FEMS Microbiology Reviews 103 (1992) 1–28

Published by Elsevier

FEMSRE 00229

The Entner-Doudoroff pathway: history, physiology and molecular biology

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Received 6 April 1992
Revision received 19 May 1992
Accepted 19 May 1992

Key words: Entner-Doudoroff pathway; Glycolysis; Metabolism; Gene expression; Microbial diversity; Metabolic diversity

1. SUMMARY

The Entner-Doudoroff pathway is now known to be very widely distributed in nature. Biochemical and physiological studies show that the Entner-Doudoroff pathway can operate in a linear and catabolic mode, in a 'cyclic' mode, in a modified mode involving non-phosphorylated intermediates, or in alternative modes involving C_1 metabolism and anabolism. Molecular and genetic analyses of the Entner-Doudoroff pathway in *Zymomonas mobilis, Escherichia coli* and *Pseudomonas aeruginosa* have led to an improved understanding of some fundamental aspects of metabolic controls. It can be argued that the Entner-Doudoroff pathway is more primitive than Embden-Meyerhof-Parnas glycolysis.

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2. INTRODUCTION

The Entner-Doudoroff pathway was discovered in 1952 in *Pseudomonas saccharophila*. During the years that have passed since that discovery, our knowledge of the pathway has been improved by numerous biochemical studies. Although the Entner-Doudoroff pathway is generally considered to be restricted to a limited number of Gram-negative bacteria, the pathway is now known to be present in a diverse group of organisms ranging from the archaea, to bacteria, to eukarya. The widespread utility of the pathway suggests that Entner-Doudoroff metabolism is of far greater importance in nature than has been previously recognized.

Genetic studies of the pathway have revealed the genetic loci that encode the key enzymes of the Entner-Doudoroff pathway, as well as some of the genetic loci involved in regulation of expression of the relevant genes in response to physiological conditions. More recently, the use of molecular genetic approaches for study of the

Entner-Doudoroff pathway have been employed to examine in detail the regulatory mechanisms responsible for control and expression of the corresponding genes.

This, the first comprehensive review of the Entner-Doudoroff pathway, will begin with a brief history of how the pathway was discovered, to be followed by an examination of the pathway enzymes. Next, the physiological modes in which the pathway has been found to operate in nature will be summarized. The genetics and molecular biology of the Entner-Doudoroff pathway, as investigated primarily in three bacterial genera, will then be examined. Lastly, some speculations regarding the evolution of the pathway will be presented.

3. ENTNER-DOUDOROFF METABOLISM

The Entner-Doudoroff pathway can be viewed as an alternative to the Embden-Meyerhof-Parnas glycolytic pathway. The overall schemes of these two glycolytic pathways are quite similar: 6-carbon sugars are primed by phosphorylation and subsequently cleaved by aldolase enzymes into two 3-carbon intermediates. The distinguishing difference between the two glycolytic pathways lies in the nature of the 6-carbon metabolic intermediates that serve as substrates for aldol cleavage. The Embden-Meyerhof-Parnas pathway involves cleavage of fructose-1,6-bisphosphate by fructose bisphosphate aldolase to yield one molecule each of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The Entner-Doudoroff pathway involves cleavage of 2-keto-3-deoxy-6-phosphogluconate (KDPG) by KDPG aldolase to form glyceraldehyde-3-phosphate and pyruvate (Fig. 1). The reactions involving further metabolism of the triose phosphate intermediates are shared by both pathways and provide energy via substrate level phosphorylation. Equal in importance to energy yield is the ability of both glycolytic pathways to provide necessary metabolic precursors for biosynthesis.

3.1. Discovery of the pathway

In their landmark paper, received for publication on New Years Eve, 1951, Entner and

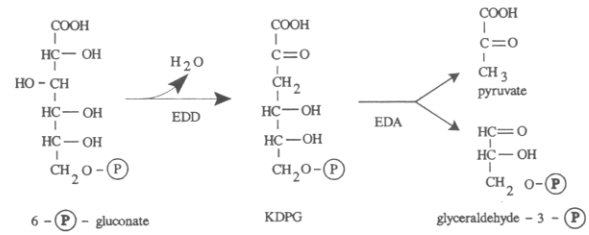

Fig. 1. The Entner-Doudoroff pathway. Abbreviations: EDD, 6-phosphogluconate dehydratase; EDA, 2-*keto*-3-deoxy-6-phosphogluconate aldolase; KDPG, 2-*keto*-3-deoxy-6-phosphogluconate.

Doudoroff [1] provided conclusive evidence for a novel pathway of glucose metabolism in the bacterium *Pseudomonas saccharophila*. Their work was based on the observation that the C_1-carbon of glucose was recovered almost entirely as CO_2. Yet, the mechanism did not appear to involve a primary decarboxylation of the glucose molecule. By using dinitrophenol-poisoned cells, they found that 1 mol of C_1-labelled glucose was degraded to 2 mol of pyruvic acid, all of the label being found in the carboxyl group of pyruvate. By using cells poisoned with iodoacetate, they showed that 1 mol of C_1-labelled glucose was converted to 1 mol of glyoxal and 1 mol of pyruvate specifically labelled at the carboxyl group. The same was found to be true when using gluconic acid as a substrate. An investigation of crude cell extracts revealed enzyme activities capable of cleaving 6-phosphogluconic acid to pyruvate and glyceraldehyde-3-phosphate. They speculated that KDPG was the likely intermediate of the cleavage of the 6-carbon compound to the two 3-carbon compounds. They were, of course, perfectly correct. The key intermediate of the Entner-Doudoroff pathway, KDPG, was subsequently crystallized from enzyme preparations that had been incubated with 6-phosphogluconate [2]. The two steps of the pathway, shown in Fig. 1, were established by separation and purification of the two key enzymes of the Entner-Doudoroff pathway, 6-phosphogluconate dehydratase [3] and KDPG aldolase [4].

3.2. Labelling patterns of the pathway

A characteristic difference in aerobic degradation of specifically labelled glucose, as detected

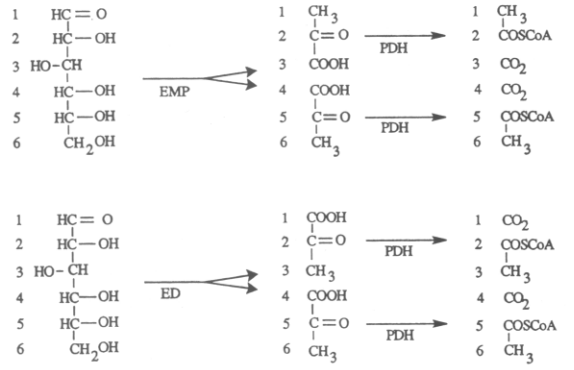

Fig. 2. Characteristic labelling patterns. CO_2 formed via Embden-Meyerhof-Parnas (EMP) glycolysis arises from C_3 and C_4 of glucose. CO_2 formed via the Entner-Doudoroff (ED) pathway arises from C_1 and C_4 of glucose. Abbreviation: PDH, pyruvate dehydrogenase complex.

by radiorespirometry, is the classical means for distinguishing Embden-Meyerhof-Parnas metabolism from Entner-Doudoroff metabolism [5]. As can be seen in Fig. 2, the C_1-labelled carbon of glucose forms the carboxyl group of pyruvate when metabolized via the Entner-Doudoroff pathway. The labelled carbon is subsequently evolved in the form of CO_2 during aerobic growth through the action of pyruvate dehydrogenase, while the remaining two unlabelled carbons of the pyruvate molecule enter the tricarboxylic acid (TCA) cycle. During Embden-Meyerhof-Parnas metabolism of C_1-labelled glucose, the methyl group of pyruvate is specifically labelled, enters the TCA cycle, and a portion of the label is assimilated into biosynthetic pathways. The remainder of the label is released as CO_2 during subsequent rounds of the TCA cycle. Thus, an early time course of appearance of labelled CO_2 from C_1-labelled glucose serves as an indication of Entner-Doudoroff metabolism. It can also be seen from Fig. 2 that the C_4 carbon of glucose will become the carboxyl group of the second pyruvic acid molecule via either of these two metabolic pathways.

3.3.6-Phosphogluconate dehydratase

The first of the two key enzymes unique to the Entner-Doudoroff pathway, 6-phosphogluconate dehydratase, catalyses a dehydration of 6-phosphogluconate to form KDPG (Fig. 1). The 6-phosphogluconate dehydratase enzyme has come to be known as the Entner-Doudoroff dehydratase, EDD, and the gene encoding the enzyme has been given the designation *edd* [6]. The EDD enzyme was partially purified for the first time from *Pseudomonas fluorescens* and characterized by Kovachevich and Wood in 1954 [3]. The K_m of this EDD enzyme for 6-phosphogluconate is 0.5 mM and the enzyme has a pH optimum of 8.0. The *P. fluorescens* EDD is activated by divalent cations, such as Fe^{2+}, Mn^{2+} and Mg^{2+} (listed in order of relative activation), and is also activated by thiol reductants, such as glutathione, cysteine, and thioglycollate (listed in order of relative activation). The enzyme is inhibited by fluoride ions. Meloche and Wood [7] provided evidence that the mechanism of action of EDD involves an enol-KDPG intermediate that undergoes spontaneous rearrangement to the keto form by a reaction sequence that is essentially irreversible. Purification of the EDD from *Zymomonas mobilis* indicated that the enzyme functions as a dimer of identical 63-kDa subunits [8]. The *Z. mobilis* EDD has a K_m for 6-phosphogluconate of 0.04 mM, a purified specific activity of 245 μmol/min/mg protein, and is inhibited by D-α-glycerophosphate, phosphate, 3-phosphoglycerate and sulfate. It was noted that the *Z. mobilis* EDD is similar to other enzymes that require ferrous ions, such as gluconate dehydratase, aconitase, and dihydroxyacid dehydratase. The finding that the *Z. mobilis* EDD contains three iron atoms per protein subunit supports the suggestion that the enzyme is an iron sulfur protein. The *Escherichia coli* EDD was found to be sensitive to superoxide, as had been predicted for other enzymes catalysing similar dehydration reactions [9]. The *E. coli* EDD was found to be stabilized by 6-phosphogluconate. A closely related enzyme, D-gluconate dehydratase was purified from *Clostridium pasteurianum* [10]. The *C. pasteurianum* enzyme shows a broad substrate range that includes 6-phosphogluconate and has otherwise similar properties to EDD, with the exception of having a higher K_m (for D-gluconate).

