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Carbohydrate Utilization Patterns for the Extremely Thermophilic Bacterium *Caldicellulosiruptor saccharolyticus* Reveal Broad Growth Substrate Preferences▽†

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Coutilization of hexoses and pentoses derived from lignocellulose is an attractive trait in microorganisms considered for consolidated biomass processing to biofuels. This issue was examined for the H_2-producing, extremely thermophilic bacterium *Caldicellulosiruptor saccharolyticus* growing on individual monosaccharides (arabinose, fructose, galactose, glucose, mannose, and xylose), mixtures of these sugars, as well as on xylan and xylogluco-oligosacchrides. *C. saccharolyticus* grew at approximately the same rate (t_d, ~95 min) and to the same final cell density (1×10^8 to 3×10^8 cells/ml) on all sugars and sugar mixtures tested. In the monosaccharide mixture, although simultaneous consumption of all monosaccharides was observed, not all were utilized to the same extent (fructose > xylose/arabinose > mannose/glucose/galactose). Transcriptome contrasts for monosaccharide growth revealed minimal changes in some cases (e.g., 32 open reading frames [ORFs] changed ≥2-fold for glucose versus galactose), while substantial changes occurred for cases involving mannose (e.g., 353 ORFs changed ≥2-fold for glucose versus mannose). Evidence for catabolite repression was not noted for either growth on multisugar mixtures or the corresponding transcriptomes. Based on the whole-genome transcriptional response analysis and comparative genomics, carbohydrate specificities for transport systems could be proposed for most of the 24 putative carbohydrate ATP-binding cassette transporters and single phosphotransferase system identified in *C. saccharolyticus*. Although most transporter genes responded to individual monosaccharides and polysaccharides, the genes Csac_0692 to Csac_0694 were upregulated only in the monosaccharide mixture. The results presented here affirm the broad growth substrate preferences of *C. saccharolyticus* on carbohydrates representative of lignocellulosic biomass and suggest that this bacterium holds promise for biofuel applications.

Cellulose and hemicellulose comprise ~70% (dry basis) of plant biomass (23) and, as such, these complex carbohydrates are promising targets for alternative energy feedstocks. For biofuel production, lignocellulose must be deconstructed into fermentable sugars by various combinations of physical, physical-chemical, chemical, and biological processing steps (33). The more efficiently and economically that this is accomplished, the more attractive this material becomes for alternative fuels (25, 28, 37, 44). For this reason, there is great interest in identifying microorganisms that can minimize or eliminate the need for physical and chemical pretreatment of plant-based biomass and are at the same time capable of simultaneously fermenting the resulting pentoses and hexoses to biofuels (17).

In environmental settings, microbial survival can be based on the capacity to utilize a variety of available carbohydrates as carbon and energy sources (19, 22). When complex polysaccharides are involved, as is the case for plant-based biomass, extracellular glycoside hydrolases depolymerize potential growth substrates, rendering them into oligosaccharides and monosaccharides that can be transported into the cytoplasm for further processing. Metabolism of these sugars is thus mediated by the existence and regulation of membrane-based molecular transporters and impacted by regulatory processes, such as carbon catabolite repression (CCR) (19, 34). The number, specificity, and control of transporters presumably reflect the nutritional needs and preferences for a given microorganism. From a biofuel perspective, microorganisms that have a broad growth substrate range are attractive, since rapid and efficient metabolism of the spectrum of sugars comprising both cellulose and hemicellulose fractions is critical for economic lignocellulosic biomass conversion (34).

Microbial uptake mechanisms for sugar transport have been studied in some detail and include phosphoenolpyruvate-dependent phosphotransferase systems (PTS), ATP-binding cassette (ABC) transporters, and proton-linked transport systems (18, 26). The distribution and number of these transport mechanisms varies from microorganism to microorganism. The genomes for several anaerobic, extremely thermophilic bacteria, such as *Thermotoga maritima* (7, 27) and *Carboxydothermus hydrogenoformans* (45), indicate a lack of a PTS in lieu of ABC transporters for sugar uptake (3, 7, 45). Alternatively, other thermophiles, such as *Thermoanaerobacter tengcongensis*, contain one PTS in addition to multiple ABC transporters (42).

