Chapter 8

The Extremely Thermophilic Genus *Caldicellulosiruptor*: Physiological and Genomic Characteristics for Complex Carbohydrate Conversion to Molecular Hydrogen

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Summary

Extremely thermophilic, carbohydrate-utilizing bacteria from the genus *Caldicellulosiruptor* should be considered for biohydrogen production to take advantage of their broad growth substrate range and high substrate conversion efficiency. In fact, *Caldicellulosiruptor* species produce molecular hydrogen at yields approaching the Thauer limit of 4 mol H_2/mol

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D. Zannoni and R. De Philippis (eds.), Microbial BioEnergy: Hydrogen Production, Advances in Photosynthesis and Respiration 38, pp. 177–195, DOI 10.1007/978-94-017-8554-9_8,
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glucose equivalent. *Caldicellulosiruptor* species can utilize pentoses, hexoses, di/oligosaccharides, as well as complex polysaccharides, including crystalline cellulose. The broad appetite of these organisms relates to the natural environment of *Caldicellulosiruptor*, where they thrive at high temperatures (65–78 °C), utilizing the variable saccharide composition of lignocellulosic biomass as growth substrate. The ability to degrade recalcitrant plant biomass and utilize a wide variety of polysaccharides in their fermentation pathways sets *Caldicellulosiruptor* species apart from many other candidate biofuel-producing microorganisms. The conversion of lignocellulose to fuels in *Caldicellulosiruptor* is driven by an array of novel multi-domain glycoside hydrolases that work synergistically to degrade plant polysaccharides into oligo/monosaccharides that enter the cytoplasm via an array of carbohydrate specific ABC sugar transporters. These carbohydrates are then processed through a series of catabolic pathways, after which they enter the EMP pathway to produce reducing equivalents in the form of NADH and Fd^{red}. The reducing equivalents are ultimately utilized by both cytoplasmic and membrane-bound hydrogenases to form molecular hydrogen. Recently completed genome sequences for a number of *Caldicellulosiruptor* species have revealed important details concerning how plant biomass is deconstructed enzymatically and shown significant diversity within the genus with respect to lignocellulose conversion strategies.

I. Introduction

The genus *Caldicellulosiruptor* is comprised of extremely thermophilic, gram-positive bacteria with optimal growth temperatures between 65 and 78 °C (Blumer-Schuette et al. 2010; Hamilton-Brehm et al. 2010). Members of the genus are associated with plant debris in high temperature terrestrial hot springs and mud flats worldwide (Fig. 8.1). Currently, eight *Caldicellulosiruptor* species have sequenced genomes, providing important insights into the metabolic and physiological traits of these extreme thermophiles (van de Werken et al. 2008; Kataeva et al. 2009; Elkins et al. 2010; Blumer-Schuette et al. 2011). Common to all species is the capability to convert complex polysaccharides into simple sugars, which are then fermented to molecular hydrogen, acetate, lactate and small amounts of alcohol (Rainey et al. 1994; Ahring 1995; Huang et al. 1998; Bredholt et al. 1999; Miroshnichenko et al. 2008; Hamilton-Brehm et al. 2010; Yang et al. 2010). *Caldicellulosiruptor* species have potential importance for biofuels production, since they produce H_2 near the Thauer limit of 4 mol H_2 per mol glucose (Ivanova et al. 2009; de Vrije et al. 2009; Zeidan and van Niel 2010; Willquist and van Niel 2012).

The long list of complex polysaccharides serving as growth substrates for members of the genus *Caldicellulosiruptor* includes α- and β-glucans, mannans, xylans, pectin and, for some species, crystalline cellulose (Rainey et al. 1994; Ahring 1995; Huang et al. 1998; Bredholt et al. 1999; Miroshnichenko et al. 2008; Hamilton-Brehm et al. 2010; Yang et al. 2010; Blumer-Schuette et al. 2012). The genus collectively contains 106 glycoside hydrolases (GH), representing 43 GH families, and an array of ATP-binding

Abbreviations: ABC – ATP binding cassette; ADH – Alcohol dehydrogenase; CAZy – Carbohydrate-active enzyme; CBM – Carbohydrate binding module; CCR – Carbon catabolite repression; CE – Carbohydrate esterase; CUT – Carbohydrate uptake; DPP – Di-peptide; EMP – Embden-Meyerhoff-Parnas; FD^{red} – Reduced ferredoxin; GH – Glycoside hydrolase; LDH – Lactate dehydrogenase; OPP – Oligo-peptide; PL – Polysaccharide lyase; PPP – Pentose phosphate pathway; PTS – Phosphoenolpyruvate-dependent phosphotransferase; SLH – S-layer homology; TCA – Tricarboxylic acid

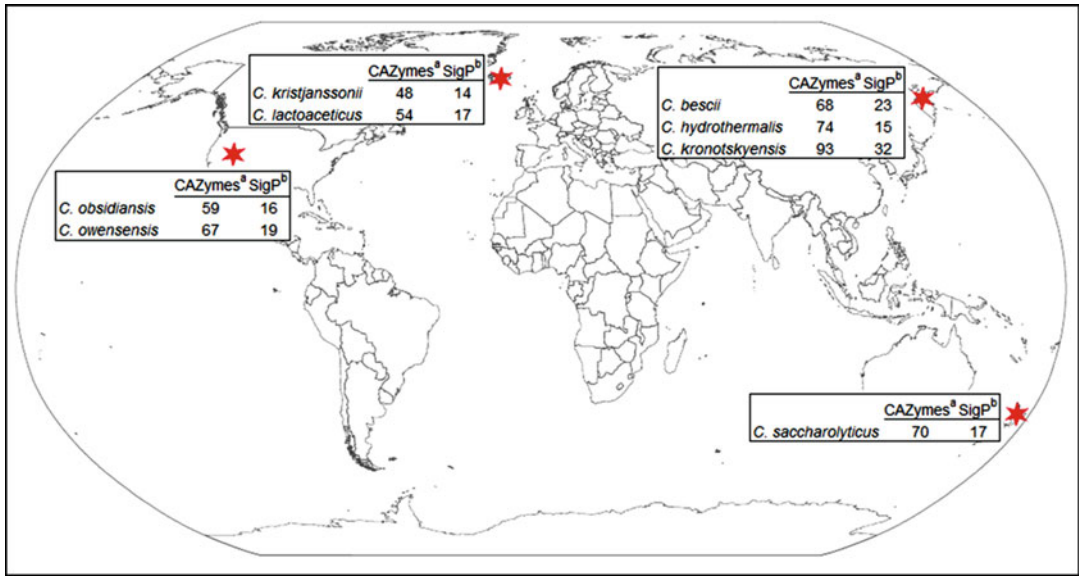

Fig. 8.1. **Geographic distribution of *Caldicellulosiruptor* species and Carbohydrate Active Enzymes** ([a] = Number of ORFs encoding either a CBM, CE, GH or PL; [b] = signal peptide is encoded in the ORF).

cassette (ABC) transporters belonging to the Carbohydrate Uptake 2 (CUT 2), Carbohydrate Uptake 1 (CUT 1), and Di/Oligopeptide (Dpp/Opp) families (Vanfossen et al. 2009). These GHs and transporters are deployed to synergistically process complex polysaccharides prior to entering into fermentation pathways (Blumer-Schuette et al. 2012). While many microorganisms preferentially utilize hexose over pentose sugars and often exhibit carbon catabolite repression (CCR) (Gancedo 1998; Brückner and Titgemeyer 2002), this is not the case for *Caldicellulosiruptor* species (Vanfossen et al. 2009). The lack of CCR makes *Caldicellulosiruptor* species especially promising in decomposing characteristically heterogeneous plant biomass to molecular hydrogen.