The *edd* gene was cloned from *Z. mobilis* [11] and from *E. coli* [12] and the sequences of both

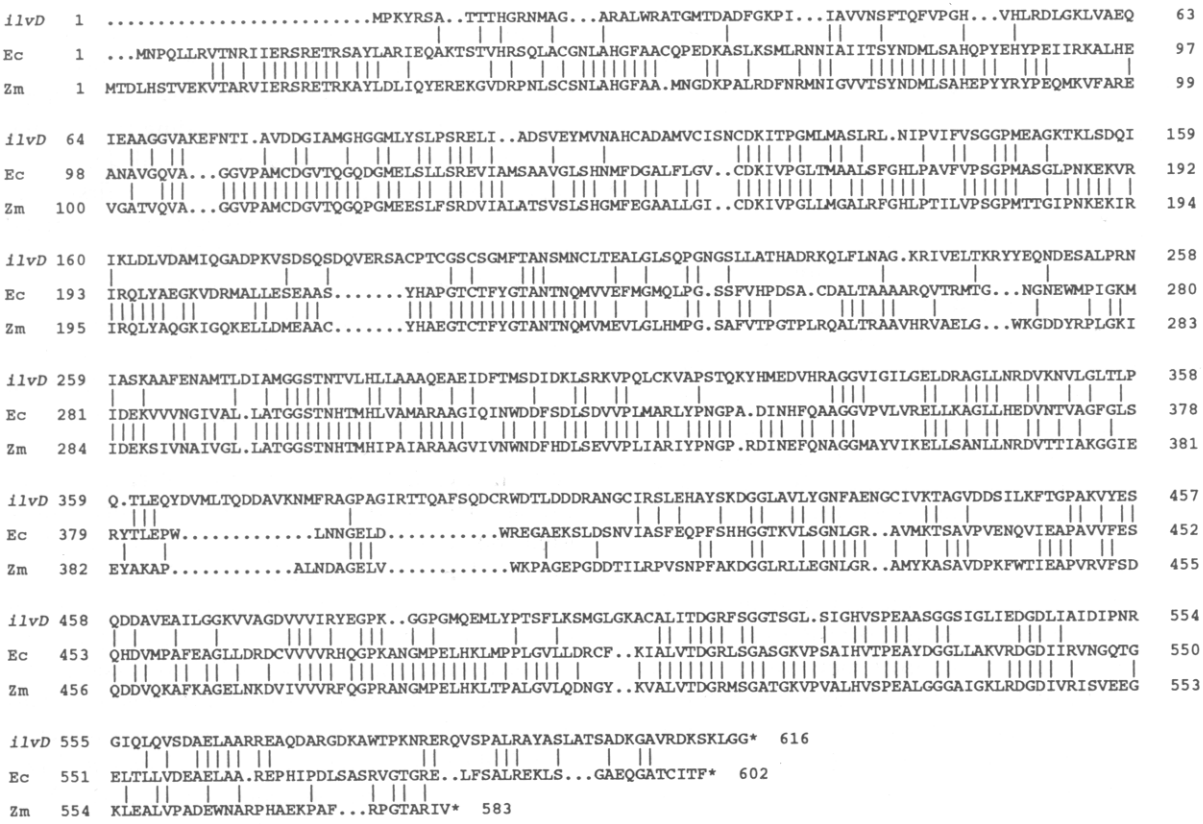

Fig. 3. Amino acid comparisons of the deduced *E. coli edd* product (Ec) with the *Z. mobilis edd* product (Zm) and the *E. coli ilvD* product (*ilvD*). Identity is indicated by (|), gaps are indicated by (.).

genes were determined. A comparison of the deduced primary amino acid sequences of EDD from the two organisms shows that they are highly conserved (Fig. 3). The two EDD enzymes exhibit 57.7% identity and 75.3% similarity (chemically conserved residues). The EDD enzymes are both related by primary sequence to the *E. coli* dihydroxyacid dehydratase, an enzyme of the isoleucine-valine biosynthetic pathway, which is encoded by the *ilvD* gene [13]. An alignment of the two *E. coli* dehydratase enzymes shows them to be 30.3% identical and 54.2% conserved (Fig. 3). A comparison of the *Z. mobilis* EDD with the *E. coli* dihydroxyacid dehydratase shows them to be 31% identical and 56% similar.

3.4. KDPG aldolase

The second of the two key enzymes of the Entner-Doudoroff pathway, KDPG aldolase, catalyses an aldol cleavage of KDPG to form pyruvate and glyceraldehyde-3-phosphate (Fig. 1). In stark contrast to EDD, the KDPG aldolase is not unique to the Entner-Doudoroff pathway. The enzyme is in fact multi-functional and is found in a wide range of organisms, including mammals, as will be described below. The KDPG aldolase enzyme has become known as the Entner-Doudoroff aldolase, EDA, and the gene encoding EDA has been designated *eda* [6]. EDA was first purified from *P. fluorescens* [4]. The K_m of the enzyme was shown to be 0.1 mM, with a broad pH optimum of 7.0–8.5. The substrate range is quite limited and does not include ketodeoxygluconate, fructose-1,6-bisphosphate, or deoxyribose-5-phosphate. The reaction equilibrium lies 64% in the forward direction. The EDA enzyme from *Z. mobilis* was purified and shown to function as a trimer of identical 23-kDa subunits. The specific activity of the purified *Z. mobilis* enzyme is 600 μmol/min/mg protein [14].

The EDA enzyme from *P. putida* was purified, crystallized and the entire primary amino sequence was determined [15]. The entire nucleotide sequence of the *Z. mobilis eda* gene was determined [16]. An enzyme that is closely related to EDA, 2-keto-4-hydroxyglutarate (KHG) aldolase, was purified from *E. coli* and the primary amino acid sequence was determined by Vlahos and Dekker [17]. The nucleotide sequence of the gene corresponding to KHG aldolase was reported [18]. The entire nucleotide sequence of the *E. coli eda* gene was determined recently [12] and is in fact identical to the *E. coli* KHG aldolase gene, indicating that EDA and KHG aldolase activities are both present in the same multi-functional enzyme. Not only is EDA able to catalyse interconversion of KDPG with glyceraldehyde-3-phosphate and pyruvate, it is also capable of catalysing the interconversion of KHG with pyruvate and glyoxylate, as well as β-decarboxylation of oxaloacetate [18].

KHG aldolase has been postulated to play a role in regulating glyoxylate levels in *E. coli*. In an elegant study, Gupta and Dekker [19] showed that glyoxylate could be mineralized to carbon dioxide via a pathway involving condensation with pyruvate, catalysed by KHG aldolase, to form KHG. The KHG was further metabolized by α-ketoglutarate dehydrogenase, citrate synthase, malic enzyme, malate dehydrogenase, and a secondary activity of KHG/KDPG aldolase — β-decarboxylation of oxaloacetate — resulting in formation of carbon dioxide and pyruvate. Readers are referred to the paper by Gupta and Dekker [19] for details. KHG aldolase has also been purified from mammals, where the enzyme is involved in hydroxyproline biosynthesis [17].

An alignment *E. coli* EDA with the same enzyme from *Z. mobilis* and *P. putida* is shown in Fig. 4. The *E. coli* enzyme is 51.5% identical and 68.4% similar to the *Z. mobilis* enzyme and 44.4% identical and 65.4% similar to the *P. putida* enzyme. Inspection of the alignment reveals the presence of only five regions of four or more consecutive identical amino acids. The active site lysine No. 133 [15] and active site arginine No. 49 [17] are conserved in all three EDA enzymes. These amino acid residues are believed to be involved in cleavage and condensation by this Schiff-base aldolase.

4. PHYSIOLOGY AND ORGANIZATION OF THE PATHWAY

The Entner-Doudoroff pathway is known to operate in several different modes. It is, perhaps, an over-simplification to categorize organisms with respect to their mode of Entner-Doudoroff metabolism, because for many organisms there are not sufficient regulatory data available. Nevertheless, an examination of the literature reveals predictable patterns of association of certain microbial lifestyles with specific modes of Entner-

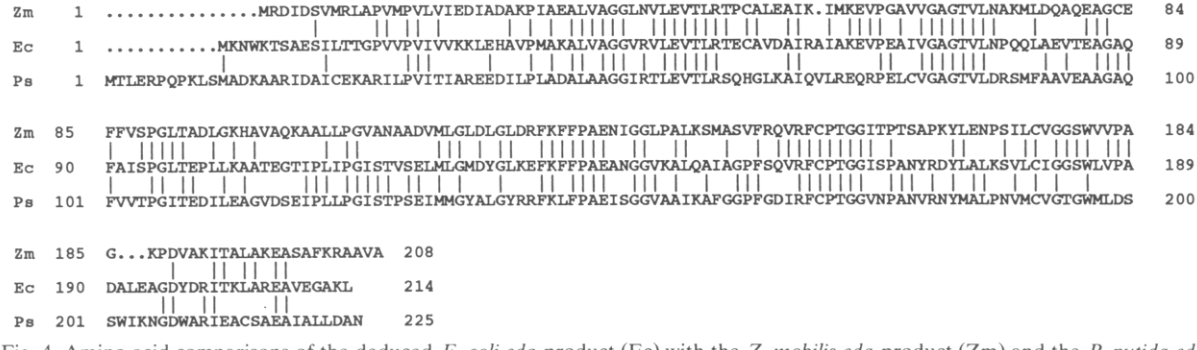

Fig. 4. Amino acid comparisons of the deduced *E. coli eda* product (Ec) with the *Z. mobilis eda* product (Zm) and the *P. putida eda* product (Ps). Identity is indicated by (|), gaps are indicated by (.).

Doudoroff metabolism. In a few organisms the Entner-Doudoroff pathway is employed in a strictly catabolic direction, is expressed constitutively, and forms the core of central metabolism. In numerous bacteria, including the enterics, the Entner-Doudoroff pathway operates in a linear fashion, is induced for growth on particular carbon sources such as gluconate, and the Embden-Meyerhof-Parnas pathway forms the core of central metabolism in these bacteria. In the majority of pseudomonads, the Entner-Doudoroff pathway forms the core of central carbohydrate metabolism, is inducible, and appears to operate in a 'cyclic' fashion, as will be described below. In still other bacteria, including some Clostridia and Archaebacteria, modified versions of the Entner-Doudoroff pathway, involving non-phosphorylated intermediates, are employed for carbohydrate catabolism. In a number of methylotrophic bacteria, the Entner-Doudoroff pathway plays a role in formaldehyde assimilation. It is also apparent that the Entner-Doudoroff pathway is present in some non-carbohydrate utilizers, where the pathway may play a role in anabolism.

