Metabolic Pathway Promiscuity in the Archaeon Sulfolobus solfataricus Revealed by Studies on Glucose Dehydrogenase and 2-Keto-3-deoxygluconate Aldolase*

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The hyperthermophilic Archaeon Sulfolobus solfataricus metabolizes glucose by a non-phosphorylative variant of the Entner-Doudoroff pathway. In this pathway glucose dehydrogenase and gluconate dehydratase catalyze the oxidation of glucose to gluconate and the subsequent dehydration of gluconate to 2-keto-3-deoxygluconate. 2-Keto-3-deoxygluconate (KDG) aldolase then catalyzes the cleavage of 2-keto-3-deoxygluconate to glyceraldehyde and pyruvate. The gene encoding glucose dehydrogenase has been cloned and expressed in Escherichia coli to give a fully active enzyme, with properties indistinguishable from the enzyme purified from S. solfataricus cells. Kinetic analysis revealed the enzyme to have a high catalytic efficiency for both glucose and galactose. KDG aldolase from S. solfataricus has previously been cloned and expressed in E. coli. In the current work its stereoselectivity was investigated by aldol condensation reactions between D-glyceraldehyde and pyruvate; this revealed the enzyme to have an unexpected lack of facial selectivity, yielding approximately equal quantities of 2-keto-3-deoxygalactonate and 2-keto-3-deoxygalactonate. The KDG aldolase-catalyzed cleavage reaction was also investigated, and a comparable catalytic efficiency was observed with both compounds. Our evidence suggests that the same enzymes are responsible for the catabolism of both glucose and galactose in this Archaeon. The physiological and evolutionary implications of this observation are discussed in terms of catalytic and metabolic promiscuity.

The hyperthermophilic Archaeon Sulfolobus solfataricus grows optimally at 80–85 °C and pH 2–4, utilizing a wide range of carbon and energy sources (1). It has become one of the most comprehensively researched model organisms of archaeal metabolism and bioenergetics (2). Central metabolism in this organism involves a modified Entner-Doudoroff pathway (3), production of acetyl-CoA by pyruvate:ferredoxin oxidoreductase (4), and the citric acid cycle coupled to oxidative phosphorylation (5). The modified Entner-Doudoroff pathway is a non-phosphorylative variant of the classic pathway and proceeds with no net production of ATP (Fig. 1). An analogous pathway has also been detected in the thermoacidophilic Archaea Sulfolobus acidocaldarius (6), Thermoplasma acidophilum (7), and Thermoproteus tenax (8), as well as strains of Aspergillus fungi (9, 10).

The first reaction of the non-phosphorylative Entner-Doudoroff pathway involves the NAD(P)-dependent oxidation of glucose to gluconate, catalyzed by glucose dehydrogenase. Gluconate is then dehydrated by gluconate dehydratase to 2-keto-3-deoxygluconate (KDG),1 which undergoes an aldol cleavage to pyruvate and glyceraldehyde, catalyzed by KDG aldolase. Glyceraldehyde dehydrogenase then oxidizes glyceraldehyde to glyceraldehyde, which is phosphorylated by glyceraldehyde kinase to give 2-phosphoglycerate. A second molecule of pyruvate is produced from this by the actions of enolase and pyruvate kinase.

Glucose dehydrogenase has previously been purified to homogeneity from cell extracts of S. solfataricus (11). It was shown to have broad substrate specificity, which implied a possible function in the oxidation of other sugars. The same enzyme was found to be responsible for galactose dehydrogenase activity in the extracts, and it was suggested that the enzymes of the non-phosphorylative Entner-Doudoroff pathway might be used for the metabolism of both glucose and galactose (11). This possibility was investigated in the current work by in vitro studies of glucose dehydrogenase and KDG aldolase. The successful cloning and expression of the KDG aldolase gene has been reported previously (12), and we now report the cloning and expression of the glucose dehydrogenase gene and kinetic analysis of both enzymes.