Caldicellulosiruptor saccharolyticus is an extremely thermophilic (optimum growth temperature [T_{opt}] of 70 to 75°C),

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gram-positive, fermentative anaerobe initially isolated from wood in the flow of a thermal spring in New Zealand (36). *C. saccharolyticus* can utilize a variety of simple (e.g., glucose, xylose, arabinose, and mannose) and complex carbohydrates (e.g., cellulose, xylan, and mannan) associated with lignocellulose as carbon and energy sources, producing hydrogen with yields approaching the Thauer limit (e.g., 4 H_2 of per mol of glucose consumed) (9, 16, 30, 41). Not surprisingly, of the 2,679 predicted coding sequences in the *C. saccharolyticus* genome, more than 177 open reading frames (ORFs) encode ABC transporter genes and at least one PTS, which together help to sequester a broad spectrum of potential growth substrates (40). Studies to date show that *C. saccharolyticus* does not exhibit glucose-based CCR and can coferment glucose and xylose (15, 40). To understand more about the growth characteristics of *C. saccharolyticus*, transcriptional response analysis was used to examine growth on monosaccharide and polysaccharides as this relates to specific regulatory and functional features of resident sugar transport systems in this bacterium.

MATERIALS AND METHODS

Growth of the microorganism on sugar substrates. *C. saccharolyticus* DSM 8903 (also ATCC 43494) was passed seven times in serum bottles shaken at 100 rpm under anaerobic conditions (N_2 headspace) on the substrate of interest in modified DSMZ 640 medium before inoculating a 400-ml screw-top, batch culture at 70°C, shaken at 100 rpm, containing either 0.5 g substrate per liter or 0.5 g of each substrate per liter. Growth was followed for 18 h. Growth substrates included D-glucose, D-mannose, L-arabinose, D-xylose, D-fructose, D-galactose, and xylan (oatspelt; Sigma-Aldrich, St. Louis, MO) and xyloglucan and xyloglucan oligosaccharides (provided by H. Brumer of the Royal Institute of Technology, Stockholm, Sweden).

Analysis of residual sugar and fermentation products. The cultures grown on the various sugars were harvested by centrifugation at 9,000 × *g*, after which the supernatants were clarified by passage through 0.22-μm-pore-size polyvinylidene difluoride filters (FisherSci, Hampton, NH). Monosaccharide and organic acid concentrations were determined by high-pressure liquid chromatography (HPLC), using a Waters 1525 binary pump with Breeze control software (Waters, Milford, MA). Sugar concentrations were determined by using a SP0810 sugar column (Showa Denko, New York, NY) operating at 80°C with water as the mobile phase. Organic acid by-products were also determined by HPLC, using an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA), with a mobile phase of 5 mM H_2SO_4 at 65°C. Peak heights for all samples, analyzed with an RI detector (Waters 2414), were integrated and the values were compared to standard curves for quantification.

Isolation of RNA. Isolation of total RNA from *C. saccharolyticus* was performed on cells that were grown on the various growth substrates at 70°C until early- to mid-logarithmic phase (mid-10^7 cells/ml). Cells were rapidly cooled to 4°C and then harvested by centrifugation at 5,000 × *g*, before storage at −80°C until they were processed further. Total RNA was isolated by using a protocol described previously (40), with the additional step of sonicating the cells after resuspension in TRIzol reagent (Invitrogen, San Diego, CA). cDNA was produced from the collected RNA using Superscript III reverse transcriptase (Invitrogen), random primers (Invitrogen), and the incorporation of 5-(3-aminoallyl)-2′-deoxyuridine-5′-triphosphate (Ambion, Austin, TX), as described elsewhere (11).

Microarray protocols. A spotted whole-genome *C. saccharolyticus* microarray was developed from 60-mer oligonucleotides based on at least 2,679 ORFs, as discussed previously (40). cDNA was labeled with either Cy3 or Cy5 dye (GE Healthcare, Little Chalfont, United Kingdom) and hybridized to Corning UltraGap II slides (Corning, Acton, MA). Samples were hybridized according to either a ten slide (see Fig. S1 in the supplemental material) or five slide loop experimental design (see Fig. S2 in the supplemental material). Microarray slides were scanned by using a Packard BioChip Scanarray 4000 scanner. ScanArray Express (v2.1.8; Perkin-Elmer, Waltham, MA) was used to quantitate signal intensities, prior to analysis with JMP Genomics 3.0 (SAS, Cary, NC), as described previously (29), using a mixed-effects analysis of variance model (43). ORFs that were differentially transcribed twofold or more and met the Bonferroni statistical criterion were considered to be up- or downregulated (43).

