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REVIEW

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Sugar utilization and its control in hyperthermophiles

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Abstract Many hyperthermophilic microorganisms show heterotrophic growth on a variety of carbohydrates. There has been considerable fundamental and applied interest in the utilization of glucose and its α- and β-polymers by hyperthermophiles. While glycolysis by Bacteria at high temperatures shows conventional characteristics, it has been found that glucose catabolism by hyperthermophilic Archaea differs from the canonical glycolytic pathways, involves novel enzymes, and shows a unique control. This review addresses these aspects with specific attention to *Pyrococcus furiosus*, which is one of the best studied hyperthermophilic Archaea, has the capacity to grow on a variety of sugars including the marine β-(1,3)-linked glucose polymer laminarin, and has been found to contain three novel glycolytic enzymes, two ADP-dependent kinases, and a ferredoxin-dependent glyceraldehyde-3-phosphate oxidoreductase.

Key words *Pyrococcus furiosus* · Glycolysis · Hyperthermophiles · Laminarin · Gene expression

Introduction

A variety of prokaryotes share the capacity to grow on carbohydrates at temperatures above 80°C (Stetter 1996). These organisms include both Archaea and some Bacteria, and more than 20 different species have been described (Kengen et al. 1996). While some of these hyperthermophiles are capable of growing on monomeric sugars, in many cases efficient growth is only obtained with polymers of glucose, such as starch, glycogen, or laminarin. The utilization of these natural α- and β-linked glucose polymers has attracted considerable attention because of the application potential of depolymerizing glycosyl hydrolases that are required for the liberation of glucose (Sunna et al. 1997). In addition, various studies have focused on the pathways that are utilized by hyperthermophiles to catabolize the generated glucose (Schönheit and Schäfer 1995). Finally, the metabolic flexibility that is provided by the use of multiple sugars can be used to analyze regulation of gene expression at high temperatures, and such studies are now materializing.

This review summarizes the recent information on the metabolic pathways for sugar utilization in hyperthermophiles, their key enzymes, genes, and control systems. Specific attention is given to *Pyrococcus furiosus*, which is among the best studied sugar-utilizing hyperthermophiles and shows efficient growth on starch, cellobiose, and laminarin.

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Metabolic pathways in carbohydrate-utilizing hyperthermophiles

Many hyperthermophilic microorganisms produce enzymes that hydrolyze the α-(1,4)-, α-(1,6)-, β-(1,4)-, and β-(1,3)-glycosidic bonds present in natural polysaccharides. The distribution, physicochemical properties, and genetic characterization of these α- and β-glycosyl hydrolases has been reviewed by Sunna et al. (1997). Although most endo-acting glycosyl hydrolases are cell-wall associated or completely secreted, it appears that exo-acting glycosidases are found to be retained within the cytoplasm. Together with the fact that several hyperthermophiles are capable of growth on disaccharides but not on glucose, this suggests that oligosaccharides with various degrees of polymerization may be transported into the cell and are subsequently hydrolyzed to glucose. So far, only a limited number of studies have addressed the question of how these oligosaccharides are transported into the cell, and only for *Thermococcus*

Table 1. Well-studied carbohydrate-utilizing hyperthermophiles, their metabolic pathways and modifications (after Schönheit and Schäfer 1995; Kengen et al. 1996; Selig et al. 1997; Brunner et al. 1998)

Species	T^a_{opt}	Metabolism[b]	Pathway[c]	Modification[d]
Archaea				
Sulfolobus acidocaldarius	75	R (O_2)	ED	Nonphosphorylated ED
Archaeoglobus fulgidus	83	R (SO_4)	Unknown	No conserved glycolytic genes
Thermoproteus tenax	88	R (S^0)	EM and ED	PP_i-PFK GAPN
Desulfurococcus amylolyticus	90	F	EM	GAPOR ACS
Thermococcus celer	87	F	EM	ADP-GLK ADP-PFK GAPOR ACS
Pyrococcus furiosus	100	F	EM	ADP-GLK ADP-PFK GAPOR ACS
Bacteria				
Thermotoga maritima	80	F	EM and ED	None

[a] Optimal growth temperature in °C.
[b] R, respiratory metabolism – complete oxidation to CO_2, with either oxygen (O_2), sulphate (SO_4), or sulphur (S^0) as electron acceptor; F, fermentative metabolism – incomplete oxidation.
[c] EM, Embden-Meyerhof pathway; ED: Entner-Doudoroff pathway. EM and ED, operation of both EM and ED pathways in a relative proportion of approximately 85%–15% as determined from ^{13}C-labeling experiments in cell suspensions.
[d] PP_i-PFK, pyrophosphate-dependent phosphofructokinase; ADP-GLK, ADP-dependent glucokinase; ADP-PFK, ADP-dependent phosphofructokinase; GAPN, NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase; GAPOR, glyceraldehyde-3-phosphate oxidoreductase; ACS, acetyl-CoA synthetase (ADP-dependent).

litoralis has a transport system with high affinity for both maltose and trehalose been described that probably represents an ABC transporter (Xavier et al. 1996).