EXPERIMENTAL PROCEDURES

Materials—Matrex gel Red A affinity medium was purchased from Millipore Ltd., Watford, UK, and the Cibacron blue F3GA dye affinity column was from Bio-Rad Laboratories, Hemel Hempstead, UK. Matrix 60 silica gel for flash chromatography was from Fluorogen Ltd., Gloucester, UK, and the expression vector pREC7/NdeI was provided by Dr. L. C. Kurz (Washington University School of Medicine, St. Louis, MO). General Procedures—Protein concentrations were determined by the method of Bradford (13) using a calibration curve constructed with bovine serum albumin. Polariometry was carried out using an AA-10 automatic polarimeter (Optical Activity Ltd., Huntingdon, UK). NMR analysis was performed on an Avance 300 machine (Bruker, Coventry, UK). HPLC analysis was performed using a Bio-Rad Aminex HPX-87H.

1 These abbreviations used are: KDG, 2-keto-3-deoxygluconate; KDGal, 2-keto-3-deoxygalactonate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; KDGPgal, 2-keto-3-deoxy-6-phosphogalactonate; HPLC, high performance liquid chromatography; MES, 4-morpholineethanesulfonic acid.
The non-phosphorylative Entner-Doudoroff pathway of *S. solfataricus*.

Organic Analysis column (300 mm × 7.8 mm) linked to an RID-10A refractive index detector (Shimadzu, Milton Keynes, UK). Samples were eluted in 8 mM H$_2$SO$_4$ at a flow rate of 0.55 ml/min$^{-1}$, and the system was calibrated with pyruvate, glyceraldehyde, p-KDG and 2-keto-3-deoxy-d-galactonate (p-KDGGal). Enzyme activities were monitored spectrophotometrically using a Lambda Bio 230V spectrophotometer and the N-terminal sequence was determined by the chain-termination method (17) on a 377 automated DNA sequencer (Applied Biosystems).

Expression and Purification of Recombinant Glucose Dehydrogenase—The glucose dehydrogenase gene was amplified from the *S. solfataricus* lambda library using gene-specific primers to introduce a unique *Nde*I site upstream of the initiating methionine codon and a unique *Bam*HI site downstream of the termination codon. The PCR products were cloned into these sites of the pREC7 expression vector and used to transform *Escherichia coli* strain JM109 (Promega). One-liter cultures were grown in LB media containing carbenicillin (50 μg/ml) at 37 °C to A$_{600}$ nm ~ 0.6, before induction with nalidixic acid (50 μg/ml) and further incubation for 21 h. Cells were harvested at 2000 × g for 10 min and resuspended at ~0.2 g/ml in 50 mM MES buffer, pH 5.5, containing 20 mM MgCl$_2$. Cells were lysed by addition of 0.1% (v/v) Triton X-100 and lysozyme (100 μg/ml). After 1 h of incubation at 37 °C, followed by five 30-s bursts of sonication at 4 °C, cell debris was removed by centrifugation. The resulting extract was heated at 80 °C for 30 min, and precipitated proteins were removed by centrifugation. The extract was further purified by a Matrex gel Red A column (2 × 30 cm) using the MES buffer with a 0.1–1.5 mM NaCl elution gradient.

Glucose Dehydrogenase Assay—Glucose dehydrogenase activity was determined spectrophotometrically by following the increase in absorbance at 340 nm, corresponding to the reduction of NADP$^+$, over 1 min at 70 °C. The standard assay mixture (1 ml) contained 0.5 mM NADP$^+$, 5 mM glucose, and 20 mM MgCl$_2$ in 100 mM HEPES buffer (pH 7.5 at 70 °C). Enzyme kinetic analysis was performed with 10 mM NAD$^+$ or 1 mM NADP$^+$ at various concentrations of glucose or galactose. Kinetic parameters were determined by the direct linear method of Eisenhal and Cornish-Bowden (18).