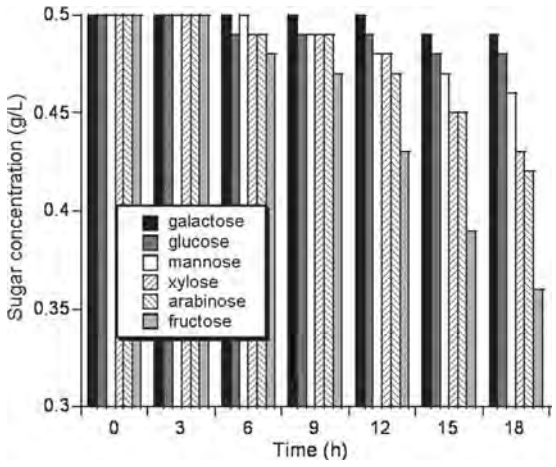

FIG. 1. Simultaneous utilization of sugars during the growth of *C. saccharolyticus* in a batch culture. Equal amounts of each of the six monosaccharides were added to cultures at the time of inoculation. The remaining concentration of each sugar is shown at various times during the growth of *C. saccharolyticus* in the batch culture. Average values from triplicate experiments are shown; the standard deviation for each sugar measured by HPLC was <0.02 g/liter.

Bioinformatic identification of sugar transporter genes. Protein sequences of putative substrate-binding proteins found in the *C. saccharolyticus* genome (http://www.ncbi.nlm.nih.gov/nuccore/CP000679) were subjected to similarity searches (BLASTP) at the National Center for Biotechnology Information website, based on the *Thermotoga maritima* MSB8 genome (http://www.ncbi.nlm.nih.gov/nuccore/AE000512), to identify ABC transporter systems involved in carbohydrate import. Similarity was defined as ≥25% identity and an E value of $\leq 10^{-5}$. Candidate ORFs were then compared to carbohydrate transporter predictions by TransportDB (31). To identify CcpA homologs, sequences from *Bacillus subtilis* 168 and *Lactobacillus brevis* ATCC 367 were used, with a threshold similarity of ≥35% identity and an E value of $\leq 10^{-5}$.

Microarray accession numbers. The microarray platform used in the present study is available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GPL6681. The raw data along with the final $\log_2$-fold changes have been deposited in the same database under accession number GSE17436.

RESULTS AND DISCUSSION

Carbohydrate utilization patterns. *C. saccharolyticus* was grown on a variety of monosaccharides (i.e., glucose, xylose, galactose, arabinose, mannose, fructose, and mixtures of the monosaccharides) commonly found in lignocellulose. On all sugars and sugar mixtures tested, cultures typically grew to final cell densities of 1×10^8 to 3×10^8 cells/ml at 70°C, with doubling times of ~95 min. Although previous studies of *C. saccharolyticus* monosaccharide cofermentation showed that xylose was used to a greater extent than glucose (15, 40), its capacity to coutilize other biomass-related sugars has not been reported. Figure 1 shows the extent of utilization for individual sugars present in a mixture of pentoses and hexoses. Several monosaccharides were utilized simultaneously; fructose was consumed to the greatest extent, followed by the pentoses, xylose, and arabinose (fructose > arabinose > xylose > mannose > glucose > galactose). Although fructose has not been a specific focus as a biofuels substrate, it is the main constituent of fructans, which are found in many plant families including wheat, barley, onion, and chicory (12). The possible source of

TABLE 1. Transcriptional response contrasts for *C. saccharolyticus* growth on monosaccharides

Sugar 1	Sugar 2	No. of ORFs upregulated ≥2-fold on[a]:	
		Sugar 1	Sugar 2
Arabinose	Xylose	20 (0)	138 (12)
	Fructose	22 (4)	80 (11)
	Galactose	10 (3)	48 (8)
	Glucose	34 (3)	45 (5)
	Mannose	32 (3)	25 (5)
	Sugar mix	5 (0)	84 (15)
Xylose	Fructose	19 (4)	22 (8)
	Galactose	70 (8)	25 (3)
	Glucose	59 (11)	23 (0)
	Mannose	113 (9)	56 (3)
	Sugar mix	62 (17)	69 (11)
Fructose	Galactose	36 (6)	25 (4)
	Glucose	32 (8)	29 (0)
	Mannose	92 (10)	29 (1)
	Sugar mix	27 (5)	29 (6)
Galactose	Glucose	21 (10)	11 (3)
	Mannose	57 (9)	51 (4)
	Sugar mix	12 (6)	27 (8)
Glucose	Mannose	174 (6)	179 (11)
	Sugar mix	13 (5)	69 (18)
Mannose	Sugar mix	58 (3)	91 (11)

[a] The numbers of transporter ORFs are indicated in parentheses.

fructans in thermal springs, the natural habitat of *C. saccharolyticus*, however, is not clear.

