity was measured using the standard assay described above.

Inhibition and activation of lactaldehyde dehydrogenase. Lactaldehyde dehydrogenase (9 μg) was incubated with either 3 mM *N*-ethylmaleimide, 3 mM iodoacetamide, and 1 mM fructose-1-phosphate or 1 mM fructose-6-phosphate and 2.5 mM NAD in 100 mM TES buffer, pH 7.5, for 5 min at room temperature. Following incubation, the reaction was initiated through the addition of 1 mM lactaldehyde and the increase in absorbance at 340 nm was monitored.

Measurement of the native molecular weight. The native molecular weights for L-fuculose-1-phosphate aldolase and lactaldehyde dehydrogenase were determined by size exclusion chromatography on a Superose 12HR column with buffer containing 50 mM HEPES, pH 7.2, 150 mM NaCl, and 1 mM EDTA at 0.5 ml/min and detection at 280 nm. Protein standards used to generate the standard curve included alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa).

pH optimum of the recombinant enzymes. The activities of lactaldehyde dehydrogenase and fuculose-1-phosphate aldolase were determined at 0.5 pH increments between pH 5 and 10 using a three-component buffer system (45) consisting of 75 mM Bis-Tris, 38 mM HEPPS [4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid], and 38 mM CHES [2-(cyclohexylamino)ethanesulfonic acid] in place of TES buffer in the standard assays.

RESULTS AND DISCUSSION

Origin of lactate and lactyl-phosphate in *M. jannaschii*. As discussed in the introduction, the most obvious pathway to lactate, the NADP-dependent reduction of pyruvate, proved not to be the route utilized in *M. jannaschii* (25). As this work progressed several different routes to lactate, needed for F_{420} biosynthesis, were considered. Possible routes considered include the formation of lactate from the reverse-Michael addition of water to acrylic acid or acrylyl-CoA. This pathway has been observed in *Clostridium propionicum*, which has been shown to reduce lactate to propionate via acrylate and a proposed acrylyl-CoA intermediate (30). The cleavage of malate to lactate, as occurs in malolactic enzyme, is another possible source of lactate (7, 50). Using isotope dilution analysis, *M. jannaschii* cell extract was tested for the formation of lactate from pyruvate and NADH, L-malate, acrylate, acrylate with CoA and ATP, acrylyl-CoA, or propionyl-CoA, under aerobic and anaerobic conditions under hydrogen or argon. An increase in lactate above the background level of ~10 nmol/mg protein was not observed in any of the experiments (data not shown). Reduction of phosphoenolpyruvate to lactyl-phosphate was also tested by GC-MS analysis of lactyl-phosphate-$(TMS)_3$; however, an increase in lactyl-phosphate was not observed (data not shown). Several recombinant enzymes likely to generate lactate were also tested for these activities, and none worked despite the fact that one of the enzymes was annotated as lactate dehydrogenase (25). Similar studies with *Methanococcus maripaludis* also failed to detect any lactate dehydrogenase activity in that organism (57).

The lack of an obvious enzyme or pathway for the formation of L-lactate or 2-phospho-L-lactate in *M. jannaschii* led us to evaluate other potential sources. One possible route to the formation of lactate that had not been previously investigated was the oxidation of lactaldehyde. An NAD-dependent lactaldehyde dehydrogenase has been identified in a number of organisms, where it is involved in the metabolism of deoxysugars (1, 8, 18).

In order to determine if lactaldehyde was a possible source for lactate in *M. jannaschii*, cell extracts were incubated for 15 min with 10 mM DL-lactaldehyde and 10 mM NAD. Analysis of the incubation mixture by isotopic dilution showed that 28.6% of the DL-lactaldehyde had been oxidized to lactate during the incubation, corresponding to a specific activity of 9.5 nmol/min/mg protein. The lactaldehyde used was a mixture of both D and L isomers. Presumably only the L configuration is utilized, as it corresponds to the correct stereochemistry of the lactyl-phosphate in F_{420}.

The *M. jannaschii* genome was searched for genes with homology to the *E. coli* lactaldehyde dehydrogenase gene (NP_418045). The closest match in *M. jannaschii* was found to be MJ1411 (NP_248414), encoding an enzyme annotated as a NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (gapN). The MJ1411 gene product is a member of the aldehyde dehydrogenase protein family (pfam00171) and the NAD-dependent aldehyde dehydrogenase conserved orthogonal group (COG1012, PutA) (37). The MJ1411 gene product was one of the 963 proteins identified in a shotgun proteomics study of *M. jannaschii* (58).