Synthesis and Analysis of D-Glyceraldehyde—D-Glyceraldehyde was synthesized by periodate cleavage of 1,2,5,6-di-O-isopropylidene-D-mannitol (19). The sample was purified by silica gel flash chromatography on a Bio-Beads S-X2 column and eluted with 1 M NaCl and *Bam*HI restriction sites. One-liter cultures of *E. coli* BL21(DE3) Novagen) containing the vector were grown overnight at 37 °C without induction. Cells were collected by centrifugation and resuspended at ~0.2 g/ml in water. They were lysed by two passes through a cell disruptor (One-shot model, Constant Systems, Warwick, UK) at 200 MPa before heat precipitation at 95 °C for 30 min. Debris was removed by centrifugation at 18,000 × g for 30 min, and the resulting KDG aldolase sample was lyophilized.

**KDG Aldolase-catalyzed Condensation between D-Glyceraldehyde and Pyruvate**—Approximately 1 g of D-glyceraldehyde was mixed with 2.2 g of sodium pyruvate (2-fold molar excess) in 100 ml of water containing 5 mg of lyophilized *S. solfataricus* KDG aldolase. The reaction was heated at 50 °C in a shaking incubator, and regular samples were taken over 9 h. 500–μl samples were added to 100 μl of 12% (w/v) trichloracetic acid and centrifuged before HPLC analysis. The remaining reaction mix was lyophilized, and the diastereomers were separated by high-performance liquid chromatography on a 0.9 × 30 cm column of Waters X-Select C$_{18}$ reversed-phase column. The band corresponding to glucose dehydrogenase activity was electroblotted onto a hydrophobic polyvinylidene difluoride membrane and the N-terminal sequence was determined using a 470 gas-phase sequencer, coupled to a 120 phospholipiodyantin analyzer (Applied Biosystems, Warrington, UK).

**Coupled Assay for S. solfataricus KDG Aldolase**—A continuous assay was adapted to assay *S. solfataricus* KDG aldolase in the cleavage direction using the l-lactic dehydrogenase from *Bacillus stearothermophilus* as a coupling enzyme (23). Assays were performed at 60 °C in 50 mM sodium phosphate buffer, pH 6.0, containing 5 mM fructose-1,6-bisphosphate and 0.2 mM NADH. Various concentrations of p-KDG or
D-KDGal were added from 1 M stock solutions, and rates were monitored by a decrease in absorbance at 340 nm. Appropriate controls were performed to ensure the requirements for coupled enzymatic analysis were met (24), and kinetic parameters were determined by the direct linear method (18).

RESULTS

Glucose Dehydrogenase from S. solfataricus—Glucose dehydrogenase was purified from cell extracts of S. solfataricus and its Km for glucose with NADP/H was found to be 1.20 (±0.02) mM. One major protein species was responsible for the activity and the Mr was found to be ~41,000 by preparative SDS-PAGE. N-terminal sequencing was successfully carried out on this protein and determined to be MKAIIVKPPNAGVQVKDVDEAE. A DNA probe corresponding to this sequence was generated by PCR amplification and, after radiolabeling, was used to identify the full gene sequence from a genomic DNA library in lambda phage. The gene was found to encode a polypeptide of 366 amino acids with a predicted Mr of 40,849.

The nucleotide sequence data were deposited in the GenBank™ sequence data base in October 1998 under the accession number AJ012093 and are annotated in Fig. 2. In addition to the open reading frame a TATA box, TTTATA, was found 28–33 nucleotides upstream of the start codon, followed by a putative Shine-Dalgarno sequence 6–14 nucleotides upstream. T-rich polypyrimidine termination sequences, as described by Reiter et al. (25), were found 21–26 and 35–41 bp downstream of the stop codon.

Characterization of Recombinant Glucose Dehydrogenase—The recombinant enzyme was successfully expressed in E. coli with a specific activity of 2 units/mg in cell extracts. It was purified to homogeneity by heat precipitation and Matrex gel Red A affinity chromatography, as assessed by SDS-PAGE. Calibrated gel filtration of the purified enzyme was consistent with a tetrameric structure with Mr of ~160,000. The enzyme was found to have activity with a number of alternative sugar substrates and to possess dual cofactor specificity, as listed in Table I. These features are shared with the glucose dehydrogenase from the related thermoacidophile T. acidophilum (26) and the enzyme from other archaeal sources (27, 28).