To examine sugar substrate preferences more carefully, cultures were grown on arabinose plus glucose and on a mixture of the hexoses (fructose, galactose, mannose, and glucose). *C. saccharolyticus* growth on arabinose plus glucose showed that the pentose was consumed to a greater extent (data not shown); a similar result was reported for growth on xylose plus glucose (15, 40). Preference for pentoses may be related to the fact that *C. saccharolyticus* lacks an oxidative pentose-phosphate pathway to convert hexoses to pentoses (40). Growth on a mixture of hexoses changed the utilization pattern to fructose > galactose > glucose > mannose (data not shown) from fructose > mannose > glucose > galactose when xylose and arabinose are present.

Monosaccharide transcriptomes. RNA from cultures grown on the six monosaccharides, both individual sugars and a mixture, was collected to examine the associated transcriptomes (see Tables S1 to S7 in the supplemental material). Table 1 summarizes the number of ORFs differentially transcribed by ≥2-fold for these experiments. Typically, fewer than ~200 ORFs were differentially transcribed (upregulated plus downregulated) when one monosaccharide was compared to another, with the exception of the comparison between glucose and mannose (353 ORFs). In the complete genome, ~25% of all ORFs (~600) were annotated as either "conserved hypothetical protein" or "protein of unknown function" (40); of these, the contrast between glucose and mannose implicated over a tenth of them. Growth on mannose triggered the transcription of two CcpA homologs: Csac_0389 and Csac_2494 (see Fig. S3 in the supplemental material). These two CcpA homologs were transcribed higher on mannose than during growth on the other individual monosaccharides. CcpA, coupled with a serine-phosphorylated Crh, can repress the expression of genes involved in the catabolism of alternative sugars by binding to the upstream catabolic repression element (*cre*) sites. However, searches for possible *cre* sites near gene promoters, based on *cre* sites found in other microorganisms such as *B. subtilis*, were unproductive.

In the presence of certain monosaccharides, several genes related to carbohydrate metabolism were differentially transcribed, as expected (Fig. 2 and 3). These genes were often also upregulated in the presence of the sugar mixture. For example, three genes in the Leloir pathway involved in galactose metabolism—galactose-1-phosphate uridylyltransferase (Csac_1510, EC 2.7.7.12), galactokinase (Csac_1511, EC 2.7.1.6), and UDP-galactose 4-epimerase (Csac_1512, EC 5.1.3.2)—were highly transcribed compared to the other five monosaccharides but not the sugar mixture. It was interesting that the monosaccharides did not stimulate transcription of many of the 53 glycoside hydrolases identifiable in the *C. saccharolyticus* genome, perhaps suggesting that these genes are triggered by oligo- and polysaccharide substrates.

ABC-type carbohydrate transporter identification in *C. saccharolyticus* genome. At least 177 putative ABC transporter genes could be identified in the *C. saccharolyticus* genome (40). These comprise 24 putative carbohydrate transporters, based on their substrate-binding protein similarity to proteins identified in the genome of *T. maritima* (7, 27) and/or prediction by TransportDB (31). Of these, two belong to the carbohydrate uptake (CUT) 2 family, two are members of the di/oligopeptide transporter family (Dpp/Opp), and the rest are CUT1 types (Table 2). In *T. maritima*, many of the transporters are colocalized with glycoside hydrolases (7), which also seems to be the case for *C. saccharolyticus*. For these transporters in *C. saccharolyticus*, predictions for sugar substrate specificity were based on one or more of the following criteria: (i) functional genomics analysis of the ORFs in the transporter showed a twofold change for a given sugar contrast; (ii) the substrate-binding protein identity was >30% compared to the homologous protein in *Escherichia coli*, *Bacillus subtilis*, or *T. maritima*; or (iii) genes located near the ABC transporter are conserved in the same relative location in another well-studied genome. As mentioned earlier, *C. saccharolyticus* genome also encodes a phosphoenolpyruvate-dependent PTS, which is annotated as fructose specific (40).