The MJ1411 gene was cloned and heterologously expressed,

TABLE 1. Kinetic parameters of *M. jannaschii* lactaldehyde dehydrogenase with lactaldehyde and propionaldehyde substrates

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
DL-Lactaldehyde	302	2	7×10^3
Propionaldehyde	284	3	1×10^4

and the purified protein was shown to catalyze the formation of lactate from lactaldehyde, with NAD serving as the oxidant. The specific activity of recombinant lactaldehyde dehydrogenase was 600 nmol/min/mg protein with lactaldehyde as substrate under the standard assay conditions. The formation of lactate by the recombinant gene product was confirmed by GC-MS analysis of the reaction product. Kinetic parameters for *M. jannaschii* lactaldehyde dehydrogenase were nearly identical with propionaldehyde and DL-lactaldehyde substrates (Table 1). It is important to note that the lactaldehyde used was a mixture of the D and L isomers. Consequently, the kinetic parameters do not reflect the possible stereospecificity of the enzyme.

Unlike the lactaldehyde dehydrogenase purified from *Saccharomyces cerevisiae* (26, 27, 39), *M. jannaschii* lactaldehyde dehydrogenase showed relatively broad substrate specificity and was able to use a variety of aldehyde substrates. *M. jannaschii* lactaldehyde dehydrogenase was found to utilize DL-glyceraldehyde and crotonaldehyde as substrates, albeit with lower levels of activity than observed with lactaldehyde, and exhibited even less activity with glycolaldehyde, acetaldehyde, acrolein, and formaldehyde (Table 2). This relaxed substrate specificity is more similar to the *E. coli* lactaldehyde dehydrogenase than to the *S. cerevisiae* enzyme (1, 48). *M. jannaschii* lactaldehyde dehydrogenase exhibited a clear preference for NAD over NADP and had only 20% activity with NADP when propionaldehyde was used as the substrate and 37% activity with lactaldehyde as the substrate. Although MJ1411 is annotated as an NADP-dependent glyceraldehyde-3-phosphate dehydrogenase gene in GenBank, *M. jannaschii* lactaldehyde dehydrogenase was not capable of reducing DL-glyceraldehyde-3-phosphate. Additionally, while *M. jannaschii* lactaldehyde dehydrogenase was capable of utilizing propionaldehyde and glyceraldehyde, the *Thermoproteus tenax* glyceraldehyde-3-phosphate dehydrogenase was not found to utilize these aldehyde substrates (5).

Properties of *M. jannaschii* lactaldehyde dehydrogenase. In addition to being more closely related to *E. coli* lactaldehyde dehydrogenase in terms of sequence similarity, *M. jannaschii* lactaldehyde dehydrogenase was also more similar to the *E. coli* enzyme than the *S. cerevisiae* enzyme in many of its physical properties. *M. jannaschii* lactaldehyde dehydrogenase showed the highest level of activity at pH 9, and activity remained level to pH 10.0, the highest pH tested. Below pH 9, the activity gradually declined, and only 17% of optimal activity was observed at pH 7.0. Lactaldehyde dehydrogenase was inactive at pH 6 or below. This is consistent with lactaldehyde dehydrogenase from *E. coli*, which showed activity only at high pH values and demonstrated no activity below pH 9.0 (48). Conversely, the yeast lactaldehyde dehydrogenase has a pH optimum at 6.5 (27). *M. jannaschii* lactaldehyde dehydrogenase activity was completely eliminated by the alkylating agents iodoacetamide and *N*-ethylmaleimide. *T. tenax* glyceraldehyde-3-phosphate dehydrogenase was found to be activated by the presence of fructose-1-phosphate and fructose-6-phosphate (5); however, activation of *M. jannaschii* lactaldehyde dehydrogenase by these sugar-phosphates was not observed. *M. jannaschii* lactaldehyde dehydrogenase has an estimated monomeric molecular mass of 50 kDa, as judged by SDS-polyacrylamide gel electrophoresis, corresponding well with the predicted molecular mass of 51 kDa. The *M. jannaschii* enzyme was found to have an estimated multimeric molecular mass of 180 kDa by gel filtration and is thus predicted to exist as a tetramer. This is also consistent with the *E. coli* enzyme (1). *M. jannaschii* lactaldehyde dehydrogenase was true to its thermophilic origins and retained 86% activity when heated to 80°C, 45% at 90°C, and 25% at 100°C for 10 minutes.

