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Analysis of the Glycolytic Pathways of the Hydrogen Producing *Caldicellulosiruptor Saccharolyticus*

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Caldicellulosiruptor saccharolyticus is an extremely thermophilic, gram-positive anaerobe which ferments cellulose-, hemicellulose- and pectin-containing biomass to acetate, CO_2, and hydrogen. Its broad substrate range, high hydrogen-producing capacity, and ability to co-utilize glucose and xylose make this bacterium an attractive candidate for microbial bioenergy production. Despite these profitable features, still little is known about the different sugar uptake systems, the subsequent pathways and the regulatory aspects determining the final hydrogen yield. It is our goal therefore, to investigate the main sugar degradation pathways and their regulation by a systems biology approach.

1 Genomics

The genome of *Caldicellulosiruptor saccharolyticus* DSM 8903 consists of one circular chromosome of 2,970,275 base pairs (bp), which has a G+C-content of 35.3% [1]. The complete genome sequence confirms the phylogenetic position of *C. saccharolyticus* as member of the class Clostridia and reveals *Thermoanaerobacter tengcongensis* as closest relative. When compared to other thermophilic hydrogen producing bacteria and archaea, like *Clostridium thermocellum, T. tengcongensis*, *Thermotoga maritima* and *Pyrococcus furiosus,* the *C. saccharolyticus* genome harbors the largest number of carbohydrate transport and metabolism genes. In fact, the *C. saccharolyticus* genome contains at least 177 ABC-transporter genes, outnumbering the 165 identified in *T. maritima* [1, 2].

1.1 Polysaccharide degrading enzymes

The capacity of *C. saccharolyticus* to hydrolyze a broad range of polysaccharides prior to fermentation differentiates this bacterium from many thermophilic anaerobes. The carbohydrate-utilizing enzymes are often clustered on the chromosome and can be assigned to substrate specific catabolic pathways for cellulose, hemicellulose and, to a lesser extent, starch and pectin. The α-1,4-glucan polymers, for instance, can be transported into the cell using a maltodextrin ABC-transport system (Csac_0427-0428/0431). An intracellular α-amylase (Csac_0426) and a 1,4-α-glucan phosphorylase (Csac_0429) further degrade the intracellular maltodextrins, releasing glucose-1-phosphate. Several starch-degrading enzymes, such as an α-amylase precursor (Csac_0408), an oligo-1,6-glucosidase (Csac_2428), a pullulanase (Csac_0689), a 4-α-glucanotransferase (Csac_0203), and a putative glucan 1,4-α-glucosidase (Csac_0130) were identified. Besides this putative starch-degrading regulon, *C. saccharolyticus* has a glycogen metabolic cluster (Csac_0780-0784), a maltose ABC-transport system (Csac_2491-3), and a second pullulanase (Csac_0671). Taken together, *C. saccharolyticus* is well-equipped for starch utilization.

C. saccharolyticus does not metabolize cellulose by means of a cellulosome as the typical molecular components of a cellulosome, *i.e.*, dockerin domains and scaffolding proteins, were not identified in the genome. Nevertheless, a gene cluster (Csac_1076-1081) containing cellulase precursors is present. These highly modular cellulases are potentially capable of degrading this plant polysaccharide. Moreover, another gene cluster (Csac_1089-1091) and an extracellular cellulase (Csac_0678) may assist in completely hydrolyzing cellulose to glucose.

C. saccharolyticus has an enzyme system to cleave the glycoside bonds and hydrolyze ester bonds in hemicellulose (Csac_2404-2411). These mostly extracellular enzymes, which are variable in domain composition as well, might be co-expressed with a smaller putative xylan-utilizing cluster (Csac_0203-0205). Furthermore, putative genes that encode enzymes to degrade galactomannan (Csac_0663-0664), galactoarabinan (Csac_1560-1562) and laminarin (Csac_2548) were identified [1].