Proposed annotation of monosaccharide transporters in *C. saccharolyticus*. Table 2 shows the proposed assignment of substrates to ABC sugar transporters in the *C. saccharolyticus* genome, based on functional genomics and bioinformatics analysis. CUT2 family transporters consist of an extracellular substrate-binding protein, one permease, and one ATPase; the CUT2 ATPase with two sets of Walker A and B sites is, in general, longer than other ATPases (18, 35). CUT2 family transporters only import monosaccharides. There are only two CUT2 family transporters identifiable in the *C. saccharolyticus* genome, (i) Csac_0238 and Csac_0240 to Csac_0242 (Csac_0238,0240-0242) and (ii) Csac_2504 to Csac_2506 (Csac_2504-2506). These may have broad specificity for mon-

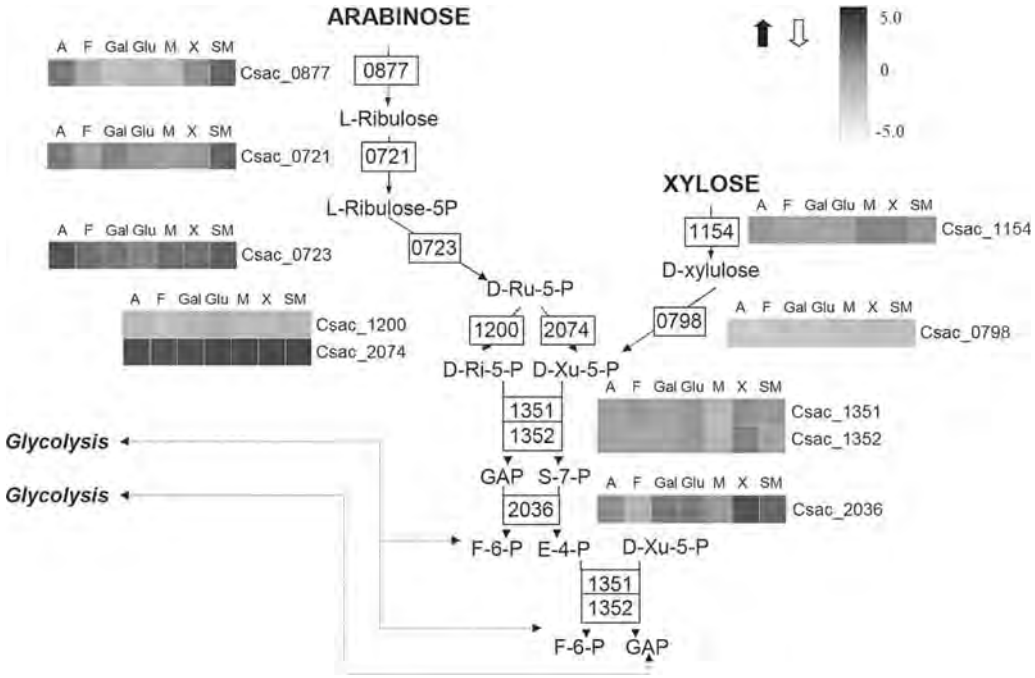

FIG. 2. Differential transcription of genes encoding enzymes involved in nonoxidative pentose phosphate pathway in *C. saccharolyticus*. Abbreviations: A, arabinose; F, fructose; Gal, galactose; Glu, glucose; M, mannose; X, xylose; SM, sugar mix; L-Ru-5-P, L-ribulose-5-phosphate; D-Ru-5-P, D-ribulose-5-phosphate; D-Ri-5-P, D-ribose-5-phosphate; D-Xu-5-P, D-xylulose-5-phosphate; GAP, glyceraldehyde-3-phosphate; S-7-P, sedoheptulose-7-phosphate; F-6-P, fructose-6-phosphate; E-4-P, erythrose-4-phosphate. Least squares mean estimates (see Materials and Methods) of the transcript level are given for selected genes (ORF numbers are boxed). Black and white denote transcript levels above (black) or below (white) the mean across all genes.

osaccharides: Csac_0238,0240-0242 was upregulated on arabinose, galactose, and xylose, whereas Csac_2504-2506 responded to xylose, glucose, fructose, and galactose. The CUT1 family ABC transporters have one substrate-binding protein, two permeases, and one ATPase; they transport both monosaccharides and di/oligosaccharides, in addition to glycerol phosphate (18). Similar to *T. maritima*, the *C. saccharolyticus* CUT1 ATPases utilized are not necessarily located in the same operon as the permeases and substrate-binding protein. Three CUT1 family transporters were differentially transcribed in the