TABLE 2. Relative activity of *M. jannaschii* lactaldehyde dehydrogenase to catalyze NAD-dependent oxidation with various aldehydes

Substrate	Relative % activity[a]
Propionaldehyde	187
DL-Lactaldehyde	100
DL-Glyceraldehyde	97
Crotonaldehyde	84
Glycolaldehyde	35
Acetaldehyde	25
Acrolein	19
Formaldehyde	4
Methylglyoxal	ND[b]
Glyceraldehyde-3-phosphate	ND

[a] The measured specific activity at 100% was 600 nmol/min/mg protein. The detection limit was 8.4 nmol/min/mg protein.
[b] ND, not detected.

Comparison of *M. jannaschii* lactaldehyde dehydrogenase and other dehydrogenases. Although the primary protein sequence of the MJ1411 gene product is most similar to nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN; E.C.1.2.1.9), sequence alignment reveals some key differences (Fig. 1). The active-site residues were identified in the crystal structure of glyceraldehyde-3-phosphate dehydrogenase from *Thermoproteus tenax* (36), and sequence alignment allowed for the identification of the corresponding residues in *M. jannaschii* and other lactaldehyde dehydrogenases. The key *T. tenax* catalytic residues Cys297 and Glu263 are conserved in all four proteins. The cysteine residue is the nucleophile that forms the thiohemiacetal prior to substrate oxidation by NADP (36). The conserved glutamate residue has been proposed to be the general base needed for activation of the catalytic cysteine residue as well as the activation of water for deacylation in aldehyde dehydrogenases (51–53). The involvement of a catalytic cysteine in *M. jannaschii* lactaldehyde dehydrogenase was confirmed by complete inactivation of the enzyme by the alkylating agents iodoacetamide and *N*-ethylmaleimide.

Although the catalytic residues are conserved in the MJ1411 protein, several substrate binding residues are not. Notably, the polar, charged residues involved in binding the phosphate moiety of glyceraldehyde-3-phosphate (*T. tenax* GAPN Arg296, Asp298, and His455) are not conserved in the *M. jannaschii* enzyme or in other lactaldehyde dehydrogenases (Fig. 1) (36). *T. tenax* glyceraldehyde-3-phosphate dehydroge-