Various genes coding for the degradation of the main pectin component, D-galacturonate could be identified, except that a tagaturonate reductase and altronate dehydratase were not detected in the genome of *C. saccharolyticus*. In contrast, a gene cluster for the conversion of glucuronic acid to KDG (Csac_2686-2689) was identified, and includes fructuronate reductase, mannonate dehydratase, a putative β-galactosidase/β-glucuronidase, and an α-glucuronidase. Glucuronic acid is a common substituent of xylan. Enzymes for the subsequent conversion of KDG to pyruvate and GAP are present as well (Csac_0355 or Csac_2720, and Csac_0345). The encoding genes of these last two steps are clustered with genes (Csac_0356-0357 and Csac_2718-2719) that both metabolize 5-keto-4-deoxyuronate, an unsaturated cleavage product from pectate, to KDG. The enzymes that are able to hydrolyze the pectate backbone and the side chains, β-galactosidase and a glycoside hydrolase with unknown substrate specificity are in proximity to these KDG metabolic enzymes as well. However, neither a pectate lyase nor a methylesterase could be definitively identified in the genome [1].

C. saccharolyticus is also able to grow on L-rhamnose and on L-fucose, thereby producing 1,2-propanediol as end product. A putative rhamnose catabolic pathway can be assigned that generates dihydroxy-acetone phosphate and 1,2-propanediol. Fucose can be processed by a similar pathway.

1.2 Central carbon metabolism

The genome sequence reveals all the components of a complete Embden-Meyerhof (EM) pathway. In addition, a gene coding for an additional phosphofructokinase was identified (Csac_2366), which might be PPi-dependent instead of ATP-dependent (PPi-PFK; Csac_1830). Moreover, a PPi-dependent pyruvate phosphate dikinase (PPDK; Csac_1955) was present as well. However, the oxidative branch (ox) of the Pentose Phosphate Pathway (PPP) and the Entner-Doudoroff (ED) pathway were not detected, which is consistent with previous reports using ^{13}C-NMR [3]. Xylose, a major constituent of hemicellulose, is apparently funneled into the non-oxidative branch of the PPP. Galactose also enters the EM via the Leloir-pathway. Remarkably, none of the established types of fructose-bisphosphatase (Class I to IV) are evident in the *C. saccharolyticus* genome. Since fructose-bisphosphatase is an essential enzyme of the gluconeogenesis, *C. saccharolyticus*

presumably uses a novel phosphatase. Moreover, a gene for the gluconeogenic PEP synthetase is also missing. Alternatively, although the conversion of pyruvate to PEP could occur via the reversible PPDK or via oxaloacetate.

Pyruvate, the end product of the EM-pathway, is subsequently decarboxylated to acetyl-CoA by pyruvate:ferredoxin oxidoreductase (Csac_1458-1461). Acetyl-CoA is used to generate acetate and ATP (Csac_2040/2041), or it enters the tricarboxylic acid (TCA) cycle for biosynthetic purposes.

1.3 Fermentation products

Reducing equivalents are produced at the level of NAD and ferredoxin. Since *C. saccharolyticus* can produce almost 4 H_2 per mol of glucose, both NADH and reduced ferredoxin should ultimately be able to transfer their reducing equivalents to protons to form hydrogen. In the genome, two hydrogenase gene clusters could be identified, which are very similar to the two related clusters in *Thermoanaerobacter tengcongensis* [4]. The first cluster (Csac_1534-1539) encodes subunits of a Ni-Fe hydrogenase (EchA-F) and various genes required for maturation of the hydrogenase complex (HypA-F; Csac_1540-1545). For *T. tengcongensis,* this Ni-Fe hydrogenase is ferredoxin-dependent, membrane-bound, and may act as a proton pump to generate a proton motive force. The second cluster (Csac_1860-1864) codes for a Fe-only hydrogenase (HydA-D), which is NAD-dependent and located in the cytoplasm, similar to the case for *T. tengcongensis*. Altogether, information available suggests that *C. saccharolyticus* is able to produce hydrogen from ferredoxin, but can also do this directly from NADH. Production of hydrogen would seem to be preferable, because under these conditions all pyruvate is converted to acetate (and CO_2), which is coupled to the synthesis of ATP.

When the hydrogen partial pressure becomes too high, hydrogen formation from NADH is no longer thermodynamically favorable. In that case, NADH is oxidized through the formation of lactate or ethanol. A gene for a lactate dehydrogenase could be identified (Csac_1027), but genes for acetaldehyde dehydrogenase and alcohol dehydrogenase were not obvious.