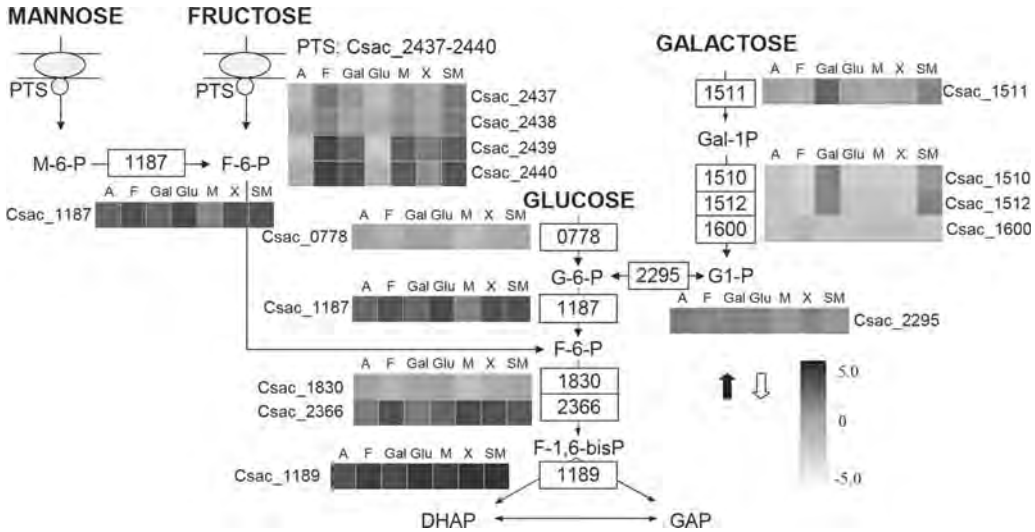

FIG. 3. Differential transcription of genes encoding enzymes involved in glycolysis in *C. saccharolyticus*. Abbreviations: A, arabinose; F, fructose; Gal, galactose; Glu, glucose; M, mannose; X, xylose; SM, sugar mix; M-6-P, mannose-6-phosphate; F-6-P, fructose-6-phosphate; Gal-1-P, galactose-1-phosphate; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; F-1,6-bisP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate. Least squares mean estimates (see Materials and Methods) of the transcript level are given for selected genes (ORF numbers are boxed). Black and white denote transcript levels above (black) or below (white) the mean across all genes.

TABLE 2. Putative substrates for sugar ABC transporters in *C. saccharolyticus*

ABC transporter (Csac_)	Group	SBP[a] (Csac_)	GH neighbor(s) (Csac_)	GH neighbor family[b]	SBP homolog in *T. maritima*	Proposed substrate(s)
0126-0128	CUT1	0126	0129, 0130	2, 15	TM0810 (*N*-acetylglucosamine or GlcNAc polysaccharide)	Unknown
0238,0240-0242	CUT2	0242			TM0114 (monosaccharides)	Arabinose, galactose, xylose
0261-0265	Dpp/Opp	0261	0258, 0259	?	TM1223 (cellobiose, barley)	Fructose, sucrose
0297-0299	CUT1	0297	0296	43	None	Unknown
0391-0394	CUT1	0391			None—proximate to two xylose isomerase-like TIM barrel proteins	Unknown
0427-0428,0431	CUT1	0431	0426	13	TM1839 (maltose, maltotriose, trehalose)	Maltodextrin (40)
0440-0442	CUT1	0440	0439, 0444	65, 65	TM1220 (cellobiose, barley)	Galactose
0679-0682	CUT1	0682	0678	5	TM0810 (*N*-acetylglucosamine or GlcNAc polysaccharide)	Xyloglucan
0692-0694	CUT1	0692	0689, 0696	13, 10	TM1120 (glycerol-3-phosphate)	Monosaccharides
1028-1032	Dpp/Opp	1032			TM1746 (β-mannans)	Monosaccharides
1357-1359	CUT1	1359	1354		None	Xyloglucan
1557-1559	CUT1	1557	1560, 1561, 1562	43, 51, 4	TM1120 (glycerol-3-phosphate)	Xyloglucan
2321-2322,2324,2326	CUT1	2326			TM0102 (CUT2 unknown)	Glucose, xylose, fructose
2412-2414	CUT1	2412	2408, 2409, 2410, 2411	10, 39, 10, 43	TM1204 (maltose, maltotriose, mannotetraose)	Xylooligosaccharides
2417-2419	CUT1	2419			TM1839 (maltose, maltotriose, trehalose)	Xylooligosaccharides
2491-2493	CUT1	2493			None	Xylose, glucose, fructose
2504-2506	CUT2	2506			TM0114 (monosaccharides)	Xylose, glucose, fructose, galactose
2514-2516	CUT1	2516	2513	30	TM0595 (*N*-acetylglucosamine or GlcNAc polysaccharide)	Glucooligosaccharides
2529-2531	CUT1	2529	2527, 2528	43, 5	None—proximate to multidomain GHs	Unknown
2542-2544	CUT1	2544	2539	20	TM0810 (*N*-acetylglucosamine or GlcNAc polysaccharide)	*N*-Acetylglucosamine, GlcNAc polysaccharide
2552-2554	CUT1	2552	2548	16f	None	Unknown
2692-2694	CUT1	2694	2689	67	None	Unknown
2724-2726	CUT1	2726	2730, 2731, 2732	88, 39f, 39f	None—proximate to multidomain GHs	Unknown
2740-2742	CUT1	2742	2734, 2748	2, 4	TM1120 (glycerol-3-phosphate)	Unknown