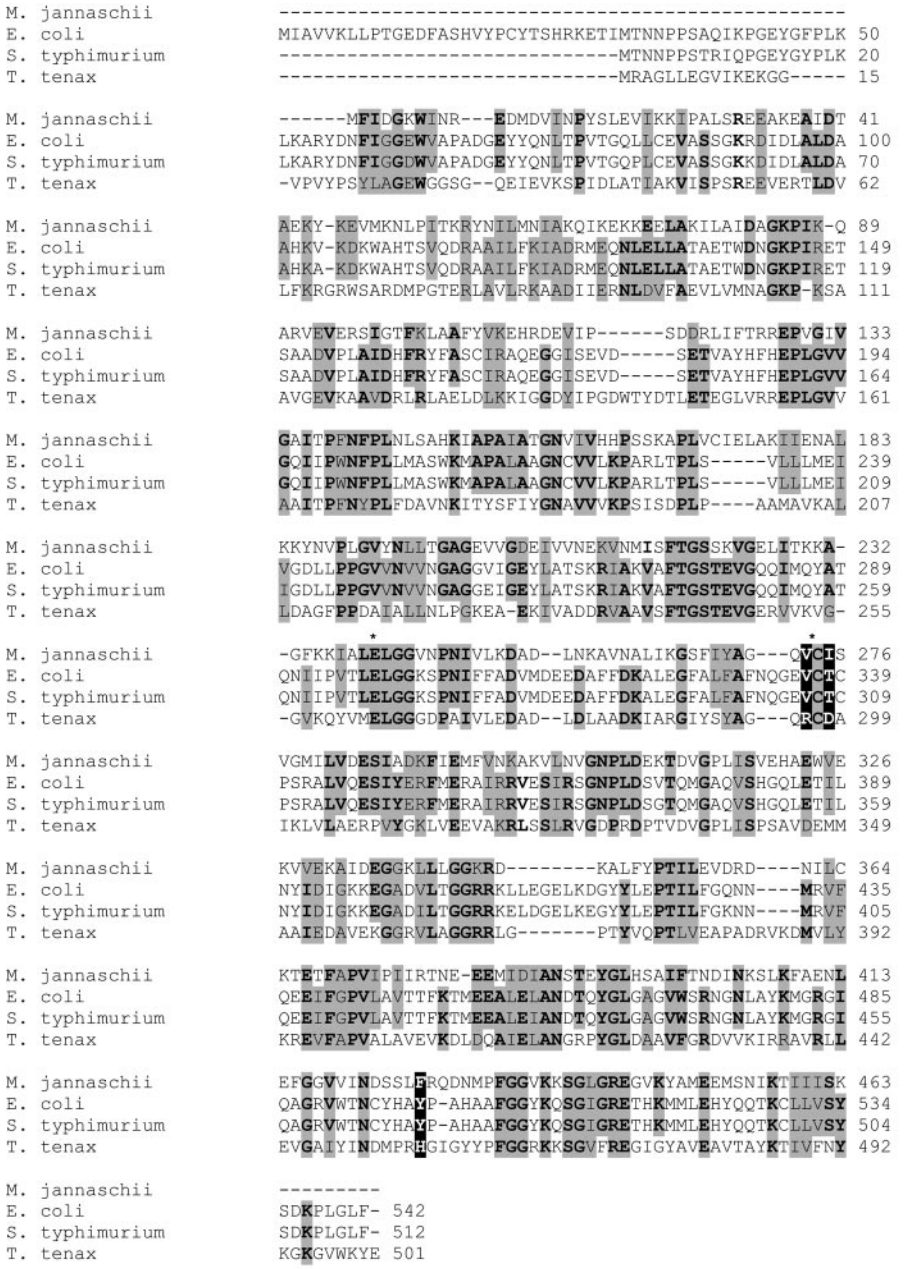


FIG. 1. Sequence alignment of *M. jannaschii* lactaldehyde dehydrogenase with lactaldehyde dehydrogenases from *E. coli* (encoded by *aldB*, NP_418045) and *Salmonella enterica* serovar Typhimurium (encoded by *aldB*, NP_462580) as well as *T. tenax* glyceraldehyde-3-phosphate dehydrogenase (T44939). Identical residues are shown in boldface, and similar residues are highlighted in gray. Conserved catalytic cysteine and glutamate residues are indicated with an asterisk. Residues proposed to be involved in glyceraldehyde-3-phosphate dehydrogenase substrate binding are highlighted in black.

nase contains an arginine residue at position 296 that is involved in binding the phosphate group of the substrate, while lactaldehyde dehydrogenases possess a valine at this position. The aspartate residue at position 298 of *T. tenax* glyceraldehyde-3-phosphate is also proposed to be involved in hydrogen bonding to the substrate phosphate; however, the lactaldehyde dehydrogenases contain either a threonine or an isoleucine at this position. These amino acid substitutions are consistent with the utilization of nonphosphorylated substrates in the lactaldehyde dehydrogenases. Another residue proposed to be involved in glyceraldehyde-3-phosphate dehydrogenase substrate binding is His455, which forms a hydrogen bond to the chiral C-2 of the substrate. Lorentzen et al. proposed that this residue is important in determining the stereoselectivity of the D substrate by glyceraldehyde-3-phosphate dehydrogenase (36). *E. coli* lactaldehyde dehydrogenase contains an aromatic residue at this position and preferentially binds L-lactaldehyde, supporting Lorentzen's proposal. The *M. jannaschii* lactaldehyde dehydrogenase also contains an aromatic residue at this position.