4.1. Constitutive, linear pathway

Z. mobilis is the only organism known to use the Entner-Doudoroff pathway anaerobically [20–22]. The pathway, as it operates in *Z. mobilis*, is shown in Fig. 5. In this bacterium, the Entner-Doudoroff pathway is expressed constitutively and forms the core of central metabolism. The key enzyme of the pentose phosphate pathway, 6-phosphogluconate dehydrogenase, does not appear to be present in *Z. mobilis* [11]. Thus, the Entner-Doudoroff pathway is the only means for glucose catabolism in *Z. mobilis*. The pathway is employed in a linear fashion, providing all the necessary metabolic precursors for biosynthesis (in conjunction with the transketolase/transaldolase system and an incomplete TCA cycle). Energy is provided by substrate level phosphorylation involving the triose phosphates. The fermentation is completed by a two step oxidation of pyruvate to ethanol and CO_2. In the environment, *Z. mobilis* is found mostly in warm, tropical climates, in association with plants that have high sugar content in their saps. The unusual

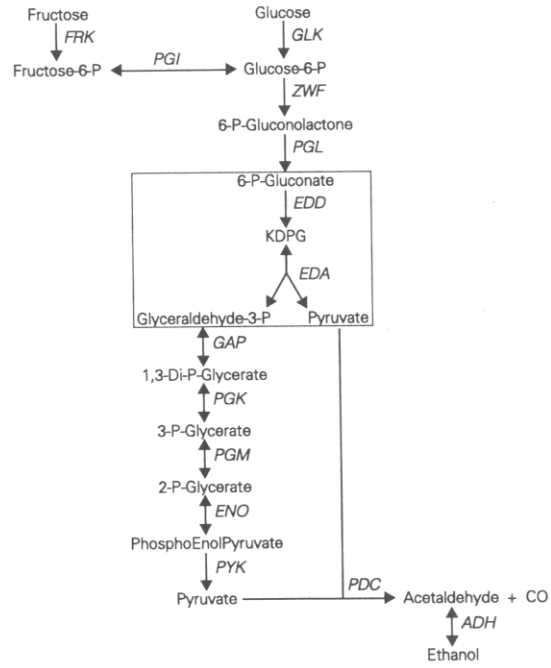

Fig. 5. Linear Entner-Doudoroff pathway as it operates in *Z. mobilis*. Abreviations: FRK, fructokinase; PGI, phosphoglucose isomerase; GLK, glucokinase; ZWF, glucose-6-phosphate dehydrogenase; PGL, phosphogluconolactonase; EDD, 6-phosphogluconate dehydratase; EDA, 2-keto-3-deoxy-6-phosphogluconate aldolase; GAP, glyceraldehyde phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglyceromutase; ENO, enolase; PYK, pyruvate kinase; PDC, pyruvate dehydrogenase; ADH, alcohol dehydrogenase.

metabolism employed by this bacterium is probably an adaptation to growth in this narrow ecological niche.

Glucose, fructose, and sucrose (which is cleaved to form glucose and fructose) are the only carbon and energy sources that support growth of *Z. mobilis*. These compounds are transported into the cell by a high-velocity facilitated diffusion system [11,23] and are primed for entry into the Entner-Doudoroff pathway by ATP-dependent phosphorylation by specific kinase enzymes [24]. The single glyceraldehyde-3-phosphate molecule generated from each hexose yields two ATP during metabolism to pyruvate. As a consequence of its inherent inefficiency, *Z. mobilis* obtains only a single mol of ATP per mol of glucose fermented via the Entner-Doudoroff pathway. Understandably, *Z. mobilis* has the lowest molar growth yield

reported for a microorganism [21]. In order to obtain sufficient energy to sustain a growth rate of 90 min, *Z. mobilis* is forced to maintain a rapid carbon flux which exceeds 1 μmol/min/mg total cell protein [25]. This rapid flux is facilitated by high level expression of the glycolytic and alcohologenic enzymes, which comprise approximately 50% of the total soluble protein [26].

Fructose enters the central pathway by the conversion of fructose-6-phosphate to glucose-6-phosphate, as catalysed by phosphoglucose isomerase [27]. 6-Phosphogluconate is formed from glucose-6-phosphate by the combined action of glucose-6-phosphate dehydrogenase and phosphogluconolactonase [11,28]. The *Z. mobilis* glucose-6-phosphate dehydrogenase is able to use NAD or NADP with approximately equal efficiency, making it possible for this enzyme to both produce reducing equivalents in the form of NADPH and to balance oxidation–reduction reactions via NADH-dependent reduction of acetaldehyde by alcohol dehydrogenase. Acetaldehyde and CO_2 are formed from pyruvate by a non-oxidative decarboxylation catalysed by pyruvate dehydrogenase [29].

Other organisms are known to utilize the Entner-Doudoroff pathway in a linear, catabolic mode. For example, in *Neisseria gonorrhoeae*, glucose is the only carbohydrate that can serve as an energy source and is catabolized by combined action of the Entner-Doudoroff (87% of carbon flux) and pentose phosphate pathways [30,31]. The end products of glucose metabolism are acetate and CO_2, formed presumably via pyruvate dehydrogenase with acetyl CoA as an intermediate. These end products are formed in growing cultures only under aerobic conditions [32]. The Entner-Doudoroff pathway appears to be expressed constitutively in *Xanthomonas campestris*, an organism that also uses the pathway in combination with the pentose phosphate pathway. The EDD and EDA enzyme levels do not change whether the organism is grown on gluconate, citrate, or glycerol [33].

4.2. Inducible, linear pathway

In numerous bacteria, including the enterics, the Entner-Doudoroff pathway plays only a peripheral role in central metabolism, being induced for metabolism of certain carbohydrates, such as gluconate. This pathway is depicted in Fig. 6 as it is known to operate in *E. coli*. Ordinarily, carbohydrate metabolism proceeds via the Embden-Meyerhof-Parnas and pentose phosphate pathways. When gluconate is available, the enzymes of the Entner-Doudoroff pathway are synthesized, in addition to the gluconate transport system and gluconokinase [6,34,35]. In *E. coli*, gluconate is actively transported into the cell by a proton symport system [36]. The gluconate is phosphorylated by gluconokinase to form 6-phosphogluconate, the substrate of EDD. This pathway can be used either aerobically, in conjunction with the TCA cycle and respiratory metabolism or anaerobically in a fermentative capacity [35]. Under fermentative conditions the pathway operates in much the same way as in *Z. mobilis*. Energy is produced via substrate level phosphorylation and oxidation–reduction reactions are balanced by a mixed acid fermentation mode of pyruvate dissimilation [5].

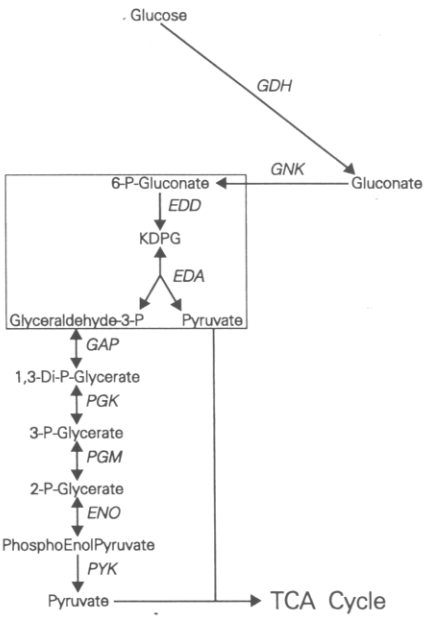

Fig. 6. Inducible Entner-Doudoroff pathway as it operates in *E. coli*. Abbreviations as in legend to Fig. 5 and: GDH, glucose dehydrogenase; GNK, gluconokinase; TCA cycle, tricarboxylic acid cycle.

The major flow of carbon from gluconate is via the Entner-Doudoroff pathway with a minor flow of carbon through the pentose phosphate pathway [34]. The failure of glyceraldehyde-3-phosphate dehydrogenase mutants to grow on gluconate indicates that the enteric Entner-Doudoroff pathway operates in a strictly linear, catabolic direction [37]. The reasons for unidirectional Entner-Doudoroff metabolism in *E. coli* are not obvious, since all of the enzymes that allow cyclic operation of the pathway in *Pseudomonas*, as described below, are present. One difference between *E. coli* and *Pseudomonas* is the presence of phosphofructokinase in *E. coli*. Despite expressing significant levels of both phosphofructokinase and fructose bisphosphatase, futile cycling of fructose-6-phosphate and fructose-1,6-bisphosphate, as measured by isotope equilibration, does not appear to occur in *E. coli* [38]. Moreover, the absence of back-labelling of the C_1 carbon of fructose bisphosphate from C_6 labelled glucose indicates that triose phosphates and fructose bisphosphate are not in equilibrium, suggesting that either fructose bisphosphate aldolase or triose phosphate isomerase is not readily reversible in vivo [39]. Whatever the reason, it is clear that cyclic operation of the Entner-Doudoroff pathway in *E. coli* is prevented by the physiology of the cell.

Until recently, the physiological role of Entner-Doudoroff metabolism in *E. coli* was unclear [6]. The discovery of a PQQ-dependent glucose dehydrogenase, which catalyses the periplasmic oxidation of glucose to gluconic acid, suggested an alternate route for glucose catabolism in *E. coli* [40]. *E. coli* phosphotransferase mutants (PTS) are able to grow on glucose in the presence of exogenous pyrroloquinoline quinone (PQQ), the cofactor for the glucose dehydrogenase apoenzyme [41]. Paradoxically, wild-type *E. coli* does not synthesize PQQ [40]. More recently, *E. coli* mutants capable of producing PQQ were isolated [42]. In a recent study, we showed that growth of wild type *E. coli* in the presence of glucose plus PQQ leads to induction of the Entner-Doudoroff pathway (unpublished data). Hence, *E. coli* possesses a pathway for oxidative glucose metabolism. In the presence of PQQ, the glucose dehydrogenase apoenzyme becomes functional, resulting in formation of gluconate from glucose [42]. This creates a metabolic branch point, in the periplasm, for glucose catabolism. The alternative branches consist of: (1) PTS-mediated glucose transport, or (2) glucose oxidation to gluconate and subsequent transport, phosphorylation and catabolism via the gluconate-inducible Entner-Doudoroff pathway. This pathway would of course be functional only in the absence of catabolite repression of the Entner-Doudoroff enzymes, which has been reported [44]. Eisenberg and Dobrogosz [34] showed that gluconate and glucose can be degraded simultaneously by *E. coli*.