Analysis of the translated amino acid sequence has shown that the enzyme is a putative member of the medium-chain alcohol/polyol dehydrogenase/reductase branch of the superfamily of pyridine-nucleotide-dependent alcohol/polyol/sugar dehydrogenases (29). These enzymes are characterized by a chain length of 350–375 residues and conserved structural zinc-binding and nucleotide-binding sites. They possess a characteristic GXXGXXG/A fingerprint motif (30) in the classic nucleotide-binding fold (31). In T. acidophilum glucose dehydrogenase the dual cofactor specificity has been rationalized by the presence of asparagine and histidine residues at positions 215 and 217, in conjunction with a GXXGXXA motif (32). The enzyme from S. solfataricus was found to have a GXXGXX motif from residues 188–193 and a structurally equivalent asparagine residue at position 211 (Fig. 2). Arg-213 is likely to take the place of His-217, allowing stabilization of the adenosine 2-phosphate of NADP. These features provide a plausible structural explanation for the observed dual cofactor specificity of the S. solfataricus enzyme.

S. solfataricus glucose dehydrogenase was found to possess four conserved cysteine residues at positions 93, 96, 99, and

![Fig. 2. Nucleotide sequence of the glucose dehydrogenase gene from S. solfataricus and its flanking regions. The nucleotide sequence encoding the glucose dehydrogenase gene is numbered with its amino acid translation below. The proposed active-site residues (Cys-93 and His-66) are double underlined and in boldface type. The conserved cysteine residues (Cys-93, Cys-96, Cys-99, and Cys-107) of the putative structural zinc-binding site are underlined and in boldface type, and the conserved glycine residues (Gly-188, Gly-190, and Gly-193) of the cofactor-binding site are indicated by boxes. The putative TATA box, Shine-Dalgarno sequence, and termination sequences are highlighted by boldface type in the flanking DNA sequence.](image-url)
TABLE I  
*Table displaying metabolic pathway promiscuity in S. solfataricus*  

| Substrate | Relative rate | NAD<sup>+</sup> | NADP<sup>+</sup> |
|-----------|--------------|----------------|-----------------|
| d-Glucose | 83           | 100            |                 |
| d-Galactose | 53         | 12             |                 |
| d-Allose | 0            | 0              |                 |
| d-Mannose | 0            | 0              |                 |
| d-Idose | 3            | 1              |                 |
| d-Xylose | 72           | 8              |                 |
| t-Arabinose | 95         | 99             |                 |
| d-Ribose | <1           | <1             |                 |
| d-Lyxose | 6            | 5              |                 |
| d-Arabinose | 0           | 0              |                 |
| t-Lyxose | 0            | 0              |                 |
| d-Threose | 0            | 0              |                 |
| d-Glucosamine | 9   | 2              |                 |
| 2-Deoxy-d-glucose | 2 | 2 | |
| 6-Deoxy-d-glucose | 49 | 57 | |
| d-Fucose | 51           | 10             |                 |
| Maltose | 1            | <1             |                 |
| Lactose | <1           | 1              |                 |
| Sucrose | 1            | 1              |                 |

107, which are equivalent to the residues involved in the coordinate binding of a zinc atom in the *T. acidophilum* enzyme (32). In addition, it was found to possess the catalytic zinc-binding residues Cys-39 and His-66, which align well with highly conserved Cys and His residues present throughout the whole alcohol dehydrogenase family (33). In support of this mechanism, activity in the standard assay was reduced by 60% in the presence of 10 mM EDTA, although there was no significant increase in the presence of ZnCl₂, CaCl₂, or MgCl₂, at final concentrations of 0.1 mM.

As listed in Table I, the glucose dehydrogenase from *S. solfataricus* has activity with a number of alternative aldose sugars. Activity was found with d-galactose, the C4 epimer of d-glucose, although not with d-allose or d-mannose, the C3 and C2 epimers. Activity was also observed with d-xylose, an aldopentose sugar with identical configuration to glucose at C2, C3, and C4. Good activity was observed with its C4 epimer t-arabinose but not with its C3 or C2 epimers d-ribose or d-xylose. It therefore appears that the enzyme has a preference for the glucose-specific stereo-configuration at C2 and C3 but accepts either configuration at C4. The C2 derivatives d-glucosamine and 2-deoxy-d-glucose showed very little activity, which may indicate an interaction with the enzyme at this position. The fact that aldopentoses serve as substrates and that 6-deoxy-d-glucose has activity, shows the diminished importance of the configuration at C5 and C6. d-Fucose, the 6-deoxy derivative of d-galactose also had activity, which again confirms that the enzyme can accept either configuration at the C4 position.