Metabolic origins of lactaldehyde. Although the conversion of lactaldeyde to lactate answered the initial question of the source of lactate for F_{420} biosynthesis; the biosynthetic origins of lactaldehyde in *M. jannaschii* still were not known. Biological origins of lactaldehyde may include isomerization of acetol, reduction of methylglyoxal, and/or the aldolase cleavage of fuculose-1-phosphate. Each of these possible precursors was tested for conversion to lactaldehyde in *M. jannaschii* cell extracts. *M. jannaschii* cells have been found to contain 48 μM acetol and 88 μM acetol-phosphate (54). Isomerization of acetol or the consecutive dephosphorylation, then isomerization, of acetol-phosphate could be a source of lactaldehyde. *M. jannaschii* cell extracts incubated with acetol, however, failed to isomerize it to lactaldehyde (data not shown).

The second proposed source of lactaldehyde is through reduction of methylglyoxal. Methylglyoxal has been shown to form chemically and enzymatically from glyceraldehyde-3-phosphate (46, 47), and the chemical transformation is accelerated at the high temperatures that are optimal for *M. jannaschii* growth. In addition, a methylglyoxal synthase has been detected in halophilic archaea (42). Methylglyoxal has been shown to be toxic to cells and has been proposed to be involved in the regulation of cellular division (26, 39). The methylglyoxal pathway is involved in the detoxification of methylglyoxal generated from dihydroxyacetone phosphate or the catabolism of threonine (40) and results in the formation of lactate from lactaldehyde (27). Analysis of *M. jannaschii* cell extracts showed the presence of 0.07 mM methylglyoxal, which would correspond to an intracellular concentration of 0.35 mM. In addition we found that methylglyoxal is readily formed from glyceraldehyde-3-phosphate by cell extracts with a specific activity of 5.5 nmol/min/mg protein (unpublished results). This high concentration of methylglyoxal suggested that it is a plausible precursor for lactaldehyde. Incubation of *M. jannaschii* cell extract with methylglyoxal and NADPH was found to result in the formation of lactaldehyde. This was established by the formation of the *O*-(4-nitrobenzyl)hydroxylamine derivative of generated lactaldehyde. The derivative was purified by preparative TLC and the amount quantitated by measuring the absorbance at 275 nm. Based on the amount of methylglyoxal added, a 7% yield of product was recorded. This corresponds to a specific activity of 1.9 nmol/min/mg protein.

Lactaldehyde has been shown to be produced from methylglyoxal by several enzymes including methylglyoxal reductase and glycerol dehydrogenase (6, 27, 35). A survey of the *M. jannaschii* genome did not reveal any genes encoding proteins with predicted sequence similarity to methylglyoxal reductase; however, a gene, MJ0712, was found that encodes a protein annotated as a glycerol dehydrogenase. Nishihara et al., however, predicted that this gene codes for a glycerol-1-phosphate dehydrogenase (41). Glycerol dehydrogenase from *Enterobacter aerogenes* was found to catalyze the NADH reduction of methylglyoxal to L-lactaldehyde at 56% of the level of activity observed with glycerol as a substrate (6). In order to test if the *M. jannaschii* glycerol dehydrogenase may be capable of a similar reduction, the MJ0712 gene was cloned and the recombinant protein overexpressed in *E. coli*. The partially purified protein was found to catalyze the NADH-dependent reduction of DHAP, as predicted by Nishihara et al. (41), but showed no activity with methylglyoxal or dihydroxyacetone as substrates. Thus, this gene product was confirmed to be a glycerol-1-phosphate dehydrogenase and is not apparently involved in the formation of lactaldehyde from methylglyoxal in *M. jannaschii*.

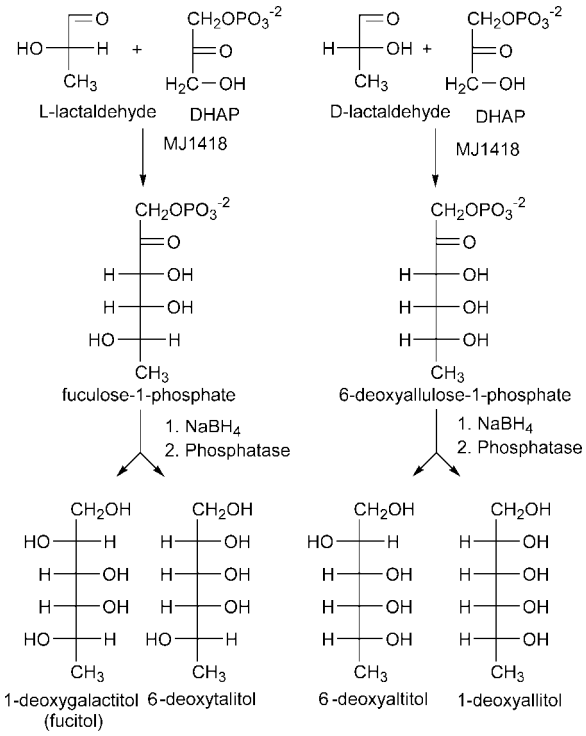
A second enzyme that was tested for the formation of lactaldehyde from methylglyoxal is malate dehydrogenase. MdhI from *M. jannaschii*, encoded by MJ0490, has been previously cloned and found to be a malate dehydrogenase with relaxed substrate specificity (25, 32). It is not uncommon for a dehydrogenase to exhibit relaxed substrate specificity (28), and so we hypothesized that the *M. jannaschii* MdhI may also be capable of reducing methylglyoxal to lactaldehyde; however, this activity was not observed.