Although the discovery and initial isolation of *Caldicellulosiruptor* species (*C. saccharolyticus* formerly *Caldocellum saccharolyticum*) occurred more than 20 years ago (Donnison et al. 1986; Rainey et al. 1994), it was only within the past 5 years, concomitant with the increased interest in biofuels, that these bacteria have received intense interest. An overview of current progress in studying *Caldicellulosiruptor* is provided here, with an eye towards how these bacteria produce molecular hydrogen from complex carbohydrates, especially lignocellulosic biomass.

II. Extracellular Deconstruction of Lignocellulosic Biomass

The production of molecular hydrogen from plant biomass begins with extraction and deconstruction of the carbohydrate content of lignocellulose into fermentable sugars (Fig. 8.2).

A. Lignocellulose Composition and Recalcitrance

Lignocellulose is primarily composed of cellulose, hemicellulose and lignin; the physical and chemical properties of these polymers varies between plant species, stages of growth, and environmental conditions (Reddy and Yang 2005). Cellulose, the major structural component, is a long chain of glucose molecules linked by β–1,4 glycosidic bonds (van Wyk 2001). Hydrogen bonds between the polysaccharide chains form

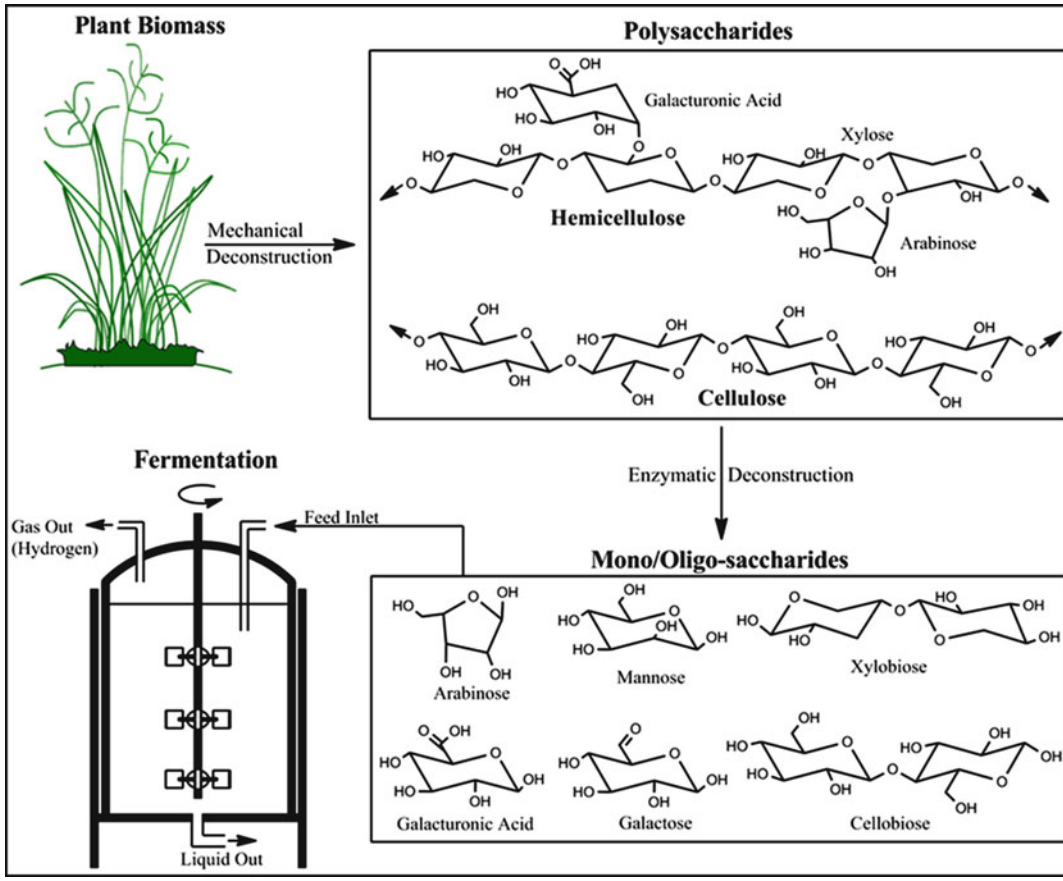

Fig. 8.2. **Conversion process of plant biomass to fuels**. Plant biomass is first mechanically degraded to yield long chained polysaccharides, which are then enzymatically deconstructed to shorter chained mono/oligo saccharides. Shorter chained saccharides are fermented to produce biofuels.

crystalline cellulose, conferring an increased resistance to degradation (Rubin 2008). Hemicellulose is a heteropolymer, consisting of xylose, mannose, galactose, glucose, arabinose and glucuronic and galacturonic acids. These sugars are linked primarily by β-1,4 and β-1,3 glycosidic bonds. Lignin is an amorphous, water-insoluble heteropolymer, consisting of phenylpropane units joined by different types of linkages. Lignin acts as molecular "glue", conferring structural support, impermeability and resistance to microbial attack (Fig. 8.3) (Pérez et al. 2002; Rubin 2008). The antimicrobial characteristics of lignin and crystallinity of cellulose are the two major challenges in the lignocellulosic deconstruction process. Microorganisms that can overcome the recalcitrance of cellulose in the context of potentially toxic lignin moieties are especially interesting for biofuels production.

B. Enzymatic Lignocellulose Deconstruction

The deconstruction of lignocellulose by *Caldicellulosiruptor* initially involves extracellular enzymatic attack of the plant biomass substrate. Members of the genus utilize an array of extracellular glycoside hydrolases (GHs), polysaccharide lyases (PLs), and carbohydrate esterases (CEs) that break the glycosidic linkages of long-chained polysaccharides to eventually yield oligosaccharides and simple sugars (Blumer-Schuette et al. 2012). These are then transported into the cell for utilization in metabolic pathways.

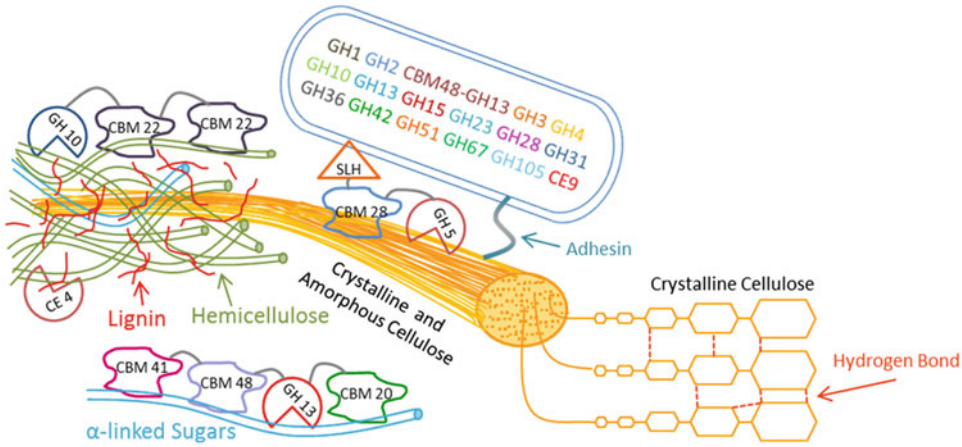

Fig. 8.3. **Lignocellulose microfibril with *Caldicellulosiruptor* core extracellular enzymes**. Hemicellulose and lignin form a protective sheath around cellulose. Core enzymes have activity against α-linked sugars, xylan and amorphous cellulose.