A natural role can be suggested for oxidative glucose metabolism in the enterics. It is known that the oxidative glucose pathway is operable only under aerobic conditions [41]. Furthermore, low phosphate in the growth medium greatly reduces the lag-phase for growth on glucose plus PQQ, suggesting that induction of the PhoE porin facilitates PQQ uptake into the periplasm [41]. The nature of induction of this pathway in enteric bacteria suggests an important role in aerobic aquatic habitats which contain free PQQ and are low in carbon and phosphate. Perhaps this pathway allows survival during the extra-intestinal, aquatic phases of *E. coli*'s existence. Limiting phosphate in aquatic environments should result in uptake of PQQ, turning on glucose dehydrogenase and resulting in glucose oxidation to gluconate. Thus, phosphate limitation leads indirectly to induction of the Entner-Doudoroff pathway. Oxidative glucose metabolism may indeed provide a bioenergetic advantage in the environment, since it is found in a large number of enteric and free-living, aquatic microorganisms [45]. It seems reasonable that *E. coli* should be able to harness the energy of electron transfer via PQQ-dependent glucose dehydrogenase, which is supported by the finding that growth on glucose plus PQQ results in higher cell yields than growth on glucose alone [41].

In *Rhodobacter capsulata* [46,47] and *R. sphaeroides* [47,48], levels of the Entner-Doudoroff enzymes and glucose-6-phosphate dehydrogenase increase significantly under het-

erotrophic growth conditions and when carbohydrates are available. Both species contain an inducible 1-phosphofructokinase that is utilized for fructose metabolism. This enzyme channels carbon into the Embden-Meyerhof-Parnas pathway, which is solely responsible for fructose catabolism in *R. capsulata* [47], and used in conjunction with the Entner-Doudoroff pathway in *R. sphaeroides* [48]. Interestingly, endogenous fructose released by sucrase when growing on sucrose, is metabolized via the Entner-Doudoroff pathway in *R. capsulata* [49]. It is not known if the pathway operates in a linear or cyclic fashion in *Rhodobacter*.

4.3. 'Cyclic' pathway

The role of the Entner-Doudoroff pathway in *Pseudomonas* metabolism has been the subject of two excellent reviews, the first by Lessie and Phibbs [50] and the second by Phibbs [51]. Pseudomonads, in general, are devoid of 6-phosphofructokinase and thus are unable to catabolize carbohydrates via the Embden-Meyerhof-Parnas pathway. The core of central carbohydrate metabolism is formed by the Entner-Doudoroff pathway, along with the gluconeogenic enzymes triose phosphate isomerase, fructose 1,6-diphosphate aldolase, fructose 1,6-diphosphatase, phosphoglucose isomerase, and glucose-6-phosphate dehydrogenase (Fig. 7). There is substantial evidence for cyclic operation of the Entner-Doudoroff pathway, involving recycling of triose phosphates via the gluconeogenic enzymes, as will be described below. Pseudomonads typically display diauxic growth, succinate being preferred over carbohydrates. Recent progress with respect to the mechanism of non-cyclic AMP-dependent catabolite repression in *P. aeruginosa* has been made [52]. Glucose is metabolized under aerobic conditions via an oxidative pathway. The enzymes for carbohydrate metabolism are inducible, although the enzymes induced, and the inducer molecules, show considerable species variations.

Cyclic operation of the Entner-Doudoroff pathway in the pseudomonads is especially intrigueing. The glyceraldehyde-3-phosphate formed by EDA-dependent cleavage of KDPG is apparently recycled to 6-phosphogluconate via the

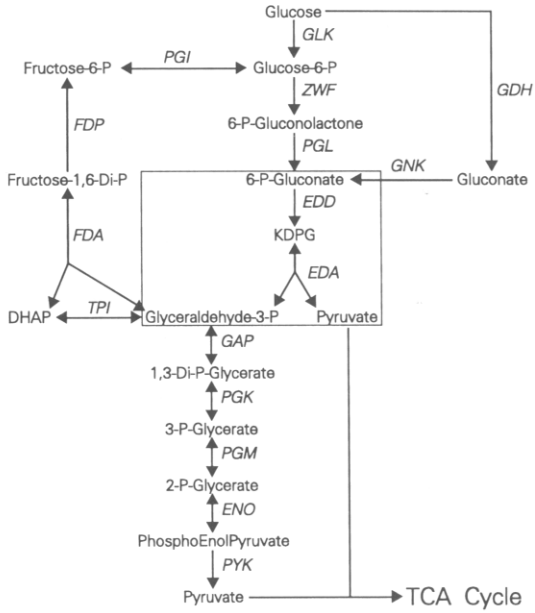

Fig. 7. Cyclic Entner-Doudoroff pathway as it operates in *P. aeruginosa*. Abbreviations as in legends to Figs. 5 and 6 and: TPI, triose phosphate isomerase; FDA, fructose diphosphate aldolase; FDP, fructose diphosphatase.

gluconeogenic enzymes. The evidence supporting cyclic operation of the pathway can be summarized as follows:

EDD$^-$ mutants are impaired for growth, not only on glucose and gluconate, but also on numerous carbohydrates that feed into central metabolism via peripheral pathways that form dihydroxyacetone phosphate (i.e. glycerol), fructose 1,6-diphosphate (i.e. fructose), and fructose-6-phosphate (i.e. mannitol) [50,51]. It is interesting that a class of glycerol-positive EDD revertants which regained only a limited capacity to metabolize 6-phosphogluconate are still glucose-negative [53]. Phosphoglucose isomerase mutants and glucose-6-phosphate dehydrogenase mutants [51], as well as fructose diphosphate aldolase mutants [54] are also impaired for growth on glycerol, fructose, etc. These experiments indicate that formation of 6-phosphogluconate from glycerol, fructose, and mannitol via the gluconeogenic enzymes is an important step in catabolism.

The impaired ability of the gluconeogenic and Entner-Doudoroff mutants to grow on glycerol is particularly interesting since all of the enzymes

necessary for conversion of glycerol to pyruvate via the triose phosphate portion of the pathway are expressed. Moreover, mutants with defects in either glyceraldehyde phosphate dehydrogenase or phosphoglycerate kinase are able to grow normally on glycerol, or for that matter, on glucose and gluconate [54]. Likewise, mutants defective in either enolase or pyruvate kinase grow normally on fructose, glycerol, etc. [50,51]. Growth on glycerol induces glucose-6-phosphate dehydrogenase and the Entner-Doudoroff enzymes [50,51]. The inducer molecule of these enzymes in the pseudomonads is most likely 6-phosphogluconate [55]. Galactonate, a compound that is degraded to glyceraldehyde-3-phosphate via the DeLey-Doudoroff pathway independently of the Entner-Doudoroff pathway, induces glucose-6-phosphate dehydrogenase, EDD, and EDA. Moreover, EDD^- and EDA^- mutants are impaired for growth on galactonate [57]. Together, these experiments indicate that conversion of glyceraldehyde-3-phosphate can proceed directly via the triose phosphate intermediates or via recycling through 6-phosphogluconate, the latter pathway apparently being more physiologically significant.

Despite having an inducible 1-phosphofructokinase that is compatible with Embden-Meyerhof-Parnas catabolism of fructose (which is transported via the fructose-PTS), several species of *Pseudomonas* use the Entner-Doudoroff pathway as the primary route for fructose catabolism [56]. Alginate, a polysaccharide derived from the metabolic precursor fructose-6-phosphate, is formed during growth on glycerol, fructose, mannitol, glucose, gluconate, or succinate. Phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, EDD^-, and EDA^- mutants are all defective in alginate synthesis [58], as are fructose 1,6-bisphosphate aldolase mutants [59]. Labelling studies indicate that the majority of labelled carbon in alginate comes not from the pyruvate formed during glucose catabolism, but from glyceraldehyde-3-phosphate only [60]. These experiments indicate that recycling of fructose-1,6-diphosphate to 6-phosphogluconate is important physiologically and that fructose-6-phosphate is formed by recycling of triose phosphates.

Several Gram-negative, aerobic microorganisms that are related to the pseudomonads appear to utilize the Entner-Doudoroff pathway in the cyclic mode. It has been shown that incorporation of labelled glucose into alginate by *Azotobacter vinelandii* involves recycling of triose phosphates [60,61]. In *Alcaligenes eutrophus*, phosphoglycerate mutase mutants lose the ability to grow on succinate, but are still able to grow on fructose, a pattern similar to that observed for *Pseudomonas* mutants of the triose phosphate pathway [62]. Mutants of *Rhizobium meliloti* that are defective in glucose-6-phosphate dehydrogenase activity are impaired for growth on several carbohydrates and accumulate significant quantities of glucose-6-phosphate from galactose, despite almost normal growth on that sugar [63]. One common feature of each of the organisms mentioned above is that they do not utilize the Embden-Meyerhof-Parnas pathway, due to deficiency of 6-phosphofructokinase. One exception to this rule has been observed in an organism that is not related to the pseudomonads. Tabita and Lundgren [64] suggested that the unexpected early release of C_6 carbon from glucose being degraded via the Entner-Doudoroff pathway in *Thiobacillus ferrooxidans* was the result of gluconeogenic formation of glucose-6-phosphate from triose phosphates, which then enters the pentose phosphate pathway. It should be noted that this is a different recycling pathway than that employed by the pseudomonads.

In an extremely interesting experiment, Steinbuchel [65] demonstrated that it is possible to convert *A. eutrophus*, a naturally 6-phosphofructokinase-deficient organism, to Embden-Meyerhof-Parnas metabolism by expression of the *E. coli pfk* gene. Successful Embden-Meyerhof-Parnas metabolism in *pfk*-expressing strains was observed in an EDA^- background, but not in the wild type. The recombinant EDA^+ strain that could potentially utilize both the cyclic Entner-Doudoroff pathway and the Embden-Meyerhof-Parnas pathway was shown to be significantly impaired for growth on fructose. Steinbuchel [65] suggested the operation of a futile cycle resulting from the combined presence of the native fructose 1,6-bisphosphatase and the cloned 6-phos-

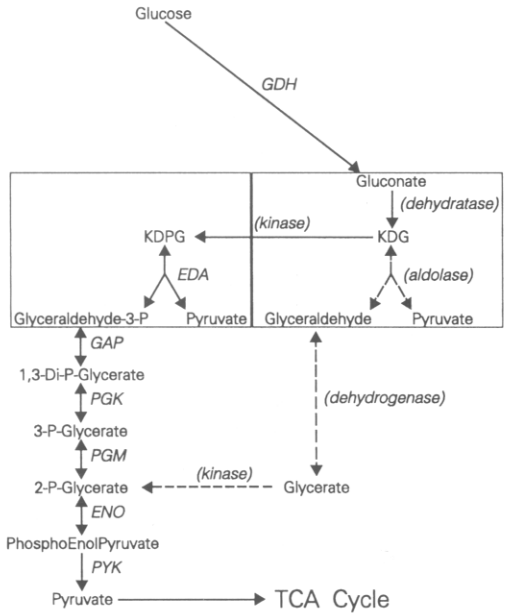

Fig. 8. Modified Entner-Doudoroff pathway. First version of the non-phosphorylated pathway as it operates in clostridia and some Archaebacteria involves conversion of KDG to KDPG by KDG kinase. Second version of the non-phosphorylated pathway as it operates in some other Archaebacteria involves cleavage of KDG by KDG aldolase, oxidation of the glyceraldehyde to glycerate by a dehydrogenase and phosphorylation by a kinase to form 2-phosphoglycerate (dashed arrows). Abbreviations as in legends to Figs. 5 and 6.

phofructokinase. The presence of these two enzymes in the cyclic Entner-Doudoroff organism, *A. eutrophus*, has a dramatically different effect on metabolism than the analogous situation in *E. coli*, as was described above. Apparently, in vivo regulation of either fructose bisphosphatase or fructose bisphosphate aldolase is very different in organisms employing the cyclic mode, by comparison to organisms such as *E. coli* that employ the linear mode of Entner-Doudoroff metabolism.