Two major pathways are known to be involved in the degradation of glucose to pyruvate in Bacteria, the Embden-Meyerhof (EM) or Entner-Doudoroff (ED) pathways. In general, these classical pathways differ in the key enzymes acting on glucose or glucose-6-phosphate and subsequently in several of the following steps that lead to the formation and subsequent aldolytic cleavage of the intermediates fructose-1,6-biphosphate (EM) and 2-keto-3-deoxy-6-phosphogluconate (ED).

The presence of the EM and ED pathways in hyperthermophiles has been studied by combining a variety of approaches, including (i) analysis of fermentation products of specifically ^{13}C-labeled glucose by ^{13}C-NMR spectroscopy, (ii) identification of intermediates of sugar degradation following conversion of ^{14}C-labeled glucose by cell extracts, (iii) determination of enzyme activities in cell extracts, (iv) characterization of purified enzymes, and (v) analysis of the genes coding for key enzymes in glycolysis and their regulation (Danson 1993; Adams 1994; Schönheit and Schäfer 1995; Kengen et al. 1996; Selig et al. 1997). The most important results from these recent studies with representative hyperthermophilic Archaea and Bacteria are summarized in Table 1. It appears that all fermentative hyperthermophiles utilize the EM pathway, while hyperthermophiles that have the capacity to completely oxidize substrates to CO_2 at least are capable of utilizing the ED pathway. In some cases both EM and ED pathways occur concurrently in the same hyperthermophile, such as in *Thermoproteus tenax* and *Thermotoga maritima*. The relative proportion of the two different pathways seems to be similar in both hyperthermophiles (approximately 85% EM and 15% ED), although it remains to be established whether this is the case under all growth conditions. While the only studied bacterial representative that is capable of utilizing sugars above 80°C, *Thermotoga maritima,* shows a conventional ED pathway, it is evident that all studied archaeal hyperthermophiles show modifications of the canonical EM and ED pathways and in many cases these include novel enzymatic conversions that are discussed here.

Respiratory glycolytic hyperthermophilic Archaea: the ED pathway and its modifications

The ED pathway is widely distributed in prokaryotes and known to have many variations (Conway 1992). Although the obligately aerobic hyperthermophile *Sulfolobus acidocaldarius* also degrades glucose via an ED pathway, this is a nonphosphorylated version involving glucose oxidation to gluconate, formation of 2-keto-3-deoxy-gluconate, and a specific kinase that phosphorylates the resulting glycerate (Selig et al. 1997). This nonphosphorylated version of the ED pathway is also found in other aerobic Archaea, such as the moderate thermophilic archaeon *Thermoplasma acidophila* (Danson 1993). *Thermoproteus tenax* also ferments glucose via a nonphosporylated ED pathway; how-

ever, this is simultaneous with an EM-type pathway that is also modified (see following).

Sugar degradation in *Archaeoglobus fulgidus* is a special case. This unique sulfate-reducing archaeon is reported to be capable of utilizing starch and glucose (Stetter 1988). However, homology analysis of the recently determined complete genome sequence of 2 178 400 bp has not revealed any of the key enzymes in the degradation of starch or even glucose (Klenk et al. 1997). Assuming that the fidelity of this genome sequence is as high as reported and excluding the possibility that a mutant has been selected during the single-cell isolation procedure used in this genome project, this would mean that *Archaeoglobus fulgidus* contains novel, hitherto undetected, genes for these key enzymes.

Fermentative glycolytic hyperthermophilic Archaea: the EM pathway and its modifications

All sugar-fermenting hyperthermophiles studied so far have been found to use the EM pathway. However, the sugar fermentation routes established in these archaeal hyperthermophiles show significant differences with the conventional EM pathway. The most important differences are observed in members of the order *Thermococcales*, including *P. furiosus,* that contain several unique enzymes including two novel ADP-dependent kinases (AMP-forming) and a ferredoxin-dependent glyceraldehyde-3-phosphate oxidoreductase (Fig. 1). The ADP-dependent kinases concern an ADP-dependent glucokinase and an ADP-dependent phosphofructokinase that are present in *P. furiosus*, *Thermococcus litoralis*, and *Thermococcus celer* (Kengen et al. 1994, 1995; Selig et al. 1997). The ADP-dependent kinase from *P. furiosus* has been purified to homogeneity and shows all physicochemical properties expected from an enzyme catalyzing this modified EM pathway (Kengen et al. 1996). One of the explanations for the presence of ADP-dependent kinases is the observation that ADP shows a higher thermostability than ATP, especially in the presence of divalent cations (Kengen et al. 1996). However, the hyperthermophile *Thermoproteus tenax* has been shown to contain a pyrophosphate-dependent phosphofructokinase kinase (Siebers and Hensel 1993).