Because of the potential metabolic significance of a dual activity with d-glucose and d-galactose, discussed later, the enzyme was subjected to further kinetic analysis with both substrates and the determined parameters are listed in Table II. Although the maximum rate with d-glucose is higher than the value for d-galactose, the lower observed *Kₘ* with the latter gives it a higher catalytic efficiency. The *Kₘ* value for d-glucose with NAD<sup>+</sup> was found to be 1.30 (±0.05) mM, which is close to that observed for the enzyme purified from *S. solfataricus* cells.

**Synthesis of d-Glyceraldehyde**—Problems were encountered establishing a reliable commercial source of d-glyceraldehyde, and it was therefore synthesized as required by the periodate cleavage of 1,2-5,6-di-O-isopropylidene-d-mannitol. This reaction affords two molecules of isopropylidene-d-glyceraldehyde, which is converted to d-glyceraldehyde by treatment with H₂SO₄. Product was shown to be free from contaminants by HPLC and 1°H NMR spectroscopy, and its enantiomeric purity was assayed by polarimetry. The determined optical rotation, [α]<sub>D</sub>₂⁰° = +9.75° (c = 2, H₂O), was well within the reported range of +7° to +14° (34). The enantiomeric purity was further confirmed by derivatization with 1-(−)-n-methylbenzylamine, which yielded diastereomically pure (S,S)-3-(1-phenylethylamine)propane-1,2-diol, as assayed by 1°H NMR spectroscopy. This was compared with the other diastereomer obtained in an equivalent reaction with l-glyceraldehyde.

**KDG Aldolase Selectivity**—d-Glyceraldehyde samples were used in an aldol condensation reaction with pyruvate, catalyzed by recombinant *S. solfataricus* KDG aldolase. The reaction progress was monitored by calibrated HPLC analysis as shown on the *graph* in Fig. 3A. Unexpectedly, the condensation resulted in the presence of two diastereometric products, indicating a lack of facial selectivity in the enzyme-catalyzed reaction. These two product peaks are clearly visible on HPLC traces, throughout the time course of the reaction (Fig. 3B). The diastereometric products, d-KDG and d-KDGal, were separated by anion exchange chromatography, and their identity was confirmed by NMR analysis of the methyl ester (21). Polarimetry was carried out on the separated products and revealed the expected specific rotation for d-KDG (35) and d-KDGal (36).

**d-KDG:** [α]<sub>D</sub>₂⁰° = −34.6° (c = 1.3, H₂O), Lit. [α]<sub>D</sub>₂⁰° = −33.1° (c = 1.3, H₂O), d-KDGal: [α]<sub>D</sub>₂⁰° = +7.0° (c = 2, H₂O), Lit. [α]<sub>D</sub>₂⁰° = +7.9° (c = 1.65, H₂O).

In an equivalent experiment with racemic glyceraldehyde, the separated diastereomers did not give the expected specific rotations for single enantiomer products. This is due to the formation of KDG and KDGal as mixtures of enantiomers. In reactions between l-glyceraldehyde and pyruvate there was a similar lack of facial selectivity as observed for d-glyceraldehyde, revealed by two peaks in HPLC analysis (data not shown). In this case the enzyme is catalyzing the formation of diastereomers l-KDG and l-KDGal. In all cases the results clearly show there is little facial selectivity in the enzyme-catalyzed aldol condensation.