4.4. Modified pathway

Several examples of microorganisms that utilize a modified version of the Entner-Doudoroff pathway, involving non-phosphorylated intermediates, are known (Fig. 8). One version of the pathway was first described by Szymona and Doudoroff [66] for *Rhodobacter (Rhodopseudomonas) sphaeroides*. This modified pathway involves conversion of gluconate to 2-keto-3-deoxygluconate (KDG) by a specific gluconate dehydratase. Further metabolism of KDG involves its phosphorylation by KDG kinase to form KDPG, followed by cleavage by EDA. This same modified pathway has been shown to occur in *Clostridium aceticum* [67] and several other species of *Clostridium* [68]. Interestingly, the halophilic Archaebacterium, *Halobacterium saccharovorum*, utilizes this same modified Entner-Doudoroff pathway for oxidative glucose metabolism via gluconate [69]. All of these latter organisms are characterized by the presence of gluconate dehydratase and KDG kinase, and by a lack of gluconokinase and EDD. The properties of the gluconate dehydratase purified from *C. pasteurianum* were described above. A large number of related non-phosphorylative pathways involving an initial dehydration reaction have been described. Pathways for metabolism of arabonate, galactonate (the DeLey-Doudoroff pathway), glucosaminic acid, mannonate, and altronate have been very nicely reviewed by Andreesen and Gottschalk [67].

A second modified version of the Entner-Doudoroff pathway was discovered in extreme thermoacidophiles. The modified pathway involving gluconate dehydratase and KDG aldolase allows metabolism of glucose to glyceraldehyde and pyruvate in *Sulfolobus solataricus* [70]. More recent studies with *Thermoplasma acidophilum* indicate that the glyceraldehyde formed via this non-phosphorylative route is converted by glyceraldehyde dehydrogenase to glycerate, which is phosphorylated to form 2-phosphoglycerate [71]. Thus, both of the modified Entner-Doudoroff pathways involving non-phosphorylated intermediates are present in the Archaebacteria. The enzymology of central metabolism in the Archaebacteria has been reviewed by Danson [72].

4.5. Involvement in methylotrophic metabolism

The ribulose monophosphate pathway (RMP) is used for formaldehyde assimilation in a number of obligate and facultative methylotrophs [73]. The formation of fructose-6-phosphate from formaldehyde and ribulose-5-phosphate occurs in stage 1. One variation of the stage 2 cleavage of

Table 1

Natural occurrence of the Entner-Doudoroff pathway

Organism	Enzymes	Reg.	Signal(*)	Mode	Other paths	Reference
Section 1.						
Treponema saccharophilum	EDA	n	n	n	EMP [a]	84
Section 2.						
Aquaspirillum itersonii	EDD EDA	i	fructose*	C	N	85
A. gracile	EDD EDA	i	glucose*	n	EMP	86
A. autotrophicum	EDD EDA	c	N	n	EMP PP [b]	87
Azospirillum brasilense	EDD EDA	c	N	n	EMP	88, 89
A. lipoferum	EDD EDA	c	N	n	EMP	89
Section 3.						
Spirosoma linguale	EDD EDA	i	gluconate*	n	EMP PP	90
Flectobacillus marinus	EDD EDA	i	gluconate*	n	EMP PP	91
Microcyclus aquaticus	EDD EDA	c	N	n	EMP PP	90
Section 4.						
Pseudomonas aeruginosa	EDD EDA	i	6-P-gluconate	C	N	77, 80, 92
P. fluorescens	EDD EDA	i	gluconate	C	PP	77, 80, 93
P. chlororaphis	EDD EDA	n	n	n	n	77, 80
P. aereofaciens	EDD EDA	n	n	n	n	77, 80
P. putida	EDD EDA	i	gluconate*	C	N	77, 80
P. stutzeri	EDD EDA	i	gluconate*	n	N	
P. mendocina	EDD EDA	i	gluconate*	n	N	57, 77, 80
P. cepacia	EDD EDA	i	6-P-gluconate	C	PP	55, 77, 80
P. acidovorans	EDD EDA	i	gluconate*	n	N	57, 77, 80
P. saccharophila	EDD EDA	i	gluconate*	n	N	1, 77, 80
P. diminuta	EDD EDA	n	n	n	n	80
P. maltophila	EDD EDA	n	n	n	n	57, 80
P. citronellolis	EDD EDA	i	gluconate*	n	N	80, 94
P. marina	EDD EDA	n	n	n	n	95
Xanthomonas campestris	EDD EDA	c	N	n	PP	33, 80
Azotobacter beijerinckii	EDD EDA	n	n	n	N	96
A. vinelandii	EDD EDA	n	n	n	N	61, 80
Azomonas agilis	EDD EDA	n	n	n	n	80
A. macrocytogenes	EDD EDA	n	n	n	n	80
Rhizobium leguminosarum	EDD EDA	i	glucose*	n	PP	80, 97
R. meliloti	EDD EDA	i	carbon [d]	C	PP	80, 98
Bradyrhizobium japonicum	EDD EDA	n	n	n	EMP	80, 99
Agrobacterium tumefaciens	EDD EDA	n	n	n	PP	80, 100
A. radiobacter	EDD EDA	n	n	n	PP	80, 100
A. rhizogenes	EDD EDA	n	n	n	PP	80, 100
A. rubi	EDD EDA	n	n	n	PP	80, 100
Methylococcus capsulatus	EDD EDA	c	N	n	n	74
Methylomonas methanica	EDD EDA	c	N	n	n	74
Acetobacter aceti subsp. *xylinum*	EDD EDA	n	n	n	n	80
Gluconobacter oxydans	EDD EDA	n	n	n	n	80
Legionella pneumophila	EDD EDA	n	n	n	n	101
Neisseria gonorrhoeae	EDD EDA	i	serum	L	PP	31
Acinetobacter calcoaceticus	EDD EDA	n	n	n	n	102
Xanthobacter autotrophicus	EDD EDA	i	carbon	n	N	103
Alteromonas macleodii	EDD EDA	n	n	n	n	95
A. haloplanktis	EDD EDA	n	n	n	n	95
A. espejiana	EDD EDA	n	n	n	n	95
A. unida	EDD EDA	n	n	n	n	95
A. communis	EDD EDA	n	n	n	n	95

Table 1(continued)

Organism	Enzymes	Reg.	Signal(*)	Mode	Other paths	Reference
A. vaga	EDD EDA	n	n	n	n	95
Flavobacterium sp.	EDD EDA	n	n	n	n	80
Alcaligenes faecalis	EDD EDA	n	n	n	n	104
A. denitrificans	EDD EDA	n	n	n	n	104
A. eutrophus	EDD EDA	i	carbon	C	N	104, 105
A. aestus	EDD EDA	n	n	n	n	95
A. cupidus	EDD EDA	n	n	n	n	95
A. pacificus	EDD EDA	n	n	n	n	95
A. venustus	EDD EDA	n	n	n	n	95
Bordetella bronchiseptica	EDD EDA	n	n	n	n	80
Paracoccus denitrificans	EDD EDA	n	n	n	PP	106
Section 5.						
Escherichia coli	EDD EDA	i	gluconate	L	EMP PP	34, 80
Salmonella typhimurium	EDD EDA	i	n	n	EMP PP	6
Klebsiella pneumoniae	EDD EDA	n	n	n	EMP PP	80
Erwinia herbicola	EDA	n	n	n	EMP PP	80
E. uredovora	EDA	n	n	n	EMP PP	80
Serratia marcescens	EDD EDA	n	n	n	EMP PP	80
Proteus mirabilis	EDD EDA	n	n	n	EMP PP	80
Yersinia pestis	EDD EDA	n	n	n	PK [c]	107
Y. pseudotuberculosis	EDD EDA	i	gluconate*	n	EMP	108
Vibrio harveyi	EDD EDA	i	gluconate*	n	EMP PP	109
Photobacterium phosphoreum	EDD EDA	i	gluconate*	n	EMP PP	109
P. leiognathi	EDD EDA	i	gluconate*	n	EMP PP	109
Plesiomonas shigelloides	EDD EDA	n	n	n	n	80
Haemophilus influenzae	EDD EDA	c	N	n	EMP PP	110
H. aegyptius	EDD EDA	c	N	n	EMP PP	0
H. parainfluenzae	EDD EDA	c	N	n	EMP PP	0
Zymomonas mobilis	EDD EDA	c	N	L	N	111
Chromobacterium violaceum	EDD EDA	n	n	n	n	80
Section 12.						
Streptococcus faecalis	EDD EDA	i	n	n	EMP PP	112
Section 13.						
Bacillus larvae	EDD EDA	c	N	n	EMP PP	113
B. stearothermophilus	EDD EDA	n	n	n	EMP PP	114
Clostridium aceticum	EDD*EDA	i	n	M	EMP	67
C. butyricum	EDD*EDA	i	n	M	EMP	68
C. formicoaceticum	EDD*EDA	i	n	M	EMP	68
C. pasteurianum	EDD*EDA	i	n	M	EMP	68
C. roseum	EDD*EDA	i	n	M	EMP	68
Section 15.						
Arthrobacter pascens	EDD EDA	n	n	n	PP	115
A. atrocyaneus	EDD EDA	n	n	n	PP	115
A. simplex	EDD EDA	n	n	n	PP	115
Section 16.						
Mycobacterium smegmatis	EDD EDA	i	glucose	n	EMP PP	116
Section 17.						
Rhodococcus rhodocrous	EDD EDA	n	n	n	n	80
Section 18.						
Rhodobacter capsulata	EDD EDA	c	N	L	PP	46
R. sphaeroides	EDD EDA	i	carbon	L, M	PP	48

Table 1(continued)