The degradation of crystalline cellulose and other recalcitrant plant polysaccharides requires the synergistic action of multiple catalytic domains, often within the same enzyme. The efficacy of these enzymes can be enhanced through the conjugation of the catalytic subunit(s) with one or more carbohydrate binding modules (CBM). The CBMs act to increase the catalytic efficiency by targeting the catalytic GH unit toward accessible polysaccharide, disrupting the polysaccharide structure, and maintaining the substrate in prolonged intimate contact with the catalytic GH (Shoseyov et al. 2006). The end goal of extracellular polysaccharide degradation is the production of carbohydrates in a transportable form, typically with six or fewer saccharide units.

The strategy used to generate small, transportable saccharides differs across the microbial world. For example, the cellulolytic fungus, *Trichoderma reesei*, utilizes extracellular enzymes, not associated with the cell, that contain a single catalytic domain and, in many cases, a single CBM (Martinez et al. 2008). The cellulosome, initially discovered in *Clostridium thermocellum*, is a multi-protein complex constructed around an enzymatically inactive scaffoldin. It contains cohesin domains for the attachment of enzyme subunits and a CBM to mediate attachment to the substrate. Enzyme subunits, which contain dockerin domains, attach to the scaffoldin via cohesin-dockerin interactions. Similarly, interactions between a dockerin domain on the scaffoldin and a cell-associated cohesin domain anchor the cellulosome complex to the cell (Bayer et al. 1983, 1998; Fontes and Gilbert 2010). Members of the genus *Caldicellulosiruptor* are non-cellulosomal, but do employ several multi-domain enzymes that mediate cellular attachment to plant biomass through S-layer homology (SLH) domains (Ozdemir et al. 2012). The S-layer containing enzymes in *Caldicellulosiruptor* are much smaller than the cellulosome, and have one or two catalytic domains coupled with one or more CBM (Blumer-Schuette et al. 2010; Dam et al. 2011; VanFossen et al. 2011). The *Caldicellulosiruptor* SLH-domain containing proteins with additional GH and/or CBM domains, contribute to biomass degradation by localizing the substrate and holding the cell in close proximity (Fig. 8.3). The majority of extracellular GHs encoded in *Caldicellulosiruptor* genomes lack SLH domains, such that they freely diffuse in the biomass-containing milieu. The presence of multiple catalytic domains within a single

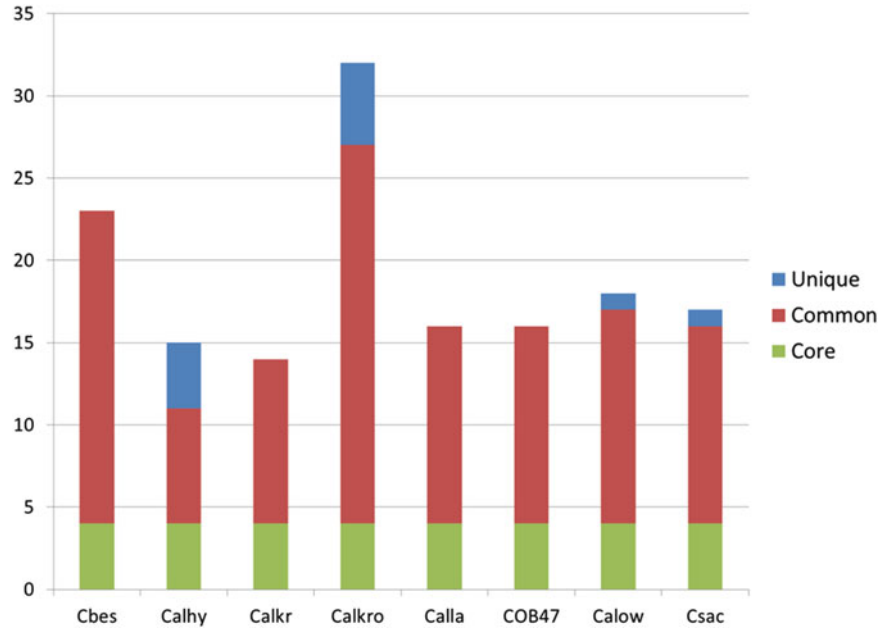

Fig. 8.4. **Extracellular glycoside hydrolases of *Caldicellulosiruptor* species**. Core GHs are common to all species. Common GHs are possessed by one or more species, while unique GHs are only in present in a particular species. Abbreviations follow the assigned locus tags and are as follows: Cbes *C. bescii,* Calhy C. *hydrothermalis,* Calkr *C. kristjanssonii,* Calkro *C. kronotskyensis,* Calla *C. lactoaceticus*, COB47 *C. obsidiansis,* Calow *C. owensensis,* Csac *C. saccharolyticus*.

extracellular enzyme imparts the capacity to degrade complex heterogeneous polysaccharides synergistically.

The pan-genome of *Caldicellulosiruptor* encodes 134 carbohydrate-active enzymes (CAZy) (GHs, CEs, PLs and CBMs), of which 106 are GHs, representing 43 GH families. However, only 26 GHs from 17 families are included in the core genome (Cantarel et al. 2009; Blumer-Schuette et al. 2012). Presumably, the core set of enzymes contains the basic catalytic capacity required for growth on plant biomass by members of the genus. It may be necessary, but not sufficient, for plant biomass deconstruction, since all *Caldicellulosiruptor* species contain additional GHs in the core genome. The core set of GHs include four out of the five known GH families that hydrolyze the β-1,4 xyloside linkages characteristic of xylan, three out of the four GH families that hydrolyze the β-1,4 mannoside linkages of mannan, and four out of the five xyloglucanase families that hydrolyze β-1,4 glucan linkages (Blumer-Schuette et al. 2010).

1. Core Caldicellulosiruptor *Hydrolytic Enzymes*

The core carbohydrate active enzyme component of the *Caldicellulosiruptor* genome includes four extracellular enzymes (Figs. 8.3 and 8.4), identified by the presence of a signal peptide at the N-terminus, directing the protein to be secreted into the extracellular environment (Navarre and Schneewind 1999). The extent to which the core set extracellular enzymes can degrade lignocellulosic substrates is based on biochemical characteristics, homology and phenotypic characteristics of the genus. Csac_0678, a bi-functional GH5 conjugated to a CBM28 and S-layer homology (SLH) domains (Fig. 8.3), has orthologs in all *Caldicellulosiruptor* genomes. As mentioned above, the S-layer homology domains of this enzyme act to anchor the enzyme to the cell surface, while the CBM facilitates attachment of the multi-domain enzyme to the substrate (Sára and Sleytr 2000). Biochemical characterization of Csac_0678 showed that the GH5 domain exhibited both