[a] SBP, substrate-binding protein.
[b] GH family indicates glycosides hydrolase family (www.cazy.org). An "f" following a number indicates a fragment of the glycoside hydrolase family. Where applicable, multiple values are given respective to the corresponding multiple values in column 4.

C. saccharolyticus genome during growth on monosaccharides. Csac_0440-0442 was upregulated only on galactose. The two other CUT1 transporters—Csac_2321-2322,2324,2326 and Csac_2491-2493—both responded to glucose, xylose, and fructose.

It is interesting that the CUT1 family transporter encoded in Csac_0692-0694 was upregulated when *C. saccharolyticus* was grown on the six-monosaccharide mixture. Directly downstream from Csac_0692-0694 is a ROK family transcriptional regulator (Csac_0695) with the highly conserved consensus sequences 1 and 2, plus the conserved DNA-binding (helix-turn-helix) motif (10, 39). Csac_0695 was highly transcribed only in the monosaccharide mixture. This was the only transcription factor in the genome that responded in this way, suggesting that it regulates this transporter. The protein encoded in Csac_0695 has 40% amino acid sequence identity to the ROK family xylose repressors found in two strains of *B. subtilis* (XylR1, P94490; XylR2, P16557), which negatively regulate the *xyl* operon by binding to the *xyl* operators in the absence of the inducer xylose (8, 32). A similar role in *C. saccharolyticus* seems likely.

Members of the Dpp/Opp family are known to transport di- and oligopeptides, simple and complex carbohydrates, nickel, and heme into the cells through an extracellular binding protein, two permeases, and two ATPases (18). The Dpp/Opp family transporter encoded in Csac_1028-1032 was highly transcribed on every monosaccharide and on the sugar mixture. The other Dpp/Opp transporter, encoded in Csac_0261-0265, was highly transcribed only on fructose. Upstream of this gene cluster is a LacI family regulator (Csac_0260), and two putative glycoside hydrolases, which were also clearly upregulated on fructose. Csac_0258, a putative intracellular β-fructosidase, may be an invertase that hydrolyzes sucrose to fructose and glucose. This suggests that Csac_0261-0265 could be a sucrose transporter; in fact, *C. saccharolyticus* grows as well on sucrose as it does on the monosaccharides examined here (unpublished results).

In the presence of fructose, the ORFs encoding a putative PTS (Csac_2437-2440) were induced 1.8- to 35-fold, compared to glucose, xylose, and arabinose. These ORFs were also upregulated in the presence of mannose. Note that a PTS can transport different sugars, with each EII being sugar-specific

(1). An inducible mannose transporter occurs in the fructose/mannose PTS in *Streptococcus mutans*, importing fructose and mannose into the cytoplasm (2). EIIA (Csac_2440) and EIIBC (Csac_2439) in the putative *C. saccharolyticus* PTS were also upregulated on galactose, but this was not the case for EI (Csac_2437) or HPr (Csac_2438). Fructose and galactose appear to have ABC transporters (Csac_0261-0265 and Csac_0440-0442) (see below), but for mannose, the only identifiable transporter is this PTS. The unusual transcriptome for mannose merits further examination.

All of the transporters discussed above that were upregulated on the individual monosaccharides were also upregulated during growth on the six sugar mixture, with the lone exception of the galactose-specific transporter (Csac_0440-0442).

Complex carbohydrate response. *C. saccharolyticus* was also grown on xylan (oat spelt), xyloglucan, and xylogluco-oligosaccharides (4) to facilitate transcriptional response comparisons to growth on the monosaccharides glucose and xylose. Final cell densities and growth rates with the complex carbohydrate substrates were comparable to the monosaccharides, although there were longer lag times on xyloglucan and xylan.