Another important and novel enzyme is glyceraldehyde-3-phosphate oxidoreductase, which is a novel tungsten-containing iron-sulfur protein (Mukund and Adams 1995). Following its discovery in *P. furiosus*, the activity of this ferredoxin-dependent enzyme was also found in *Thermococcus litoralis* and *Desulfurococcus amylolyticus* (Selig et al. 1997). The glyceraldehyde-3-phosphate oxidoreductase has been purified from *P. furiosus* and found to contain a catalytically active tungsten that is probably liganded in a pterin cofactor (Mukund and Adams 1995; van der Oost et al., in manuscript). This unusual enzyme catalyzes a novel conversion, the single-step production of 3-phospho-glycerate and reduced ferredoxin from glyceraldehyde-3-phosphate. In the conventional EM pathway this step is catalyzed by two enzymes, glyceraldehyde-3-phosphate dehydrogenase, which generates NADH, and phosphoglycerate kinase, an important enzyme involved in ATP formation. It is evident that the absence of substrate-level phosphorylation by the novel glyceraldehyde-3-phosphate oxidoreductase has important bioenergetic consequences for these fermentative Archaea (Kengen et al. 1996). This unique modification of the EM pathway may also reflect an adaptation to high temperatures because pyridine nucleotides are less thermostable than ferredoxines (Daniel and Danson 1995). However, it has been found that the hyperthermophilic *Thermoproteus tenax* also contains an alternative enzyme for this conversion, an NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase, which does depend on pyridine nucleotides (Brunner et al. 1998).

A final step in sugar fermentation by fermentative hyperthermophiles is the conversion of acetyl-CoA into acetate, giving rise to the production of ATP. Also in this crucial step, the archaeal hyperthermophiles are unique because they have a single enzyme, an ADP-dependent acetyl-CoA synthase, while in *Thermotoga maritima* and other bacteria this reaction is catalyzed by two different enzymes, phosphate acetyltransferase and acetate kinase. Two enzymes with acetyl-CoA synthase activity and a preference for GTP above ATP have recently been purified from *P. furiosus* and could both be involved in this last step in the utilization of glucose (Mai and Adams 1996; Glasemacher et al. 1997). Finally, many hyperthermophiles have the capacity to convert pyruvate into alanine, which acts as an alternative electron sink. This reaction first was described for *P. furiosus* and involves the combined activity of both alanine aminotransferase and glutamate dehydrogenase (Kengen and Stams 1994) (see Fig. 1).

Laminarin degradation and its control in *Pyrococcus furiosus*

Pyrococcus furiosus is known to grow efficiently on cellobiose, and its active β-glycosidase has been implicated in the intracellular hydrolysis of this β-(1,4)-linked dimer of glucose (Kengen et al. 1993, 1996). However, neither *P. furiosus* nor one of the other presently described hyperthermophiles is capable of growth on the widely occurring natural β-(1,4)-linked glucose polymer cellulose (Schönheit and Schäfer 1995). This raised the question as to which glucose polymer was the natural substrate for the pyrococcal β-glycosidase, which we found to be a family 1 exo-acting glycosyl hydrolase encoded by the *celB* gene (Voorhorst et al. 1995). During the analysis of a part of the *P. furiosus* genome we found a new gene cluster upstream of the *celB* gene with the order *adhA-adhB-lamA* (Fig. 2) (Voorhorst et al., in manuscript). The *adhA* and *adhB* genes were found to encode a short-chain and an iron-containing alcohol dehydrogenase, respectively (Voorhorst et al., in manuscript). Interestingly, the *lamA* gene was found to belong to the family 16 glycosyl hydrolases and only showed