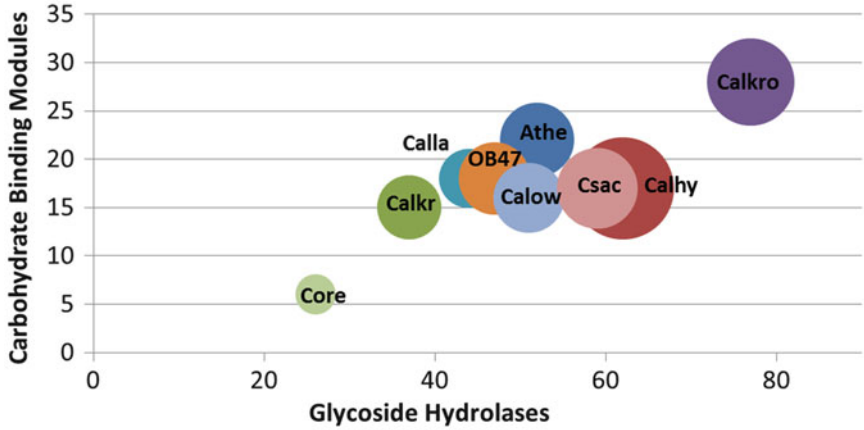

Fig. 8.5. **Number of ORFs containing glycoside hydrolases, carbohydrate binding modules and ABC transporters**. *Bubble size* correlates to the number of ABC transporters in each species.

endoglucanase and xylanase activity, while the CBM28 was required for activity and binding to crystalline cellulose (Ozdemir et al. 2012). Two other extracellular core GHs are a putative xylanase, containing a GH10 domain conjugated to two CBM22 domains, and a putative amylase with a GH13 domain conjugated to a CBM41, a CBM48 and a CBM20 (Janecek 1997; Andrews et al. 2000). The remaining extracellular core enzyme is a CE family 4 enzyme with putative xylanase activity (Caufrier et al. 2003; Cantarel et al. 2009). This core set of extracellular enzymes theoretically provides the genus with the ability to hydrolyze α- and β-glucan linkages of starch and cellulose, respectively, in addition to β-xyloside linkages of xylan. It should be noted, even though the core extracellular enzyme set of *Caldicellulosiruptor* contains biocatalysts active against the β-glucan linkages of cellulose, this does not necessarily mean crystalline cellulose deconstruction is possible, as not all species are able to efficiently hydrolyze this substrate.

2. *Cellulolytic* Caldicellulosiruptor *Enzymes*

Beyond the core genome, the presence and absence of specific types of extracellular GHs in *Caldicellulosiruptor* species correlates to the capacity to utilize crystalline cellulose (Blumer-Schuette et al. 2010). In particular, growth on Avicel and filter paper differentiates the cellulolytic members of the genus. For example, the strongly cellulolytic species: *C. bescii*, *C. kronotskyensis*, *C. saccharolyticus* and *C. obsidiansis* grow well on Avicel and filter paper, while *C. lactoaceticus* grows to a lesser extent on these substrates. The weakly cellulolytic species, *C. hydrothermalis*, *C. kristjanssonii* and *C. owensensis*, grow to a limited extent on filter paper, with no visible deconstruction of the solid substrate. Within the sequenced *Caldicellulosiruptor* genomes, *C. kronotskyensis* contains the most carbohydrate-active encoded enzymes, indicating the ability to degrade a wide range of polysaccharides (Figs. 8.4 and 8.5) (Blumer-Schuette et al. 2012). The genomes of the four strongly cellulolytic species contain a shared set of seven GHs, three of which are extracellular. These extracellular multi-domain enzymes each contain different GH domains (GH9 and GH48, GH74 and GH48, or GH9 and GH5) linked by CBM3 modules. The activity of one or more of these extracellular GHs presumably confers the ability to degrade crystalline cellulose. In order to determine which of these enzymes confers the degradation of crystalline cellulose, the weakly cellulolytic species were inspected for the presence of these four GH families. All *Caldicellulosiruptor* genomes sequenced to date harbor GH5-containing enzymes.

However, while the *C. kristjanssonii* genome encodes a putative extracellular enzyme containing GH9 and GH74 domains linked to CBM3 domains, this bacterium is weakly cellulolytic. As such, the presence of GH5, GH9 and GH74 enzyme families is not necessarily indicative of crystalline cellulose hydrolytic capacity in the genus *Caldicellulosiruptor*. On the other hand, GH48 family enzymes cannot be identified in the genomes of any of the weakly cellulolytic species, suggesting the presence of a GH48 domain is an essential determinant for the ability to hydrolyze crystalline cellulose by *Caldicellulosiruptor* species (Blumer-Schuette et al. 2010, 2012). Furthermore, the coupling of GH48 with CBM3 domains is indicative of strong cellulolytic capacity. Along these lines, CelA, the GH9-, GH48- and CBM3-containing enzyme present in the cellulolytic species, has been characterized biochemically. CelA, isolated from *C. bescii* culture supernatants, as well as specific GH domains produced recombinantly in *E. coli,* had activity against crystalline cellulose and other β-linked glucans (Te'o et al. 1995; Zverlov et al. 1998), demonstrating the importance of CelA to the cellulolytic phenotype in *Caldicellulosiruptor*. As genetic tools for this genus become available, it will be interesting to see if the insertion of a GH48-domain containing enzyme can impart a strong celluloytic capacity on the weakly celluloytic species in this genus or if the absence of CelA results in loss of capacity to degrade crystalline cellulose.

III. Carbohydrate Transport

Upon degradation of long-chained polysaccharides to di/oligosaccharides by extracellular enzymes of *Caldicellulosiruptor* species, the simpler sugars are transported into the cell via transmembrane carbohydrate transport systems for use in anabolism or catabolism (VanFossen et al. 2011). Given the wide-ranging inventory of GHs found in the various *Caldicellulosiruptor* species, it is not surprising that there is also significant variability in the number and specificity of substrate transporters across the genus. ABC and phosphoenolpyruvate-dependent phosphotransferase (PTS) carbohydrate transport systems can be identified in *Caldicellulosiruptor* genomes, although the presence of PTS transporters in the genus is sparse and variable. ABC carbohydrate transporters typically belong to one of two groups, the carbohydrate uptake transporter (CUT) family and the Di/Oligopeptide transporter family (Dpp/Opp) (Schneider 2001). The CUT-family transporters are further divided into two sub-families, differentiated in architecture and substrate specificity. CUT sub-family 1 (CUT1) systems, in *Caldicellulosiruptor,* transport both di/oligosaccharides and monosaccharides (Vanfossen et al. 2009). CUT1 transporters consist of an extracellular substrate binding protein, two membrane proteins forming the translocation path, and a single ATP binding subunit likely in the form of a homodimer. The CUT2 sub-family is solely involved in monosaccharide transport, containing a single membrane protein, presumably a homodimer, and two fused ATPase domains. The Dpp/Opp transport family has been implicated in the transport of di- and oligopeptides, nickel, heme, as well as sugars. Its architecture is a combination of CUT1 and CUT2 sub-family features, with an extracellular binding protein, two membrane domains and two ATPase domains that form a heterodimer (Koning et al. 2002). The genus *Caldicellulosiruptor* collectively contains 45 ABC transporters, with the core genome consisting solely of 6 CUT1 transporters (Fig. 8.5) (Blumer-Schuette et al. 2012). The weakly cellulolytic *C. hydrothermalis* contains the greatest number of ABC transporters, indicating carbohydrate transporter inventory is not necessarily correlated to a strongly cellulolytic phenotype (Fig. 8.5). Across the genus, CUT1 transporters appear to be responsible for the majority of carbohydrate transport into the cell, making up 37 of the 45 identifiable transporter systems in *Caldicellulosiruptor* genomes. Dpp/Opp and CUT 2 systems

account for 3 and 5 of the ABC transporters present in the genus, respectively.