Organism	Enzymes	Reg.	Signal(*)	Mode	Other paths	Reference
Section 20.						
Thiobacillus intermedius	EDD EDA	i	glucose	n	PP	117
T. perometabolis	EDD EDA	i	glucose	n	PP	118
T. ferrooxidans	EDD EDA	i	glucose	C	PP	64
T acidophilus	EDD EDA	n	n	n	PP	119
Acidiphilium cryptum	EDD EDA	n	n	n	PP	120
Caulobacter crescentus	EDD EDA	c	N	n	N	121, 122, 123
Asticcaccaulis excentricus	EDD EDA	n	n	n	N	124
Angulomicrobium tetredrale	EDD EDA	n	n	n	n	125
Section 22.						
Leptothrix discophora	EDD EDA	n	n	n	N	126
Section 23.						
Leucothrix mucor	EDD EDA	n	n	n	EMP PP	127
Section 25.						
Halobacterium saccharovorum	EDD*EDA	n	n	M	N	69, 72
H. halobium	EDD*EDA	n	n	M	N	71, 72
Sulfolobus solfataricus	EDD*EDA*	n	n	M	N	70, 72
Thermoplasma acidophilum	EDD*EDA*	n	n	M	N	71, 72
Eukaryotes						
Entamoeba histolytica	EDD EDA	n	n	n	n	128
Aspergillus niger	EDD*EDA*	i	gluconate	M	EMP PP	129
Penicillium notatum	EDD EDA	c	n	L, M	EMP PP	130

[a] EMP (Embden-Meyerhof-Parnas pathway) characterized by presence of 6-phosphofructokinase.
[b] PP (pentose phosphate pathway) characterized by presence of 6-phosphogluconate dehydrogenase.
[c] PK (phosphoketolase pathway) characterized by presence of phosphoketolase.
[d] Carbon indicates free-living or heterotrophic conditions.
Signal(*) Asterisk denotes that the signal is either the indicated carbon source or a metabolic derivative.
EDD* indicates a modified mode of Entner-Doudoroff metabolism involving gluconate dehydratase.
EDA* indicates a modified mode of Entner-Doudoroff metabolism involving KDG aldolase.
Abbreviations: n, not known; i, inducible; c, constitutive; N, none, C, cyclic mode; L, linear mode; M, modified mode.

fructose-6-phosphate to form two 3-carbon compounds involves the Entner-Doudoroff pathway. In this version of the RMP, fructose-6-phosphate is converted to glucose-6-phosphate by the action of phosphoglucose isomerase and glucose-6-phosphate is converted to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. The 6-phosphogluconate is converted to glyceraldehyde-3-phosphate and pyruvate through the actions of EDD and EDA. The glyceraldehyde-3-phosphate is then recycled to ribulose-5-phosphate. The presence of the Entner-Doudoroff pathway in several obligate methylotrophs, including *Methylococcus capsulatus*, *Methylomonas methanica*, and *Methylosinus trichosporium* was first described by Strom et al. [74]. This same version of the RMP was also found in several obligate and facultative methylotrophic species of *Bacillus* [75]. The Entner-Doudoroff pathway was shown to operate for glucose metabolism in some facultative methylotrophs, including *Methylobacterium organophilum*, in addition to operation of the RMP [76]. The use of the Entner-Doudoroff enzymes in the capacity of C_1 metabolism is indicative of their versatility and of their ability to cope with positions in metabolic pathways that experience high flux rates.

4.6. Involvement in anabolic metabolism

The presence of the Entner-Doudoroff pathway in the obligate methylotrophs, as seen above, is particularly interesting. These organisms are

incapable of growth on carbohydrates, dispelling the notion that the Entner-Doudoroff pathway is a strictly catabolic pathway. Some species of *Pseudomonas* are unable to grow at the expense of carbohydrates, but there are few other examples of bacteria that possess the enzymes of the Entner-Doudoroff pathway and are unable to utilize carbohydrates [77]. The prejudiced perspective that the pathway is employed only for the purpose of carbohydrate catabolism has apparently inhibited searches for the Entner-Doudoroff pathway in non-carbohydrate utilizers. Nevertheless, some studies suggest the involvement of the pathway in anabolic metabolism. The induction of the Entner-Doudoroff enzymes and other enzymes of central metabolism for growth of *P. aeruginosa* on glycerol, as described above, suggests an anabolic role for the pathway. Perhaps the best evidence for anabolic operation of the Entner-Doudoroff pathway comes from studies on hopanoid biosynthesis. The incorporation of labelled acetate into hopanoids in *Methylobacterium organophilum, Rhodopseudomonas palustris*, and *R. acidophila* has been shown to proceed via the Entner-Doudoroff pathway in the absence of any additional carbon sources [78]. Preiss et al. [79] have suggested that activation of ADPglucose phosphorylase from *R. sphaeroides* by fructose-6-phosphate is consistent with the involvement of the Entner-Doudoroff pathway in glycogen synthesis.

5. OCCURRENCE AND PROPERTIES

In the years that followed discovery of the Entner-Doudoroff pathway, the obvious question of distribution in the microbial world was considered. In a monumental investigation, Kersters and DeLey [80] assayed for the Entner-Doudoroff enzymes in more than 150 strains belonging to 37 genera. The Entner-Doudoroff pathway was found to be widely distributed in Gram-negative bacteria, but rare in Gram-positives. Since 1968, numerous studies of carbohydrate metabolism have revealed the presence of Entner-Doudoroff metabolism in previously unrecognized, untested organisms. Also, bacterial taxonomy and classification have changed significantly over the last 24 years. Thus, it is time to take another look at the distribution of the Entner-Doudoroff pathway.

The data presented include many of the results of the Kersters and DeLey [80] study, as well as others that have been conducted since. Where information is available, some specific aspects of the mode and regulation of the pathway are included. The organization of Table 1 is based on the classification schemes of Bergey's Manual of Systematic Bacteriology [81–83]. Where numerous species within a genus are known to contain the Entner-Doudoroff pathway, and a general description of the genus is available, these references are included in Table 1.

It is immediately obvious from the data that the Entner-Doudoroff pathway is far more widely distributed than previously recognized. The pathway, and modified versions of the pathway, are found in organisms representing most sections of Bergey's Manual including both Gram-negative and Gram-positive bacteria. Moreover, the pathway is present in several eukaryotic organisms. An enzyme that is closely related to EDA, KHG aldolase, which is involved in hydroxyproline synthesis, is present in bovine liver (see above). Nature has certainly found ways to make extensive use of the Entner-Doudoroff pathway and of the Entner-Doudoroff enzymes.

6. GENETICS AND MOLECULAR BIOLOGY

The physiology of an organism must be considered in order to fully appreciate the importance of the molecular mechanisms that govern gene expression. In the following discussion of the genetics and molecular biology of Entner-Doudoroff metabolism, the physiological constraints that have dictated the evolution of certain regulatory features are addressed. The three bacteria described below are the only organisms that have been subjected to detailed molecular analyses. This is fortuitous, since each of them employs the Entner-Doudoroff pathway in a different mode. These investigations provide some insight into the role of gene expression in coordinating the levels of metabolic enzymes.

6.1. Gene organization and expression in Z. mobilis

Z. mobilis, for the reasons discussed above, must maintain rapid carbon flux while keeping the levels of toxic metabolic intermediates low. Rapid carbon flux is allowed by high level expression of the glycolytic and alcohologenic enzymes [25,26]. Also of particular importance in the Entner-Doudoroff pathway is the intracellular concentration of the key intermediate, KDPG. This compound is known to be highly toxic for *E. coli*; mutations that lead to KDPG accumulation are lethal and secondary mutations that prevent KDPG accumulation restore viability [6]. Therefore, coordinate expression of the enzymes responsible for KDPG formation and degradation is a necessary constraint on metabolism in *Z. mobilis*. Much of the molecular genetic studies of *Z. mobilis* metabolism have focused on understanding how the genes that encode the central pathway enzymes are expressed at such high levels and how their expression is balanced appropriately. Given that each of the glycolytic genes is essential, classical genetic approaches for the study of *Z. mobilis* metabolism have not been used, nor would these approaches have been profitable. Only through the use of molecular genetic tools was the current understanding of *Z. mobilis* made possible.

In *Z. mobilis*, the *edd* gene is clustered on the genome with several other genes involving glucose metabolism (Fig. 9A). The genes that are linked to *edd* include *glf*, which encodes the glucose-facilitated diffusion transporter, *zwf* which encodes glucose-6-phosphate dehydrogenase, and *glk* which encodes glucokinase [11]. The *glf*, *zwf*, *edd*, and *glk* genes form an operon, in that order, and are co-transcribed from a single promoter [131]. The *zwf* and *edd* genes overlap by 8 bp, suggesting that balanced expression of these two genes is important. The arrangement of the genes that code for glucose transport and the first three steps of central metabolism in an

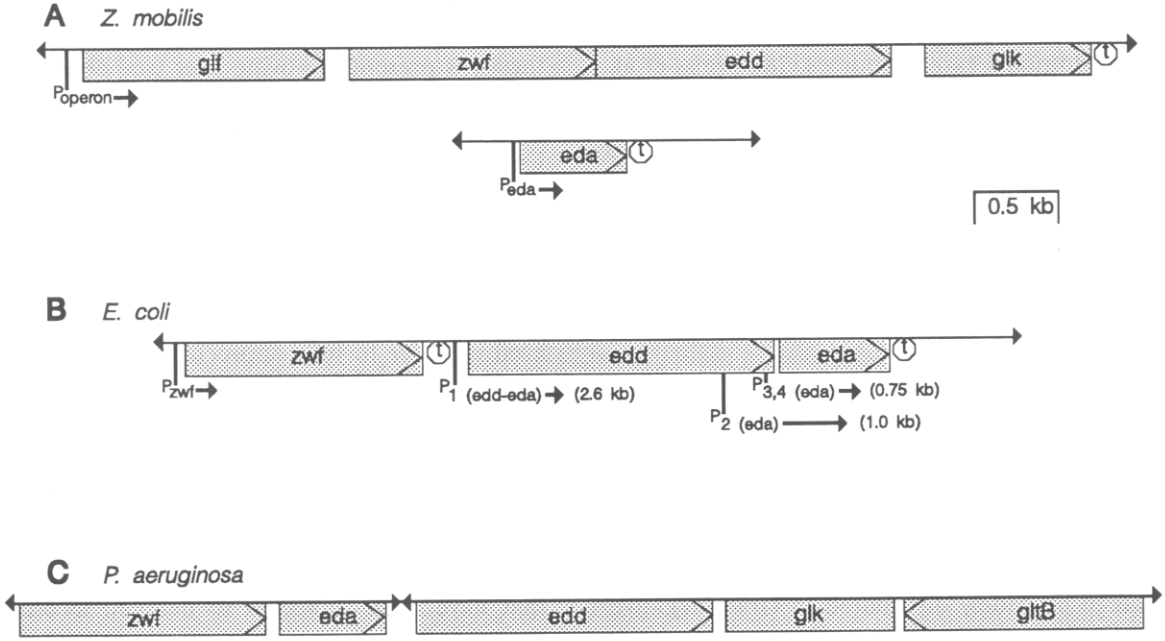

Fig. 9. Expression of the Entner-Doudoroff genes. **(A)** *Z. mobilis*. The *eda* gene is unlinked to the *glf-zwf-edd-glk* operon. **(B)** *E. coli*. **(C)** *P. aeruginosa*. Positions of mapped promoters are indicated by arrows. Transcriptional terminators are indicated by 'stop signs' (t). Where relevant, the size of transcripts is indicated at the respective promoter. Maps depicted in **A** and **B** are drawn to scale and a size bar is provided. For the map shown in **C**, the gene order is known, as is the direction of transcription of the indicated genes, but the precise gene locations and sizes are not established. The gap between *eda* and *edd* is approximately 4 kb.

operon provide the organism with a mechanism for controlling the flux of carbon to KDPG. The *eda* gene is monocistronic and is not tightly linked to the *glf* operon [16]. Equally important in maintaining low KDPG concentrations are the activities of the enzymes responsible for KDPG degradation, particularly EDA, which consumes KDPG as a substrate. EDA, along with the next five glycolytic enzymes in the pathway all have in vivo activities that are at least two-fold higher than the enzymes responsible for KDPG formation [16,22,25,26].