**KDG Aldolase Kinetics**—Purified d-KDG and d-KDGal were used for enzyme kinetic analysis in the aldolate cleavage direction using a coupled assay with the l-lactic dehydrogenase from *Bacillus steatothermophilus*. The determined kinetic parameters are listed in Table III. The lower activity with l-KDGal is partially compensated by its lower observed *Kₘ*, which increases the catalytic efficiency. The *Kₘ* for d-KDG of 25.7 (±1.2) mM is high compared with the values of 1.0 (±0.1) mM for pyruvate and 3.9 (±0.3) mM for d-glyceraldehyde (12). However, the existence of KDG and KDGal in pyranose and furanose forms (22) may have implications for in vitro studies of the aldolase-catalyzed reaction, which must operate via the straight-chain form of the sugar.

**DISCUSSION**

The cloning of the glucose dehydrogenase gene through N-terminal amino acid sequencing of the enzyme purified from *S. solfataricus* provides a valuable confirmation of the enzyme identity. An identical gene sequence has subsequently been discovered in the published genomic sequence of *S. solfataricus* strain P2 (37). The *in vitro* analysis reported herein clearly shows that glucose dehydrogenase and KDG aldolase from *S. solfataricus* have unexpectedly high activity with galactose and KDGal, respectively. The high catalytic efficiencies also suggest that the promiscuity of both enzymes may have physiological significance. When considered alongside other evidence, it appears that the same metabolic enzymes in this
organism have evolved for the catabolism of both glucose and galactose. Although *S. solfataricus* grows autotrophically in its geothermal habitat by the oxidation of elemental sulfur, it is considered to be an opportunistic heterotroph (2). Growth studies have revealed that strains of the organism can utilize both glucose and galactose as the sole carbon source, in addition to disaccharides containing them, such as cellobiose and lactose. Studies of \( /H9252\)-glucosidase from the organism have shown that the same enzyme is also responsible for \( /H9252\)-galactosidase activity (38). The catalytic promiscuity of this enzyme means it can cleave \( /H9252\)-linked glucose sugars such as cellobiose, yielding two molecules of glucose, and can also cleave \( /H9252\)-linked galactose-containing sugars such as lactose, yielding one molecule of glucose and one molecule of galactose. Two independent studies of sugar transport in *S. solfataricus* have revealed convincing evidence that a single, high affinity transporter is responsible for the uptake of both glucose and galactose into the cells (39, 40). Taken alongside the evidence for glucose dehydrogenase and KDG aldolase reported herein, it appears that at no point during uptake and catabolism does the organism distinguish between the two sugars.

There remains a question over the gluconate dehydratase activity in this metabolic pathway. Investigation into dehy-

| Substrate | Cofactor | \( K_m \) (mM) | \( V_{max} \) (units/mg) | \( k_{cat} \) (s\(^{-1}\)) | \( k_{cat}/K_m \) (s\(^{-1}\) mM\(^{-1}\)) |
|-----------|----------|----------------|-------------------------|----------------|----------------------|
| d-Glucose | NAD\(^+\) | 1.50 (±0.05)   | 110 (±5)                | 74.9           | 49.9                 |
|           | NADP\(^+\) | 1.30 (±0.05)   | 70 (±2)                 | 47.7           | 36.7                 |
| d-Galactose | NAD\(^+\) | 0.57 (±0.01)   | 90 (±1)                 | 61.3           | 107.5                |
|           | NADP\(^+\) | 0.44 (±0.01)   | 55 (±1)                 | 37.4           | 85.1                 |

**Fig. 3.** KDG aldolase-catalyzed condensation between d-glyceraldehyde and pyruvate. The reaction was carried out as described under “Experimental Procedures,” and progress was monitored by HPLC. A time course of the reaction is shown in A, with an inset showing the enzyme-catalyzed reaction. HPLC traces are shown in B after 0, 5, and 9 h.
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TABLE III

| Substrate | \(K_m\) | \(V_{max}\) | \(k_{cat}\) | \(k_{cat}/K_m\) |
|-----------|---------|------------|-----------|----------------|
| D-KDG     | 25.7 (±1.2) | 51.4 (±2.5) | 28.2 | 1.1 |
| D-KDGal   | 9.9 (±0.4)  | 12.3 (±0.4) | 6.8 | 0.7 |

The discovery of a "promiscuous" pathway in *S. solfataricus* defines an unusual metabolic phenomenon when compared with related pathways in other organisms. Selectivity studies of the aldolases are central to investigating this because of their position at the critical branch-point of these pathways. In the aldolate cleavage reaction C6 sugar acids are converted to two C3 compounds with the concomitant loss of one chiral center.