Currently, none of the *Caldicellulosiruptor* ABC transporters have been biochemically characterized. Even with the lack of specific biochemical knowledge, bioinformatics analysis can be used to map transport substrates and transport mechanisms through homology with other characterized transporters. VanFossen et al. (2009) analyzed the transcriptomes of *C. saccharolyticus* grown on glucose, fructose, mannose, xylose, arabinose, galactose and a mixture of all these sugars, in addition to xylan, xylose, xyloglucan and xylogluco-oligosaccharides. These data-sets, using metrics developed with previous work on *Thermatoga maritima*, a heterotrophic hyperthermophile (Conners et al. 2005), could be used to predict carbohydrate preference of the majority of transporters in *C. saccharolyticus*. It was concluded that the genome of *C. saccharolyticus* contained transporters for all the substrates tested. The carbohydrate specificities of the ABC transporters had either limited specificity for only one substrate, as is often observed with oligosaccharide transporters, or broad specificity for a variety of substrates, as is often the case with monosaccharide transporters. Ultimately, *C. saccharolyticus* is able to transport and utilize the wide variety of carbohydrates, simple or complex, that result from lignocellulosic biomass hydrolysis.

Phenotypic and genotypic differences can provide insight into the role of specific ABC transporters in carbohydrate transport. For example, *C. lactoaceticus* is incapable of growth on glucose, even though it hydrolyzes cellulose, raising the prospect that glucose catabolism could be transport-limited. *C. lactoaceticus* also has the fewest number of carbohydrate ABC transporters within the genus (Fig. 8.5) (Blumer-Schuette et al. 2012). Closely related *C. kristjanssonii* is capable of growth on glucose and only contains three ABC transporters not present in the *C. lactoaceticus* genome, suggesting that one of these three transporters imparts the capacity for glucose transport. Two of these transporters are members of the CUT1 and CUT2 transporter families with orthologs in all other *Caldicellulosiruptor* species. VanFossen et al. (2009) predicted that these two transporters are involved in glucose, fructose and xylose transport. In fact, these are the only transporters identified to transport glucose into *C. saccharolyticus*. Taken together, these transporters seem to enable growth on glucose by *C. kristjanssonii* and most likely other *Caldicellulosiruptor* species.

The pan-genome of *Caldicellulosiruptor* contains one identified PTS (Blumer-Schuette et al. 2012). In many organisms, the PTS is bi-functional, playing roles in carbohydrate transport and as a starting point in regulating carbon catabolism (Stulke and Hillen 2000; Kotrba et al. 2001; Brückner and Titgemeyer 2002). The PTS consists of two cytosolic energy coupling proteins (Enzyme I and histidine-containing protein (HPr)) and carbohydrate specific, Enzyme II, which catalyzes concomitant carbohydrate translocation and phosphorylation at the expense of PEP (Kotrba et al. 2001). In *Caldicellulosiruptor*, the PTS is currently the only identified mannose transporter and has been implicated in fructose transport (Vanfossen et al. 2009). The possible role of the PTS in carbohydrate catabolite regulation in *Caldicellulosiruptor* has not been established, although in a mixture of saccharides including galactose, glucose, mannose, xylose, arabinose and fructose, *C. saccharolyticus* utilized fructose to the greatest extent, followed by arabinose and xylose (Vanfossen et al. 2009). Whether the fructose specific PTS plays a role in regulation of substrate utilization is not known. In addition, genomes of *Caldicellulosiruptor* species encode the genes required for the carbon control protein A (CcpA)-dependent CCR present in *B. subtilis* and other gram-positive bacteria. The components of the CcpA-dependent CCR signaling cascade present in *Caldicellulosiruptor* include the fructose specific PTS transporter, HPr(Ser) kinase, catabolic repression HPr protein (CrH) and the CcpA (Warner et al. 2003; van de Werken et al. 2008). Though *Caldicellulosiruptor* does not exhibit traditional CCR, the combi-

nation of the fructose specific PTS, the genes encoding CcpA-dependent CCR, and proclivity for fructose utilization implies that this system plays a role in the carbohydrate preferences of these bacteria.

IV. Intermediary Metabolism

The genus *Caldicellulosiruptor* can utilize a range of carbohydrates for growth, as such, an array of metabolic pathways are implicated in bioenergetics. Genome sequence data and ^{13}C-NMR analysis revealed *C. saccharolyticus* contains a complete Embden-Meyerhoff-Parnas (EMP) pathway (de Vrije et al. 2007; van de Werken et al. 2008). The EMP pathway, which serves as the primary generator of ATP and reducing equivalents, is conserved within the genome sequenced members of the genus. The central role of the EMP pathway in *Caldicellulosiruptor* metabolism requires all carbohydrate growth substrates be directly or indirectly fed into the pathway for energy generation. Glucose, liberated from cellulose and starch, can be oxidized directly by the EMP pathway. The hydrolysis products of hemicellulose, such as xylose, pectin and galactose, must first be processed through alternative pathways. The products of these sub-pathways are then funneled into the EMP pathway at different levels (Fig. 8.6). These sub-pathways are often incomplete and have varying levels of conservation across the genus. The ability to metabolize xylose is conserved within the genus. Xylose, the major constituent of hemicellulose, is readily available during growth on lignocellulosic biomass. Xylose enters the non-oxidative branch of the pentose phosphate pathway (PPP) via conversion by a xylose isomerase and xylulokinase. Arabinose, often associated with xylan, is also funneled into the non-oxidative branch of the pentose phosphate pathway. Unlike xylose, it is converted into PPP intermediates by means of a bifunctional L-fucose/D-arabinose isomerase and a L-ribulokinase that are not conserved in the North American or Icelandic *Caldicellulosiruptor* species (Figs. 8.1 and 8.6) (van de Werken et al. 2008). The lack of these enzymes in the Icelandic species correlates with their inability to grow on arabinose (Ahring 1995; Bredholt et al. 1999). In contrast, the North American species are capable of growth on arabinose, indicating the presence of alternative enzymes for arabinose metabolism (Huang et al. 1998; Hamilton-Brehm et al. 2010). Metabolism of xylose and arabinose through the non-oxidative PPP yields β-D-fructose-6P or glyceraldehyde-3P, early metabolites in glycolysis (Fig. 8.6). Though members of the genus *Caldicellulosiruptor* rely on the non-oxidative PPP for the metabolism of many carbohydrates, the oxidative branch of the PPP is not present, akin to other anaerobic biomass degraders in the class Clostridia (Hemme et al. 2011). The oxidative branch of the PPP pathway in many organisms is the sole generator of NADPH, the primary source of reducing equivalents for cellular biosynthetic pathways (Kruger and von Schaewen 2003). There appear to be other enzymes in *Caldicellulosiruptor* with the capability for generating NADPH, but the exact physiological roles of these enzymes is unclear (van de Werken et al. 2008). This raises questions as to the mode and extent of NADPH generation within the cell.

Uronic acids, the building blocks of pectin, are primarily composed of galacturonic acid (Ridley and O'Neill 2001). All *Caldicellulosiruptor* species have been described to support growth on pectin (Rainey et al. 1994; Ahring 1995; Huang et al. 1998; Bredholt et al. 1999; Miroshnichenko et al. 2008; Hamilton-Brehm et al. 2010; Yang et al. 2010). Galacturonate, the anion of galacturonic acid, enters metabolism through isomerization to tagaturonate. Upon conversion to tagaturonate, the pertinent metabolic pathway becomes unclear, as tagaturonate reductase and altronate hydrolase, have not been identified in the genus. This implies the use of a novel pathway or unidentified enzymes for the conversion of galacturonate.