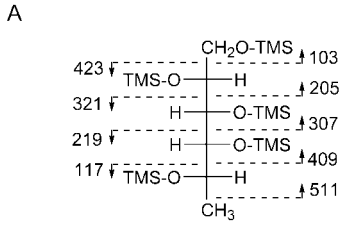
Aldolase cleavage of fuculose-1-phosphate to lactaldehyde and dihydroxyacetone phosphate. An alternate metabolic source of lactaldehyde that was considered is fuculose-1-phosphate, which could yield lactaldehyde through an aldolase type cleavage. Fuculose was detected as free fucitol in *M. jannaschii* cell extract following acid hydrolysis in 1 M HCl for 10 min at 100°C. In addition, 8 μM DL-fuculose-1-phosphate has been detected in *M. jannaschii* cells upon incubation with glucose-6-phosphate (R. H. White, unpublished results). Both of these observations indicate that fuculose-1-phosphate may be a plausible source of lactaldehyde. *M. jannaschii* cell extracts incubated with DHAP and DL-lactaldehyde were found to form fuculose-1-phosphate, detected by GC-MS of the reduced and dephosphorylated $(TMS)_5$ derivatives, with a specific activity of 0.04 nmol/min/mg protein. This indicates the presence of the desired activity.

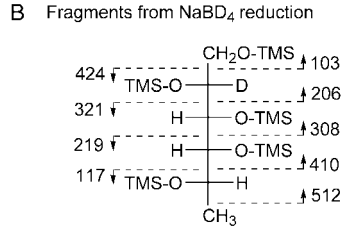
Characterization of the MJ1418 gene product and proof of structure and stereochemistry of L-fuculose-1-phosphate. A search of the *M. jannaschii* genome revealed a gene that was annotated as a fuculose-1-phosphate aldolase gene, MJ1418. MJ1418 has previously been cloned and the catalytic mechanism of the aldolase studied (9, 31, 56). In these studies glyceraldehyde was utilized as the acceptor substrate for the aldol condensation. We wanted to confirm that the MJ1418 gene product also utilized DL-lactaldehyde as predicted.

The MJ1418 gene was cloned and heterologously expressed in *E. coli*. The purified MJ1418 gene product was found to catalyze the condensation of lactaldehyde with dihydroxyacetone phosphate to generate four deoxyhexose sugars, which were detected as the $(TMS)_5$ derivatives by GC-MS. The four sugars arose from the condensation of DHAP with either D- or L-lactaldehyde, producing 6-deoxyallulose-1-phosphate and fuculose-1-phosphate (6-deoxytagatose-1-phosphate), respectively (Fig. 2). Reduction of these two ketoses with $NaBH_4$ and removal of the phosphates with phosphatase formed the four expected 6-deoxypolyols that were detected by GC-MS of their $(TMS)_5$ derivatives. The deoxyallitol and deoxyaltritol isomers arose from 6-deoxyallulose-1-phosphate, and the galactitol and talitol isomers arose from the fuculose-1-phosphate (Fig. 2). The identities of the galactitol and talitol peaks were confirmed by coinjection with authentic galactitol and a galactitol/talitol mixture, which was generated through the epimerization of fucose. That the reduction was in fact occurring at C-2 was confirmed by the analysis of the fragmentation pattern of the sugar products after reduction with $NaBD_4$. As shown in Fig. 2B, this analysis demonstrated that the deuterium was incorporated at C-2.