In addition to *S. solfataricus*, the non-phosphorylative Entner-Doudoroff pathway is also found in strains of *Aspergillus* fungi, including *Aspergillus niger* (9). A condensation reaction between D-glyceraldehyde and pyruvate using glucose-grown mycelia resulted in the synthesis of D-KDG in a large diastereomeric excess (41), indicating that the KDG aldolase in this organism does exhibit facial selectivity. An equivalent, inducible pathway exists in the organism for the catabolism of galactose, involving a separate KDGal aldolase with the opposite facial selectivity (42). The KDGal aldolase has also been investigated in *Aspergillus terreus* and shown to produce diastereometrically pure D-KDG in a condensation between D-glyceraldehyde and pyruvate (43). The classic Entner-Doudoroff pathway is widely distributed throughout both eukaryotes and bacteria (44, 45). In this pathway glucose is phosphorylated to glucose 6-phosphate, which is oxidized to 6-phosphogluconate before being dehydrated to 2-keto-3-deoxy-6-phosphogluconate (KDPG). KDPG aldolase then catalyzes the reversible aldolate cleavage of KDPG to glyceraldehyde-3-phosphate and pyruvate. Studies of KDPG aldolase from various organisms have shown that this enzyme also exhibits facial selectivity and consequently does not have activity with 2-keto-3-deoxy-6-phosphogalactonate (KDPGal) (46). In many cases the Delay-Doudoroff pathway exists in these organisms as an equivalent route for the catabolism of galactose (47). The enzymes of this pathway are often inducibly expressed and include an alternative KDGal aldolase with the opposite facial selectivity (48). In the case of *S. solfataricus* the lack of facial selectivity in the aldolase-catalyzed reaction is unusual and permits the same enzyme to be used for the cleavage of KDG and KDGal, both yielding glyceraldehyde and pyruvate.

Strains of *Sulfolobus* have been found to grow autotrophically (49) and contain glycogen stores (50). Both these features require gluconeogenesis, the nature of which has not been elucidated in the organism (2). It is now clear that a gluconeogenic sequence involving the promiscuous Entner-Doudoroff pathway would result in the formation of a mixture of glucose and galactose, because of the non-selective KDG aldolase-catalyzed reaction. This implies that gluconeogenesis in *Sulfolobus* is likely to occur via a reversal of the classic Embden-Meyerhoff-Parnas glycolytic pathway and not by a reversal of the Entner-Doudoroff pathway.

It seems likely that the metabolic promiscuity of *S. solfataricus* will extend to closely related thermoaacidophilic Archaea, such as *T. acidophilum*. This is supported by study of the glucose dehydrogenase in *T. acidophilum*, which also has a high relative activity with galactose (26). It is also possible the phenomenon extends to the part-phosphorylative Entner-Doudoroff pathway of halophilic Archaea, which would explain the failed attempts of Tomlinson et al. (51, 52) to isolate separate enzymes for the breakdown of glucose and galactose. This pathway involves the KDG kinase-catalyzed phosphorylation of KDG, which is then cleaved to glyceraldehyde-3-phosphate and pyruvate by KDPG aldolase. If the enzymes of this pathway are also responsible for galactose metabolism, it requires KDG kinase and KDPG aldolase to have activity with KDGal and KDPGal, respectively.

Hyperthermophiles constitute the deepest-branched organisms in the rRNA-based universal tree of life, and this still holds in phylogenies constructed using whole genome sequence data, despite evidence of large-scale horizontal gene transfer (53). Enzyme catalytic promiscuity is predicted to play a critical role in the evolution of new enzyme activities (54), and it is conceivable that promiscuity of an entire metabolic pathway is indicative of an early evolutionary state. However, it is unclear whether this promiscuous pathway in *S. solfataricus* reflects a primitive metabolic route or whether it is simply an adaptation to the hostile thermoacidophilic environment of the organism, allowing it to scavenge efficiently for energy substrates.

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