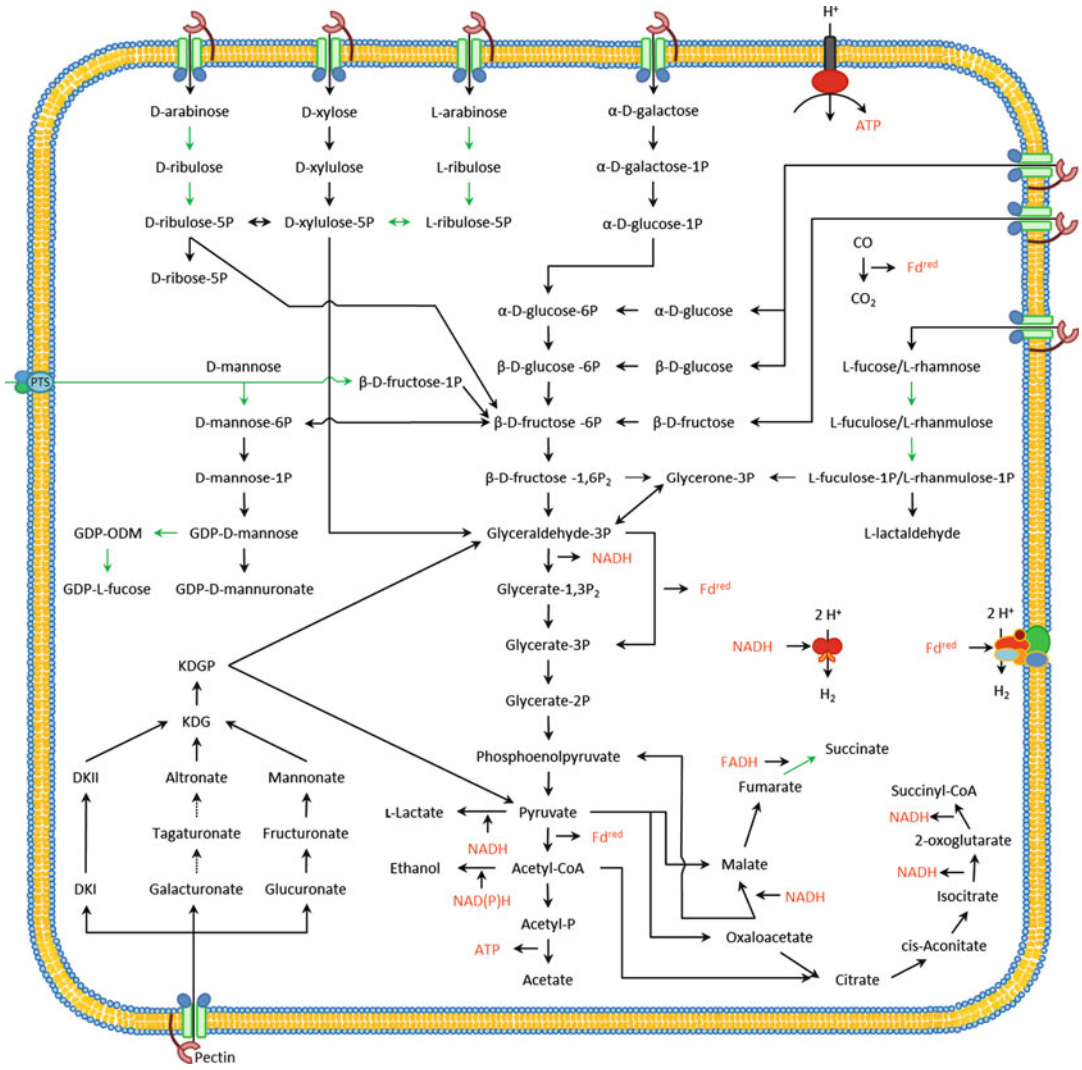

Fig. 8.6. **Metabolic features of *Caldicellulosiruptor* species**. *Green arrows* indicate reactions not conserved in all species. Abbreviations: *DKI* 5-keto-4-deoxyuronate, *DKII* 2,5-Diketo-3-deoxy-D-gluconate, Fd^{red} reduced ferrodoxin, *KDG* -2-Dehydro-3-deoxy-D-gluconate, *KDGP* KDG phosphate, *NADH* reduced nicotinamide adenine dinucleotide, *P* phosphate.

Similar to the conversion of xylose and arabinose, the metabolism of the deoxysugars, such as fucose and rhamnose, is variable within the genus. Fucose, is found as a subunit of xyloglucans (Hisamatsu et al. 1991) and rhamnose is a common component of pectin (Komalavilas and Mort 1989; Ridley and O'Neill 2001). Icelandic *Caldicellulosiruptor* species are incapable of growth on rhamnose (Ahring 1995; Bredholt et al. 1999), so it was not surprising to correlate the lack of rhamnose isomerase and rhamnulokinase from their genome sequences to this physiological trait. Limited information is available for growth of *Caldicellulosiruptor* species on fucose, which presumably requires fucose isomerase for this phenotype. North American and Icelandic species both lack fucose isomerase and consistent with this observation, *C. obsidiansis* is unable to utilize fucose as a growth substrate (Hamilton-Brehm et al. 2010). Mannose and galactose are also found as constituents of hemicellulose, but in

smaller amounts than xyloglucan. Galactose is metabolized to glucose-6P through the Leloir pathway (Holden et al. 2003). The Leloir pathway is conserved in all sequenced *Caldicellulosiruptor* species. Mannose is typically carried across the cell membrane via a PTS transporter where it is phosphorylated to mannose-6P. Several *Caldicellulosiruptor* species (*C. kristjanssonii*, *C. lactoaceticus* and *C. obsidiansis*) lack homologs to a PTS, yet have the ability of growth on mannose. An alternative system for mannose phosphorylation has not yet been reported in the genus. Fructose can also be transported via the same PTS to yield fructose-1P, which is then shuttled directly into the EMP pathway.

While the tricarboxylic acid (TCA) cycle is not involved directly in substrate utilization, it is important because essential precursors to biosynthetic pathways are produced. The TCA cycle in *Caldicellulosiruptor* species is incomplete, however all species have an oxidative branch to succinyl-CoA and a reductive branch to Fumarate (Fig. 8.6). The incomplete TCA cycle present in *Caldicellulosiruptor* likely functions to generate amino acid biosynthesis precursors, such as 2-oxoglutarate (alpha-ketoglutaric acid) and oxaloacetate, rather than reducing equivalents. The production of excess reducing equivalents in the TCA cycle could overwhelm the fermentative *Caldicellulosiruptor* without the presence of an aerobic electron transport chain.

V. Metabolism of Fuel Production

The degradation of recalcitrant plant biomass and subsequent utilization of polysaccharides in *Caldicellulosiruptor* fermentation pathways produces several metabolic products including ethanol and molecular hydrogen.