The molecular mechanisms responsible for coordinating balanced, high-level expression of the pathway enzymes are beginning to be understood. Accumulating evidence indicates that several layers of genetic control, perhaps in a hierarchical arrangement, act in concert to determine the relative abundance of the glycolytic enzymes. The genes all possess canonical ribosome binding sites and display high codon bias [16,132]. The relative stabilities of the individual mRNAs are directly proportional to the abundance of the glycolytic gene products [133]. The promoters of the glycolytic genes share common structural features that appear to impart high transcription rates [131]. Modulation of promoter activity, presumably by transcription factors, has been observed [131]. Lastly, post-transcriptional mRNA processing appears to be important for dictating an appropriate balance of the glycolytic enzymes [134,135].

High-level expression of the glycolytic genes can be attributed, in part, to the very efficient translational initiation provided by high-quality ribosome binding sites. Several poorly expressed *Z. mobilis* genes possess poor ribosome binding sites, by comparison [16]. Interestingly, an examination of the highly expressed *Z. mobilis* genes indicates that the relative quality of the ribosome binding sites of these genes might play an important role in determining the relative level of enzyme synthesis [16]. Each of the highly expressed *Z. mobilis* glycolytic genes exhibit very high codon bias, while there is little codon bias in poorly expressed genes [132,136]. This is clearly the case for the poorly expressed *Z. mobilis lig* gene that codes for DNA ligase (Shark, K. and Conway, T., unpublished data). The use of seven extremely rare codons in the glycolytic gene set (average of 10 structural genes) is common for *lig*. Moreover, codon usage for the *lig* gene is biased toward these rare codons of the glycolytic gene set. It is clear that the structures of the *Z. mobilis* Entner-Doudoroff genes are conducive to efficient translation.

It appears likely that high-level expression of the glycolytic genes is also coordinated at the level of mRNA abundance. The relative abundance of specific mRNAs is regulated by the relative rates of transcription and message decay. Transcript stabilities and protein abundance have been determined for eight of the glycolytic enzymes [133]. The transcripts are all unusually stable, with half-lives ranging between 8 and 18 min. Among the eight glycolytic genes and gene products studied, protein abundance is directly proportional to transcript stability. Thus, differential transcript stability is hypothesized to be a primary mechanism for balancing the levels of individual enzymes of the *Z. mobilis* glycolytic pathway. Also, high transcript stability is proposed to be a dominant feature in distinguishing between the highly expressed glycolytic genes and the vast majority of poorly expressed biosynthetic and housekeeping genes.

A single site of transcriptional initiation for the polycistronic *glf-zwf-edd-glk* message was mapped by primer extension and nuclease S1 protection analysis (Fig. 9; [131]). The *eda* promoter was also mapped to a single transcriptional initiation site (Fig. 9; [16]). The *glf* operon and *eda* promoter regions share significant identity to several other highly expressed *Z. mobilis* promoters, but not to consensus promoters from other bacteria. The highly expressed *Z. mobilis* promoter set is characterized by two independent, overlapping, conserved sequences that extend from approximately -100 to $+15$ with respect to the transcriptional start sites [131]. It is quite possible, perhaps even likely, that the structures of these highly expressed promoters identify them as members of a separate, high-level expression class.

Various experiments have shown a carbon source-dependent stimulation of phosphoglucose

isomerase, fructokinase, and the enzymes of the *glf-zwf-edd-glk* operon when growing on fructose [27,131,137]. The other enzymes of central metabolism do not show carbon source-dependent regulation. The physiological role of increasing phosphoglucose isomerase and fructokinase activities is to increase flux of fructose into the pathway. The increase in *glf* expression when growing on fructose suggests that the same transporter is used for both glucose and fructose transport, although the apparent affinity of the transporter is far greater for glucose than for fructose [23]. The increase in *pgi*, *frk* and *glf-zwf-edd-glk* expression in *Z. mobilis* cells grown on fructose is the result of an increased rate of transcription, rather than differential mRNA stability or differential mRNA processing on the two carbon sources [27,131,137]. This increase in transcription rate leads to a three-fold increase in the abundance of the *pgi*, *frk* and *glf-zwf-edd-glk* mRNAs. These experiments suggest the involvement of a transcription factor that serves to modulate transcription rates in the presence of fructose. It will be interesting to learn whether additional transcriptional control(s) of Entner-Doudoroff gene expression exist.

The full-length 6.14-kb polycistronic *glf-zwf-edd-glk* mRNA from *Z. mobilis* appears to be processed by endonucleolytic cleavage, resulting in the formation of several discrete transcripts [134]. Northern analysis of the fate of the 6.14-kb transcript following inhibition of transcription by rifampicin shows that the abundance of shorter, more stable transcripts increases at the expense of longer, less stable transcripts. The most abundant 5′ and 3′ transcript ends lie within secondary structures that probably impart stability to the most abundant mRNAs. The transcript mapping data reveal that the processed transcripts correspond to functional mono-, di-, or tricistronic messages. The relative abundance of the processed transcripts is a function of their respective decay rates.

The relative amounts of glucose-6-phosphate dehydrogenase, EDD, and EDA in *Z. mobilis*, calculated as intracellular enzyme concentrations, are 0.10 mM, 0.17 mM, and 0.34 mM, respectively [16]. These intracellular enzyme concentrations parallel the relative abundance of the gene-specific transcripts [133,134]. The purified specific activity of glucose-6-phosphate dehydrogenase is two-fold higher than that of 6-phosphogluconate dehydratase, but the measured in vivo activities of these two enzymes are equal in *Z. mobilis* [16]. The higher relative abundance of the *edd* transcript appears to ensure that the two enzyme activities are balanced. The overlapping coding regions of the *zwf* and *edd* genes suggests that the two genes might be co-translated [11]. This, however, does not appear to play a major role in coordinating expression of the two genes since only one-third of the *edd* messages also contain the upstream *zwf* coding region [131] and since the *edd* message can be translated independent of *zwf* in *E. coli* [11]. Independent expression of the monocistronic *eda* gene appears to ensure a significantly higher in vivo EDA activity [16].

6.2. Gene organization and expression in E. coli

In *E. coli*, the *edd* and *eda* genes are expressed differentially [34]. Expression of *edd* is induced for growth on gluconate and is virtually absent in cells grown on glucose [43,138]. On the other hand, high basal expression of *eda* occurs regardless of carbon source [36,43,139]. Although *eda* expression is constitutive, three-fold induction occurs for growth on gluconate and two-fold induction occurs for growth on hexuronic acids. The latter is consistent with the role of EDA as a key enzyme of the peripheral pathway for hexuronic acid metabolism, a function independent of the Entner-Doudoroff pathway [36].

The *edd* and *eda* genes are tightly linked to the *zwf* gene (Fig. 9B; [140]). The location of these genes on the *E. coli* physical map has been reported [141]. Despite tight linkage, *zwf* and the two Entner-Doudoroff genes are controlled differently. Expression of *zwf* is controlled in a growth rate-dependent fashion [142]. Expression of *edd*, as well as *gntT* and *gntU* (gluconate transport) and *gntK* (gluconokinase), is controlled in a negative fashion by the *gntR* product, which presumably encodes a repressor protein [35]. Catabolite repression of *gntT*, *gntU*, and *gntK* has been demonstrated [44]. Expression of

eda is subject to control by the *kdgR* product, as are the permease and kinase for 2-keto-3-deoxygluconate [36]. These genetic studies form the basis for much of what is understood about regulation of the Entner-Doudoroff pathway in *E. coli*. The molecular details of this regulation are being addressed.

The complete nucleotide sequence of the *E. coli zwf* gene is published and the promoter has been located [143]. Genetic and physical analyses of *zwf* expression have established that the *zwf* gene is monocistronic and subject to growth rate-dependent regulation at the transcriptional level [144]. Just 256 bp downstream of *zwf* lies the *E. coli edd–eda* operon. The Entner-Doudoroff genes are separated by only 36 bp [12]. A single 2.6-kb transcript was detected by Northern analysis with both *edd*- and *eda*-specific DNA hybridization probes [12]. The *zwf–edd* intergenic region contains a transcriptional terminator-like structure immediately upstream of P_1 for *edd–eda* (Fig. 9B). The entire *edd–eda* region contains only one additional transcriptional terminator-like structure, found immediately downstream of *eda* [12]. Molecular and computer characterization of *edd–eda* transcription revealed the presence of four putative promoters in this region (Fig. 9B; [12]). The 2.6-kb message transcribed from P_1 encodes both *edd* and *eda*. The fact that this transcript was observed only in cells grown on gluconate argues strongly that P_1 is regulated by gluconate (unpublished data). Thus, P_1 is hypothesized to be the primary gluconate-inducible promoter responsible for induction of the Entner-Doudoroff pathway, as mediated by the *gntR* product [35].

Constitutive expression of *eda* is due to three *eda*-specific promoters within the upstream *edd* gene [12]. The level of the 1.0-kb message that is transcribed from P_2 is regulated in response to growth on glucuronic acid (unpublished results). Thus, P_2 is hypothesized to be the glucuronic acid-responsive promoter responsible for induction of *eda*, as mediated by the *kdgR* product [36]. The P_3 and P_4 promoters give rise to the 0.7-kb transcript observed on Northern blots. Increased levels of the 0.7-kb transcript are induced by growth on gluconate, arguing in favor of the hypothesis that a gluconate-regulated promoter is present in this region. Whether one or both of the mapped 5′ ends in this region is a true transcriptional initiation product is not known [12]. It should be noted that, although the quality of the P_2, P_3, and P_4 promoters is uniformly poor, all three are located within the *edd* structural gene. The sequences that form these promoters must ultimately provide enzyme function also. In fact, P_2 is located within a region of highly conserved amino acid residues. Regulation of P_1, P_3 and P_4 by gluconate might be mediated by a conserved nine base sequence that overlaps the '−35 regions' of these gluconate-responsive promoters [12]. There is a need for further characterization of the *E. coil edd–eda* promoter regions before the molecular details of induction of the Entner-Doudoroff pathway can be fully understood.