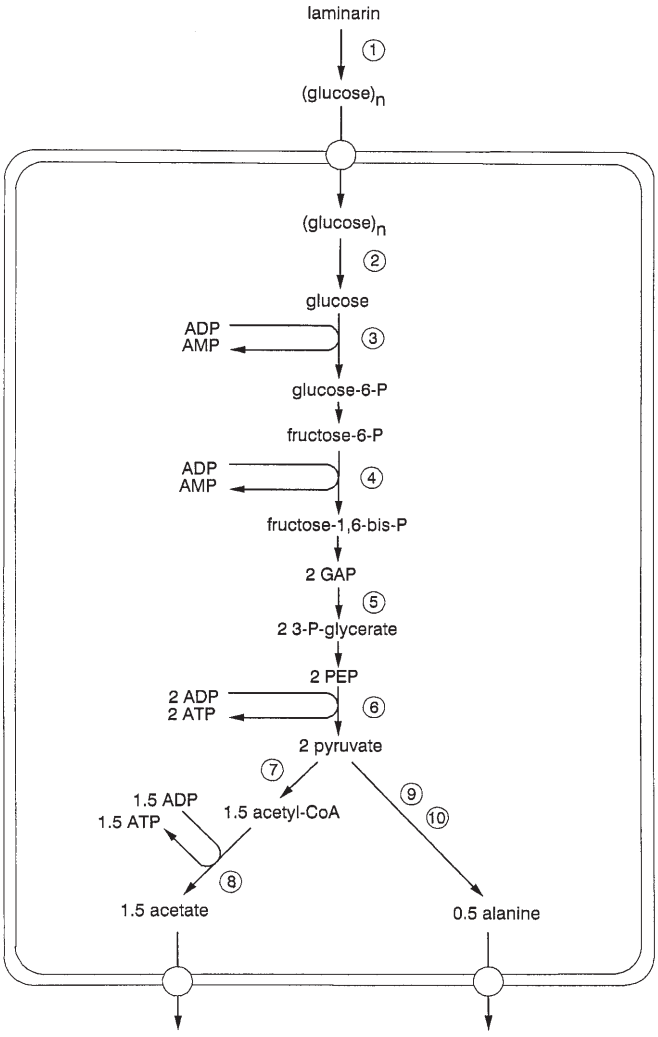

Fig. 1. Proposed pathway for laminarin degradation in *Pyrococcus furiosus* shows the enzymes discussed: *1*, *endo*-1,3-β-glucanase (LamA); *2*, β-glycosidase (CelB); *3*, ADP-dependent glucokinase; *4*, ADP-dependent phosphofructokinase; *5*, glyceraldehyde-3-phosphate:ferredoxin oxidoreductase; *6*, pyruvate kinase; *7*, pyruvate:ferredoxin oxidoreductase; *8*, acetyl-CoA synthetase (ADP-dependent); *9*, glutamate dehydrogenase; *10*, alanine aminotransferase

activity on glucose polymers that contain β-(1–3)-linkages (Gueguen et al. 1997). Furthermore, it was shown that the CelB has high β-(1–3)-glycosidase activity and that the combined action of CelB and LamA resulted in the complete degradation of laminarin. Moreover, *P. furiosus* was found to grow well on laminarin (Gueguen et al. 1997). Finally, a variety of molecular approaches showed that the *adhA-adhB-lamA* genes form an operon (the *lamA* operon), which is induced by laminarin, just as is the *celB* gene (Voorhorst et al., in manuscript). Laminarin is a naturally occurring β-(1–3)-linked glucose polymer present in the cell wall of various marine organisms such as eukaryal algae. Because *P. furiosus* is a marine isolate, it is likely that laminarin is a natural substrate that is degraded via the modified EM pathway described in Fig. 1. In the absence of

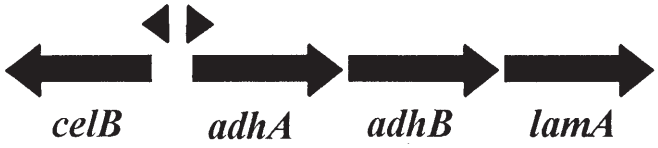

Fig. 2. Schematic representation of the locus of *P. furiosus* encoding hydrolases (CelB and LamA) involved in laminarin degradation. Promoters are indicated by the *arrows*

data on sugar transport in *P. furiosus*, it is presently not possible to indicate the degree of polymerization of the β-(1–3)-linked glucose polymers that are entering the cell.

A remarkable observation was made recently when the *gor* gene for the glyceraldehyde-3-phosphate oxidoreductase was analyzed (van der Oost et al., in manuscript). Unexpectedly, the transcription of the *gor* gene is induced by growth on β-linked sugar polymers. In contrast, the expression of the *gap* gene for glyceraldehyde-3-phosphate dehydrogenase is not induced. This result confirms the involvement of the glyceraldehyde-3-phosphate oxidoreductase in the modified version of the EM pathway and reveals a novel site for glycolytic control.

Concluding remarks

It is evident that the catabolism of glucose polymers by hyperthermophilic Archaea does not proceed via the canonical glycolytic EM and ED pathways and may involve a series of novel enzymes. Moreover, the first studies on the control of sugar utilization have revealed unique control sites in *Pyrococcus furiosus*. Genome sequences of a variety of hyperthermophiles are now emerging, including those of three *Pyrococcus* species. It may be expected that comparative genomics, forward and reversed genetics, in combination with biochemical and physiological studies, will contribute to a complete understanding of sugar polymer utilization and its control studies at temperatures around the boiling point of water.

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