2 Transcriptomics

One of the beneficial features of *C. saccharolyticus* for hydrogen production is its ability to degrade cellulosic substrates as well as hemicellulose. Moreover, mixtures of glucose and xylose can be fermented simultaneously suggesting that classical Carbon catabolite repression (CCR) by glucose does not occur. To elucidate the central carbon metabolic pathways and their regulation, transcriptome analysis was performed after growth on glucose, xylose and a 1:1 mixture of both substrates [1]. L-Rhamnose, which was likely to follow another pathway, was used as a reference substrate. The transcriptional data clearly show that glucose, xylose and the glucose:xylose mixture all trigger up-regulation of genes in the EM pathway, when compared to rhamnose. In particular, the fructose-bisphosphate aldolase, GAP dehydrogenase, PPDK and POR are significantly stimulated. The ultimate acetate-forming acetate kinase is also highly up-regulated. A catabolic role for PPDK is intriguing, since it normally is associated with gluconeogenesis, and PEP is usually converted by pyruvate kinase.

None of the identified putative CCR genes were differentially transcribed, confirming the fact that catabolite repression by glucose was not a factor [1, 2].

Transcriptional response to growth on monosaccharides enabled the identification of genes and groups of adjacent genes, that were specifically up-regulated in response to either glucose or xylose. On glucose, several genes coding for α-glucan hydrolases responded. On xylose, several gene clusters required for xylan or xylose conversion were up-regulated (Csac0692-0696; Csac0240-0242; Csac2416-2419). These clusters encode ABC transport systems, transcriptional regulators and endo-xylanases. In addition, genes, specifically required for growth on rhamnose, were highly up-regulated during growth on rhamnose, thus indicating the utilization pathway for this sugar.

3 Pyrophosphate as Alternative Energy Carrier

To manipulate the pathways leading to hydrogen it is important to know their exact composition and how the individual metabolic steps are regulated. Although the operation of the EM pathway has been confirmed for *C. saccharolyticus*, our genome and transcriptome data also suggested that PPi-dependent steps may be involved. Interestingly, recent studies on the acetate-lactate metabolic shift in *C. saccharolyticus* revealed that PPi is a strong modulator of the lactate dehydrogenase [4]. These observations motivated us to investigate the role of inorganic pyrophosphate in the energy metabolism of *C. saccharolyticus* [5].

The two genes that were annotated to code for a phosphofructokinase (Csac_1830 and Csac_2366) were analyzed more closely and it could be demonstrated that Csac_1830 contains the typical ATP-dependent PFK amino acid combination (G_{104} and G_{124}) and that Csac_2366 contains the typical PPi-dependent PFK amino acid residues (D_{104} and K_{124}). These results strongly suggest that Csac_1830 is an ATP-dependent PFK and that Csac_2366 is a PPi-dependent PFK. As mentioned previously, *C. saccharolyticus* also has the genes coding for two alternative enzymes for the conversion of PEP to pyruvate, viz. the more common pyruvate kinase (PK) and the PPi-dependent PPDK. In addition we also identified a gene coding for a membrane-bound proton-pumping pyrophosphatase (PPase), whereas a cytosolic PPase encoding gene was absent. To confirm the genome sequence based predictions, we performed enzyme assays on crude cell extracts. Activities of PK, PPDK, PPi-PFK, ATP-PFK and membrane-bound PPase could all be detected in cell extracts of *C. saccharolyticus*. Under the specified assay conditions the average PPDK activity (0.4 U/mg) was twice the PK activity. For the ATP- and PPi-dependent PFKs no significant difference was observed in the activity levels (~0.05 U/mg). The presence of PPi-dependent enzymes in the central metabolic pathway suggested the involvement of PPi as an energy carrier in the metabolism of *C. saccharolyticus*. Therefore, the levels of both ATP and PPi were determined during growth. PPi levels were relatively high (approx. 4±2 mM), while ATP levels were remarkably low (0.43±0.07 mM). Strikingly, PPi was recently shown to act as allosteric effector of the pyruvate kinase and the lactate dehydrogenase (LDH) of *C. saccharolyticus* [4, 5].

Altogether, the presented results indicate that PPi has a central role in the metabolism of the hydrogen-producing *C. saccharolyticus*. This type of metabolism agrees well with the observed physiology with respect to its sugar utilization [2]. The wide range of high-affinity

sugar uptake systems and the absence of carbon catabolite repression suggests that *C. saccharolyticus* is not fastidious, but rather has evolved to conserve energy in many different ways.

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