A. Ethanol

The genus *Caldicellulosiruptor* has the ability to produce small amounts of ethanol, indicating pathways to this fermentation product exist or ethanol the result of promiscuous enzymes. Instead of ethanol production, most carbon is directed toward acetate, and as a consequence, large quantities of molecular hydrogen are produced as a fermentation product (Fig. 8.6). The primary role of hydrogen and ethanol production in anaerobic metabolism is to re-oxidize reducing equivalents generated during the fermentation of sugars. The production of these compounds is dependent on environmental conditions and growth state. Ethanol production occurs via the reduction of acetyl-CoA by alcohol dehydrogenase (ADH). Ethanol production serves as an efficient means to recycle reducing equivalents in many other organisms, but ethanol has only been detected in very low to trace levels in *Caldicellulosiruptor* (Rainey et al. 1994; Ahring 1995; Huang et al. 1998; Bredholt et al. 1999; Hamilton-Brehm et al. 2010; Yang et al. 2010) and thus, has not been studied in detail.

Caldicellulosiruptor species contain several putative ADHs, but the specific enzyme responsible for the conversion of acetyl-CoA to ethanol is unknown. In *Thermoanaerobacter psuedethanolicus* (formerly *Thermoanaerobacter ethanolicus* 39E) ethanol production is NADPH-dependent, through the activity of a bi-functional alcohol dehydrogenase/acetyl-CoA thioesterase (Burdette and Zeikus 1994). A putative ADH in *C. saccharolyticus* (Csac_0395) contains a NADPH-binding domain and sequence similarity to the bi-functional enzyme from *T. pseudethanolicus* (van de Werken et al. 2008). This suggests that ethanol production in *Caldicellulosiruptor* is NADPH-dependent, and targeted to oxidizing NADPH, rather than the NADH generated during glycolysis. However, due to the lack of an oxidative branch of the PPP, the mode of generation and levels of NADPH are unknown. It is likely that the amount of NADPH produced is limited, leaving NADPH regeneration to $NADP^+$ to biosynthetic pathways, resulting in minimal NADPH levels available for ethanol production. In another example, ADH activity of an ethanol adapted mutant strain of *Clostridium thermocellum*

shifted from NADH to NADPH dependence, suggesting similarities for ethanol tolerance mechanisms and redox homeostasis (Brown et al. 2011). The specific role of ethanol production in *Caldicellulosiruptor* has not been explored; it is not likely a means of controlling the cellular redox balance, since homeostasis is maintained through hydrogen and lactate production.

B. Hydrogen

Caldicellulosiruptor species produce significant amounts of molecular hydrogen as a fermentation product, such that the maximum yield of H_2 is among the highest for hydrogen-producing microorganisms. *Caldicellulosiruptor* species employ the EMP pathway to achieve a maximum theoretical yield (Thauer limit) of 4 moles H_2 per mol glucose (Thauer et al. 1977). *Caldicellulosiruptor* can utilize both the less energetic NADH and preferential reduced ferredoxin (Fd^{red}) for the reduction of protons to produce molecular hydrogen. Both of these reducing equivalents are generated during the oxidation of sugars in the EMP pathway. Fd^{red} is generated from the oxidation of pyruvate to acetyl-CoA by pyruvate: ferredoxin oxidoreductase (PFOR) and the oxidation of glyceraldehyde-3P to glycerate-3P by aldehyde ferredoxin oxidoreductase. Alternatively, glyceraldehyde-3P can be oxidized to glycerate-3P via glyceraldehyde phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase to generate NADH and ATP (Fig. 8.6).

1. Hydrogen Production and Carbohydrate Transport

Hydrogen production in *Caldicellulosiruptor* may be linked to the primary use of ABC transporters for carbohydrate translocation. The translocation of substrate by ABC transporters, and subsequent phosphorylation, requires two molecules of ATP. Alternatively, import of monosaccharides by PTS requires phosphoenolpyruvate as a phosphate donor to achieve transport and phosphorylation in one step. Currently, as mentioned above, there is one orthologous PTS identified in some species of the genus. *Caldicellulosiruptor* relies primarily on ABC transporters for carbohydrate transport, making the generation of a supplementary source of ATP molecules for carbohydrate transport advantageous to the cell. The oxidation of sugars to acetate generates an extra ATP (2 moles/mole hexose), offsetting the consumption by ABC transporters, while at the same time generating Fd^{red}. The production of H_2 is then used to re-oxidize Fd^{red} generated as a byproduct of ATP generation for carbohydrate transport.

2. Hydrogenases in Caldicellulosiruptor

In *Caldicellulosiruptor* species, the reduction of protons to molecular hydrogen occurs via two distinct hydrogenases, a cytoplasmic Fe-only hydrogenase (HydA to HydD), and a membrane-bound Ni-Fe hydrogenase (EchA to EchF). Though neither of these hydrogenases have been biochemically characterized, homologs in *Caldanaerobacter subterraneus* subsp. *tengcongensis* (formerly *Thermoanaerobacter tengcongensis*) were found to be NADH- and Fd^{red}-dependent, respectively (Fardeau et al. 2004; Soboh et al. 2004). A third, putative hydrogenase cluster, containing an NADH-binding protein, also exists, but the function of this cluster is unknown and is theorized to be redundant (van de Werken et al. 2008); however, this remains to be confirmed experimentally. The production of H_2 from Fd^{red} is energetically favorable; making H_2 production by the membrane bound Ni-Fe hydrogenase preferable. In contrast, the utilization of the NADH-specific, Fe-only hydrogenase is less favorable; only under a very limited set of conditions is the production of hydrogen from NADH thermodynamically favorable (Verhaart et al. 2010). It is interesting that this Fe-only hydrogenase has approximately 50 % amino acid sequence identity to a bifuricating hydrogenase in *T. maritima*. This bifuricating hydrogenase uses the exer-

gonic oxidation of ferredoxin to drive the unfavorable oxidation of NADH to produce H_2 (Schut and Adams 2009). If the Fe-only hydrogenase of *Caldicellulosiruptor* is, indeed, bifuricating, NADH would serve as an energetically favorable substrate for the reduction of protons to H_2.

C. Growth Conditions and Hydrogen Production

During exponential growth, *Caldicellulosiruptor* produces H_2, CO_2 and acetate, almost exclusively as fermentation products (Van Niel et al. 2002; Zeidan and van Niel 2009). However, there are additional fermentation end products that are produced under specific physiological conditions. For example, increased H_2 concentrations and the transition to stationary phase, modulates NAD^+ regeneration and metabolic flux of pyruvate toward lactate formation via lactate dehydrogenase (LDH) (Willquist and van Niel 2010). Lactate formation consumes NADH and bypasses the production of Fd^{red} and ATP (Fig. 8.6). The regulation of flux at the pyruvate node is a function of LDH and hydrogenase activity.

1. Regulation of Lactate Dehydrogenase

The activity of LDH plays a key role in cellular ATP levels and redox potential, making its regulation important and complex. LDH is regulated by metabolic energy carriers: inorganic phosphate (PPi), ATP and NAD^+. The utilization of the energy carrier PPi is an alternative strategy used in *Caldicellulosiruptor* and other bacteria, to conserve energy (Mertens 1991; Bielen et al. 2010). The primary source of PPi is anabolic reactions, such as poly-nucleic acid biosynthesis and the activation of fatty acids and amino acids for lipid and protein synthesis (Heinonen 2001). Regulation of LDH occurs by both activation and inhibition; competitive inhibition occurs by PPi and NAD^+ and allosteric activation by fructose 1,6-bisphosphate, ATP and ADP (Willquist and van Niel 2010). The multitude of pathways generating and consuming these molecules results in variable activity of LDH. LDH activity has been shown to follow PPi levels and growth phase. For example, during exponential growth, high anabolic flux leads to increased generation of PPi, thereby inactivating LDH, and maximizing flux to acetate and hydrogen. As growth factors trigger stationary phase, PPi levels decrease and ATP levels increase (Bielen et al. 2010), enhancing the affinity of LDH to NADH redirecting carbon flux to lactate.