6.3. Gene organization and expression in P. aeruginosa

The genetics of the Entner-Doudoroff pathway in *P. aeruginosa* have been reviewed recently [51]. The *zwf*, *eda*, *edd* and *glk* genes have been mapped, in that order [145], and cloned [146]. The order of these genes on the genome is indicated in Fig. 9C. At least some of these genes are tightly linked, as indicated by the isolation of a 4.6-kb genomic DNA fragment containing *gltB* (glucose binding protein), *glk*, and *edd*. A second 7.0-kb DNA fragment containing *zwf* and *eda* has also been isolated [147]. Evidence from work in progress (P.V. Phibbs, East Carolina University, personal communication) indicates that *zwf* and *eda* may be co-transcribed. The *eda* and *edd* genes are separated by approximately 4 kb and the *gap* gene lies between the two Entner-Doudoroff genes. The *edd* and *gap* genes are tightly linked and appear to be transcribed from divergent promoters. It is well established that *zwf*, *eda*, *edd*, and *glk* are co-inducible, with the available evidence implicating 6-phosphogluconate as the physiological inducer [55,147]. Although initial studies suggest that regulation of the Entner-Doudoroff genes in *P. aeruginosa* is complex, additional molecular genetic analyses of these genes are necessary [147].

7. EVOLUTION OF THE PATHWAY

One of the more intriguing questions related to metabolic biology has to do with the evolution of glycolytic pathways. On the basis of arguments that are described in much greater detail elsewhere, it is possible to fix the point on the evolutionary time scale at which the Entner-Doudoroff pathway arose in relation to the series of evolutionary events leading from the primordial soup to the present. It is equally possible, using similar arguments, to describe the current distribution of the Entner-Doudoroff pathway in nature in a way that satisfactorily explains its presence in the face of the obviously more efficient Embden-Meyerhof-Parnas pathway. Readers are referred to writings by Gest and Schopf [148], by Fothergill-Gillmore [149], and by Quayle and Ferenci [150] for extensive reviews on the subject of metabolic evolution. Romano and Saier [151] have summarized the arguments, supported by experimental evidence, which suggest that the Entner-Doudoroff pathway predates the Embden-Meyerhof-Parnas pathway. These ideas are recapitulated to a certain extent here, with the addition of still further supportive arguments. The speculations in this section are included solely for the purpose of provoking thought.

Although some controversy remains, it is generally accepted that the primitive Earth atmosphere was anaerobic, consisting of inorganic chemicals that could give rise to carboxylic acids, amino acids, aldehydes, sugars, purines, etc. [148,150]. Sugars would have been formed through a series of abiotic reactions involving formaldehyde [150]. Glucose, being the most stable of the hexoses, would have had the greatest opportunity to accumulate in the primordial soup [148]. Early biological carbohydrate chemistry, resembling reactions catalysed by aldolases, isomerases, epimerases and transaldolases, etc. would have been patterned after the abiotic formose reactions [148]. As abiotically formed precursors for biosynthesis were depleted through polymerization by primitive biological systems, the demand for biochemical synthesis of these precursors would have grown. The concomitant demand for biochemical energy transduction would have supplied significant evolutionary pressure for the creation of a substrate level phosphorylation mechanism involving intermediates necessary for biosynthesis [148,150]. These criteria were most probably met by the glycolytic reactions from glyceraldehyde phosphate through pyruvate which are catalysed by the most highly conserved set of enzymes known [149].

In the absence of oxygen, fermentations were probably the first complete metabolic pathways to become operative in the closed system imposed by living cells [148,150]. It seems reasonable to predict that energy transduction involving complex structures such as electron transport chains and photosystems would only have arisen subsequently [150]. The present day microorganisms that are often mentioned as representing 'primitive' fermentations are the clostridia and *Streptococcus faecalis* [148,152]. The critical question is: which glycolytic pathway evolved first? The notion, thought by many to be set in concrete, that ubiquity of the Embden-Meyerhof-Parnas in nature is an indication of antiquity [153] must be dispelled. The distribution of the Entner-Doudoroff pathway in nature, particularly in microorganisms that are capable of primitive fermentations, is sufficient to suggest that either pathway could have predated the other. Since the lack of plausible chemical mechanisms for phosphorylation in aqueous solutions makes it difficult to predict the stage at which sugar-phosphorylating systems evolved [150], the presence of the modified Entner-Doudoroff pathway, involving non-phosphorylated intermediates, in clostridia suggests the possibility that the earliest fermentations involved this mode of Entner-Doudoroff metabolism.

It is generally regarded that the early fermentative organisms depleted the primordial soup of precursors for biosynthesis, creating the need for metabolic pathways that could form these compounds from simpler chemicals such as formaldehyde. Quayle and Ferenci [150] have eloquently argued that methylotrophy, involving the ribulose monophosphate cycle (RMP), would have been key to this next phase of evolution. Gluconeogenesis and the Calvin cycle for CO_2 could not have filled the need for biosynthetic precursor forma-

tion because of the requirement of these pathways for energy input. The RMP cycle does not require energy input for net formation of pyruvate from formaldehyde. Interestingly, several extant methylotrophs utilize the Entner-Doudoroff pathway for the cleavage phase of the RMP cycle [150].

It has been suggested that the Entner-Doudoroff pathway co-evolved as a means for rapid and direct formation of pyruvate from sugars, in conjunction with the TCA cycle and aerobic respiration [151,153]. Direct formation of pyruvate certainly occurs, but the virtually instantaneous equilibration of triose phosphates makes the need for more rapid formation of pyruvate from glyceraldehyde phosphate questionable. The presence of the Entner-Doudoroff pathway in strictly fermentative organisms such as *Z. mobilis* and in the strictly anaerobic clostridia brings the implied requirement for aerobic Entner-Doudoroff metabolism into question. It can be argued that anaerobic use of the pathway is the simpler and least efficient glycolytic route, with the implication of being most primitive. Evolution of the more efficient Embden-Meyerhof-Parnas pathway was perhaps inevitable. The predominant distribution of the Entner-Doudoroff pathway as the core of central metabolism among present-day microorganisms which possess efficient means for energy transduction, such as aerobes and photosynthetic bacteria, might reflect persistence of the pathway under conditions where use of the Embden-Meyerhof-Parnas pathway would make only a minimal difference in energy yield [151].

The presence of the Entner-Doudoroff pathway in organisms that are thought to have a deep phylogenetic root is consistent with the idea that this pathway is primitive [151]. Woese [154] has suggested that thermophily is an ancestral characteristic of eubacteria. It should be noted that the Entner-Doudoroff pathway has been found in *Bacillus stearothermophilus* [114] and in thermophilic Archaebacteria [72]. Interestingly, the *Z. mobilis* glyceraldehyde phosphate dehydrogenase enzyme more closely resembles the analogous enzyme from *B. stearothermophilus* and *Thermus aquaticus* than from *E. coli* [155]. The presence of the pathway in extremophiles is consistent with the role of Entner-Doudoroff metabolism under conditions that may have existed when the primordial soup was first formed.

8. CONCLUSIONS

Central metabolic pathways must be sufficiently complex to meet the needs of a complex cell and yet be sufficiently simple to allow their ubiquitous distribution in nature. The Entner-Doudoroff pathway fulfills the two vital roles of central metabolism by providing metabolic precursors for biosynthesis and energy via substrate level phosphorylation and/or respiration. Entner-Doudoroff metabolism is far more widely distributed in nature than has been generally recognized. The two enzymes of the pathway are both highly conserved. The Entner-Doudoroff dehydratase is unique and hence the key enzyme of the pathway, whereas the Entner-Doudoroff aldolase also plays other metabolic roles, including hexuronic acid metabolism and hydroxyproline biosynthesis. It is worth noting that the key enzyme of the Embden-Meyerhof-Parnas pathway is clearly 6-phosphofructokinase, since fructose bisphosphate aldolase is a participant in central metabolism of numerous bacteria that lack 6-phosphofructokinase.

Versatility of the Entner-Doudoroff pathway, in terms of the variety of carbohydrates that can enter the pathway from peripheral metabolism, may depend on the available mechanisms for handling electron flow. Intracellular metabolism involving conversion of hexoses to hexonic acids may depend upon the ability of glucose-6-phosphate dehydrogenase to utilize NAD, the enzyme cofactor that is most compatible with other central pathways for carbon and electron flow. The glucose-6-phosphate dehydrogenases from *Z. mobilis* and *P. aeruginosa* are able to utilize both NAD and NADP and metabolism of hexoses can proceed via the Entner-Doudoroff pathway in these organisms, whereas the *E. coli* glucose-6-phosphate dehydrogenase utilizes NADP specifically. The parameters which dictate whether the Entner-Doudoroff pathway is employed in a cyclic or linear mode are not so easily discerned. It

seems possible that all organisms which lack 6-phosphofructokinase and employ the Entner-Doudoroff pathway aerobically in conjunction with the TCA cycle are able to do so in the cyclic mode. Some experiments suggest that the key difference between cyclic and linear Entner-Doudoroff organisms might involve the in vivo operation of fructose bisphosphatase and/or fructose bisphosphate aldolase.

The molecular mechanisms that serve to coordinate balanced expression of the Entner-Doudoroff enzymes appear to have evolved to cope with the demands of significant carbon flux involving toxic metabolic intermediates. Coordinated expression of the enzymes responsible for KDPG formation and degradation in *Z. mobilis* is an excellent example. Constitutive synthesis of EDA in *E. coli*, in contrast to inducible synthesis of EDD, provides another example in which genetic regulation and promoter placement complement physiological constraints on metabolism. Molecular genetic studies of the Entner-Doudoroff pathway have provided windows through which the evolution of metabolic control at the genetic level can be viewed. The potential for more interesting results from similar studies of the pseudomonads, purple non-sulfur bacteria and methylotrophs promises that still further mysteries of Entner-Doudoroff metabolism will be revealed.

ACKNOWLEDGEMENTS

This paper is dedicated in grateful memory to Edward Alan Grula for his lifelong enthusiasm. The author wishes to thank P.V. Phibbs, A.H. Romano, and R.E. Wolf, Jr., for helpful discussions. The author is supported by grants from the US Department of Energy (DE-FG02-90ER 20002) and the Nebraska Energy Office (UNR-11).

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