The transporter encoded in Csac_0692-0694, which was upregulated only during growth on the monosaccharide mixture, was not induced on the complex carbohydrates, suggesting that it is involved in general monosaccharide transport into the cell. ABC transporters encoded in Csac_0679-0682 and Csac_1357-1359 were highly transcribed on the complex carbohydrates. Both are located near putative glycoside hydrolases (Csac_0678, GH family 5; Csac_1354, GH family 31) involved in the breakdown of glucans or xylan, suggesting a role in the uptake of degradation products of these carbohydrates. The xylan utilization cluster in *C. saccharolyticus* (5, 24) contains the ABC transporters (Csac_2412-2414 and Csac_2417-2419) and, as expected, the components were transcribed at higher levels on xylan compared to the other sugars. Growth on the xylogluco-oligosaccharide mixture triggered transcription of components of the transporter Csac_2514-2516, indicating a role in the uptake of this substrate.

Transporters with unknown function. Several putative transporters could not be assigned specific substrates, based on transcriptional response and bioinformatic analysis. One of these putative transporters, Csac_1557-1559, did not respond during growth on the simple or complex carbohydrates used here. However, this transporter is colocalized in the genome with glycoside hydrolase family 4, 43, and 51 enzymes and may transport a different type of glucan- and/or xylan-based saccharide. Further examination of the genome showed that the putative sugar transporter encoded in Csac_2542-2544 is downstream of a *N*-acetylglucosamine-6-phosphate deacetylase (Csac_2538) and a *N*-acetyl-β-hexosaminidase (Csac_2539). Based on this, and its homology to TM0810, this transporter could be associated with *N*-acetylglucosamine-based polysaccharides. The other loci encoding ABC sugar transporters (see Table 2) that did not respond to any of the substrates tested and were, furthermore, consistently transcribed at very low levels. These transporters (Csac_0126-0128, Csac_0261-0265, Csac_0391-0394, Csac_2529-2531, Csac_2552-2554, Csac_2692-2694, Csac_2724-2726 and Csac_2740-2742) are located in the genome near GH families 2, 4, 5, 15, 16, 39, 43, 67, and 88 or near carbohydrate metabolism genes, so a role in carbohydrate acquisition cannot be ruled out, especially given *C. saccharolyticus*' varied carbohydrate appetite.

No evidence of CCR. Here, there was no indication of CCR. This physiological characteristic, as observed in low G+C content, gram-positive bacteria, typically involves three components: *cis*-acting sequences or catabolite responsive elements, catabolite control protein A (CcpA), which binds to the catabolite responsive element, and the Crh (catabolite repression HPr) (21). CCR has been noted in various gram-positive bacteria, including *B. subtilis* (13), *Lactococcus lactis* (6), and *Streptococcus pneumoniae* (14), and is mediated by transcriptional control by a CcpA/HPr-Ser-P complex binding to *cre* (38). In the standard low G+C, gram-positive model of CCR, the primary sugar triggers the transcriptional repression of genes encoding saccharolytic components, related to other sugars, through the serine-phosphorylated HPr. With *C. saccharolyticus*, there was specific induction of carbohydrate catabolism genes for growth on individual sugars, although the carbohydrate uptake systems were, in general, differentially transcribed during growth on individual sugars. When the organism was presented a mixture of monosaccharides, including glucose, genes within these inducible loci were not impacted. Examination of the *C. saccharolyticus* genome identified an HPr homolog (Csac_1163), with the active His-15 site replaced with a serine residue. Thus, it cannot be involved in PTS transport. It could, however, be phosphorylated at the active site Ser-46 and participate in CCR in *C. saccharolyticus* as Crh. A number of putative CcpA homologs were found, but these candidates contain most of the residues that are typical for LacI-type transcription factors but are missing residues which are typical for CcpAs (see Fig. S3 in the supplemental material) (20). As yet, their role in *C. saccharolyticus* is unclear, since no *cre* sequences in promoter-operator regions are present. All of these results point to no involvement of a global regulatory system in *C. saccharolyticus* for carbohydrate utilization.

Summary. The results here clearly demonstrate that *C. saccharolyticus* can metabolize a number of monosaccharides simultaneously, a process apparently mediated by a system of transporters with low specificity and an absence of CCR. This phenotype is advantageous to bacteria coping in natural environments where substrate availability and composition is variable. Given its broad appetite for plant-based carbohydrates, *C. saccharolyticus* is an attractive candidate for the fermentation of biomass-derived substrates to biofuels, such as hydrogen.

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