2. Hydrogen Concentration Affects Hydrogen Production

If the removal of metabolic H_2 from the growth environment is insufficient, levels of dissolved hydrogen in liquid and partial pressure in the gas phase will begin to increase. Increasing levels of H_2 severely inhibit hydrogen production through product inhibition (Ljunggren et al. 2011; van Niel et al. 2003). The decrease in hydrogen production results in accumulation of reducing equivalents, requiring changes in metabolic flux to balance the reactive species. The critical threshold value of hydrogen partial pressure varies with growth phase and study to study (Ljunggren et al. 2011; Willquist et al. 2011), but is typically 10–20 kPa, as determined in batch cultures of *C. saccharolyticus* (van Niel et al. 2003). H_2 inhibition is more directly related to dissolved H_2 concentrations. Ljunggren et al. (2011) found a critical dissolved H_2 concentration of 2.2 mmol/L results in complete inhibition of hydrogen production. Gas sparging can be used to alleviate rising H_2 concentrations (Chou et al. 2008), and specifically, N_2 sparging can increase hydrogen yields (Zeidan and van Niel 2010; Ljunggren et al. 2011; Willquist and van Niel 2012). However, at a process level, inert gas sparging is expensive and economically unfavorable. Alternatively, CO_2 is readily available from many industrial processes and can be relatively easily separated in downstream processing of the gas stream (Hallenbeck and Benemann 2002). However, sparging with CO_2 negatively affects growth and H_2 production in *C. saccharolyticus*. Dissolved CO_2, in the form of bicarbonate and protons, inhibits growth

Table 8.1. Reported hydrogen yields of *Caldicellulosiruptor* species.

Culture Type	Species	Substrate	Yield[a] (mol H_2/mol C_6)	Reference
Continuous	*saccharolyticus*	Glucose	3.8	Willquist et al. (2011)
Continuous	*saccharolyticus*	Glucose[b]	3.5	Willquist and van Niel (2012)
Trickle bed reactor	*saccharolyticus* (non-sterile)	Sucrose	2.8	van Groenestijn et al. (2009)
Batch	*saccharolyticus*	Miscanthus hydrolysate	3.4	de Vrije et al. (2009)
Batch	*saccharolyticus*	Paper sludge hydrolysate	2–3.8	Kádár et al. (2004)
Batch	*saccharolyticus*	Wheat straw	3.8	Ivanova et al. (2009)
Batch	*saccharolyticus*	Pretreated maize leaves	3.7	Ivanova et al. (2009)
Continuous	*kristjanssonii*	Glucose	3.5	Zeidan et al. (2010)
Batch	*kristjanssonii*	Glucose + Xylose	3.0	Zeidan and van Niel (2009)
Batch	*owensensis*	Glucose[b]	4.0	Zeidan and van Niel (2009)
Batch	*owensensis*	Xylose[b]	3.5	Zeidan and van Niel (2009)
Batch	*owensensis*	Glucose + Xylose	2.7	Zeidan and van Niel (2009)
Continuous	*saccharolyticus* + *kristjanssonii*	Glucose	3.7	Zeidan et al. (2010)
Continuous	*saccharolyticus* + *kristjanssonii*	Glucose + Xylose	3.6	Zeidan et al. (2010)
Batch	*saccharolyticus* + *kristjanssonii*	Glucose + Xylose	3.8	Zeidan and van Niel (2009)
Batch	*saccharolyticus* + *owensensis*	Glucose + Xylose	3.3	Zeidan and van Niel (2009)

[a]Maximum hydrogen yield reported at varying culture conditions (dilution rate, gas sparging etc.)
[b]Defined growth medium

through a decrease in pH and an increase in osmotic pressure, rendering CO_2 sparging infeasible (Willquist et al. 2009). Ljunggren et al. (2011) found an osmolarity between 0.27 and 0.29 mol/L to be inhibitory to the growth of *C. saccharolyticus*. Engineering *Caldicellulosiruptor* strains to be insensitive to increased osmotic pressures and pH changes and/or hydrogenases with a greater hydrogen tolerance will likely be a requirement of a *Caldicellulosiruptor*-based H_2 production process.

3. Hydrogen Yields

Experimental studies of hydrogen production in *Caldicellulosiruptor* have looked at both batch (Ivanova et al. 2009; Zeidan and van Niel 2009, 2010; Willquist and van Niel 2012) and continuous (de Vrije et al. 2007; Willquist et al. 2009; Zeidan et al. 2010) cultures. Hydrogen yields vary with species, substrate and growth conditions. Yields obtained in these experiments generally range from 80 to 95 % of the 4 mol H_2/mol C_6 theoretical maximum. Note that a batch culture of *C. owensensis* in defined medium, with glucose as substrate, achieved the Thauer limit of 4 mol H_2/mol C_6 sugar using continuous N_2 sparging (Table 8.1) (Zeidan and van Niel 2010). The maximum stoichiometric yield of H_2 from glucose is 12 mol H_2 per mol of glucose (Thauer et al. 1977), even so yields in vivo have not exceeded the Thauer limit. In vitro studies, using enzymes of the pentose phosphate pathway and a $NADP^+$ dependent hydrogenase from *P. furiosus*, achieved 11.6 mol H_2 per mol glucose-6-phosphate demonstrating the ability to produce near maximum H_2 yields in biological systems (Woodward and Mattingly 1996).

In continuous culture, H_2 production varies with dilution rate (i.e. growth rate), such that lower dilution rates result in lower growth rates and an increase in H_2 yield, albeit with a decrease in productivity. The inverse is true at higher dilution rates (de Vrije et al. 2007). At low growth rates, the majority of substrate is directed toward cell maintenance, during which many biosynthetic pathways remain dormant directing many of the reducing equivalents generated during glycolysis toward H_2 production. Thus, maximizing hydrogen production is a balance between the high productivities of fast growth rates and the high yields of slow growth rates. A proposed solution to increase both yield and productivity is to inoculate slow growing cultures at high cell densities (Chou et al. 2008). *Caldicellulosiruptor* species have also been found to persist in H_2-producing co-cultures. These co-cultures have shown synergy, such that the co-culture had higher hydrogen yields than the monoculture (Table 8.1) (Zeidan and van Niel 2009; Zeidan et al. 2010). For example, continuous co-culture of *C. saccharolyticus* and *C. kristjanssonii* found that both species persisted for 70 days with a hydrogen yield 6 % greater than either species alone. More importantly, cell-free growth supernatants of *C. saccharolyticus* were found to enhance the growth of *C. kristjanssonii* by decreasing its lag phase and increasing the maximum cell concentration by 18 % (Zeidan et al. 2010). Hydrogen yields from various *Caldicellulosiruptor* species have reached the Thauer limit (Zeidan and van Niel 2010). Increasing H_2 productivity in these bacteria, while maintaining high yields, will be a significant challenge in the development of a *Caldicellulosiruptor* bio-hydrogen production process, and may be possible through strategic metabolic engineering of these bacteria.

Acknowledgements

This work was funded in part by the BioEnergy Science Center, a U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science.

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