The specific activity of the recombinant fuculose-1-phos-







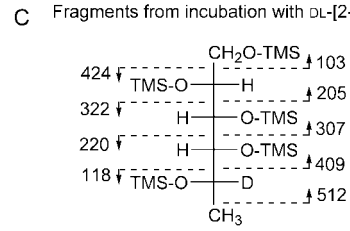


FIG. 2. Fuculose-1-phosphate aldolase reaction products. Shown is an analysis of the reduced and dephosphorylated reaction products resulting from the condensation of D- and L-lactaldehyde with DHAP. The four peaks observed were generated by reduction of the two products resulting from the condensation of DHAP with L- and D-lactaldehyde, respectively. (A) GC-MS fragmentation pattern of unlabeled TMS_5 products. (B) GC-MS fragmentation pattern of products reduced with borodeuteride. (C) GC-MS fragmentation pattern of product formed from lactaldehyde labeled with deuterium on C-2. Although the fragmentation pattern is drawn for the fucitol $(TMS)_5$ derivative, an identical fragmentation pattern was seen in the corresponding peak for each stereoisomer.

TABLE 3. Relative activity of *M. jannaschii* fuculose-1-phosphate aldolase with various aldehyde acceptor substrates and dihydroxyacetone phosphate as donor substrate

Acceptor substrate	Relative % activity[a]
Glyceraldehyde	100
DL-Lactaldehyde	88
Glycolaldehyde	75
Acrolein	50
Formaldehyde	38
Methylglyoxal	38
Acetaldehyde	25
Crotonaldehyde	13
Propionaldehyde	ND[b]

[a] The measured specific activity at 100% was 570 nmol/min/mg protein. The detection limit was 7.4 nmol/min/mg protein.
[b] ND, not detected.

phate aldolase-catalyzed condensation of DHAP with DL-lactaldehyde was 570 nmol/min/mg protein under the standard assay conditions. The monomer of fuculose-1-phosphate aldolase was estimated to be 20 kDa by SDS-polyacrylamide gel electrophoresis, which corresponded well with the predicted molecular mass of 20.5 kDa. The native molecular mass of fuculose-1-phosphate aldolase was approximately 90 kDa by gel filtration chromatography, indicating that, like *E. coli* fuculose-1-phosphate aldolase (13), the *M. jannaschii* enzyme exists as a tetramer.

The stereochemistry of the hexose product of class II aldolases (2, 14, 43) is consistent with the formation of fuculose-1-phosphate by the *M. jannaschii* enzyme as shown in Fig. 2. Fuculose-1-phosphate aldolase was incubated with DL-lactaldehyde labeled with deuterium on C-2, and the resulting hexose sugars were analyzed by GC-MS. The fragmentation pattern of each of the four labeled deoxyhexitols was consistent with the expected labeling pattern for the predicted product from an aldol addition of lactaldehyde with DHAP (Fig. 2C).

Testing substrate specificity of L-fuculose-1-phosphate aldolase. Aldolases in general, including *E. coli* fuculose-1-phosphate aldolase, have been shown to have broad substrate specificity (17, 55). In order to determine if the *M. jannaschii* enzyme has a similar profile for acceptor substrates, we tested for the ability of *M. jannaschii* fuculose-1-phosphate aldolase to condense a variety of aldehyde substrates with DHAP. As previously shown, we found that fuculose-1-phosphate aldolase was able to utilize glyceraldehyde as the acceptor substrate (9, 31). In addition, the *M. jannaschii* enzyme was also capable of utilizing lactaldehyde, glycolaldehyde, and several other aldehydes as acceptor substrates for the aldol condensation (Table 3). *M. jannaschii* fuculose-1-phosphate aldolase has a similar specificity profile as the *E. coli* enzyme (17).

Conclusion. The results presented here show that the pathway used for the production of lactate in *M. jannaschii* utilizes enzymes known to be involved in the metabolism of deoxyhex-

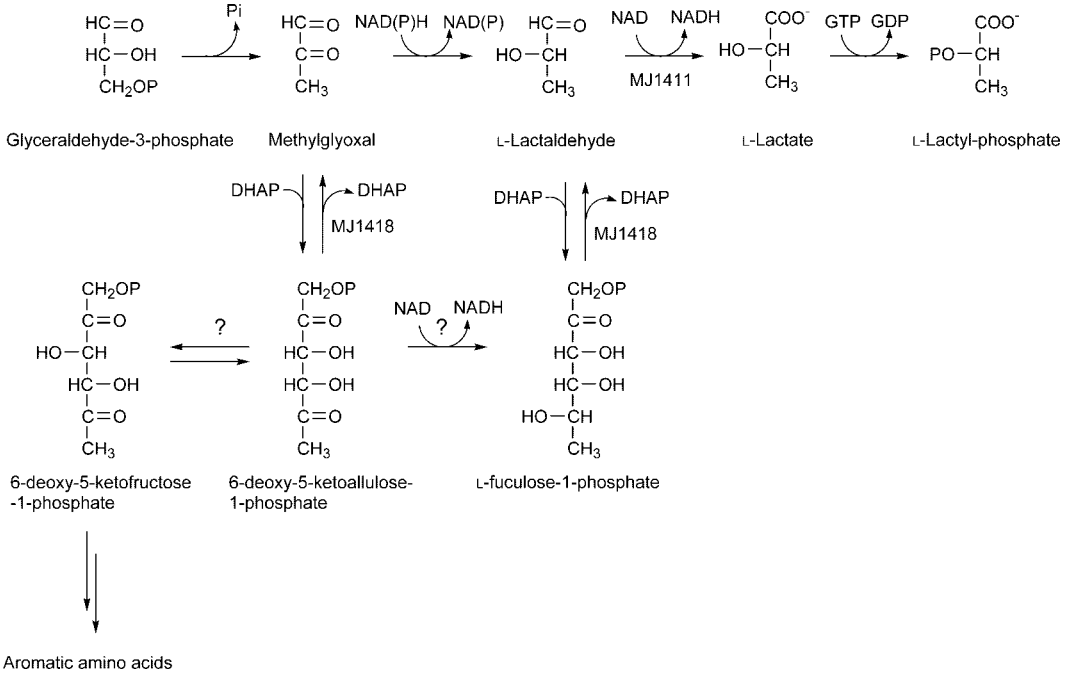


FIG. 3. Pathway for lactate formation in *M. jannaschii*.

oses in *E. coli* (Fig. 3) (18). Coenzyme F_{420} is found at 0.2 to 3.5 nmol/mg protein in methanogenic bacteria (10), and so the specific activities for the described pathway to lactate in *M. jannaschii* cell extract (ranging from 0.04 to 5.5 nmol/min/mg protein) would be adequate to provide for the coenzyme F_{420} requirements of the cells. Additional support for the described pathway comes from the identification of two of the described enzymes, MJ1411- and MJ1418-derived proteins, in a shotgun proteomics study of *M. jannaschii* (58).

Cell extract studies have showed that lactaldehyde is formed from methylglyoxal; however, the enzyme involved in this transformation has not yet been identified. Alternately, lactaldehyde may be formed through the aldol cleavage of fuculose-1-phosphate in a reaction catalyzed by the MJ1418 gene product. The lactaldehyde is then reduced to lactate by lactaldehyde dehydrogenase, encoded by MJ1411. The question then arises as to the biosynthetic origins of the deoxyhexoses in *M. jannaschii*. The metabolically significant direction for the aldolase reaction may be the formation of fuculose-1-phosphate, although the role of this compound in *M. jannaschii* is not known. Deoxyhexoses are not part of any currently known archaeal polysaccharide but have been recently identified as being involved in the biosynthesis of the aromatic amino acids (54). The observed use of methylglyoxal as a substrate by fuculose-1-phosphate aldolase (Table 3) could provide an additional route for the removal of that toxic compound. The resulting 6-deoxy-5-ketoallulose-1-phosphate could be converted into other deoxy sugars through two routes (Fig. 3). A epimerization at C-3 would result in the formation of 6-deoxy-2,5-diketofructose-1-phosphate, required for aromatic amino acid biosynthesis in *M. jannaschii*. Alternately, a reduction at C-5 would give fuculose-1-phosphate, which could be decomposed to form lactaldehyde and DHAP by fuculose-1-phosphate aldolase (Fig. 3). A more in-depth study of hexose metabolism in *M. jannaschii* is being conducted in our laboratory to try and establish these connections.

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