Relaxed control of sugar utilization in *Lactobacillus brevis*

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Prioritization of sugar consumption is a common theme in bacterial growth and a problem for complete utilization of five and six carbon sugars derived from lignocellulose. Growth studies show that *Lactobacillus brevis* simultaneously consumes numerous carbon sources and appears to lack normal hierarchical control of carbohydrate utilization. Analysis of several independent *L. brevis* isolates indicated that co-utilization of xylose and glucose is a common trait for this species. Moreover, carbohydrates that can be used as a single carbon source are simultaneously utilized with glucose. Analysis of the proteome of *L. brevis* cells grown on glucose, xylose or a glucose/xylose mixture revealed the constitutive expression of the enzymes of the heterofermentative pathway. In addition, fermentative mass balances between mixed sugar inputs and end-products indicated that both glucose and xylose are simultaneously metabolized through the heterofermentative pathway. Proteomic and mRNA analyses revealed that genes in the *xyI* operon were expressed in the cells grown on xylose or on glucose/xylose mixtures but not in those grown on glucose alone. However, the expression level of XylA and XylB proteins in cells grown on a glucose/xylose mixture was reduced 2.7-fold from that observed in cells grown solely on xylose. These results suggest that regulation of xylose utilization in *L. brevis* is not stringently controlled as seen in other lactic acid bacteria, where carbon catabolite repression operates to prioritize carbohydrate utilization more rigorously.

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INTRODUCTION

The fermentation of renewable agricultural biomass by lactic acid bacteria (LAB) is an attractive alternative to the use of limited petrochemical resources for the production of lactic acid (Dien *et al.*, 2002) and other platform chemicals. Since hydrolysis of lignocellulose results in a mixture of carbohydrates, including glucose, arabinose, xylose and galactose, LAB selected to carry out this fermentation need to possess broad substrate utilization ability, tolerate a range of stresses (from end-products or inhibitors), and minimally produce by-products, among other desired traits (Galbe & Zacchi, 2002; Ingram *et al.*, 1999). One common problem in the fermentation of lignocellulosic biomass is the inefficient use of all the sugars present (Hofvendahl & Hahn-Haegerdal, 2000). In many micro-organisms, fermentation of mixed carbohydrates is achieved sequentially, whereby the utilization of glucose, commonly the preferred carbon and energy source, represses consumption of alternative sugars. This

hierarchical control, termed carbon catabolite repression (CCR), is accomplished by a complex regulatory mechanism and is a common phenomenon in LAB (Deutscher *et al.*, 2006; Deutscher, 2008; Görke & Stülke, 2008; Saier, 1998; Stülke & Hillen, 1999; Titgemeyer & Hillen, 2002). A heat-stable phosphocarrier protein (Hpr), an Hpr kinase and a carbon catabolite protein A (CcpA) all play a role in CCR in Gram-positive bacteria. A histidine-phosphorylated Hpr (P-His-Hpr), which transfers a phosphate group onto incoming sugars, inhibits phosphoenolpyruvate:sugar phosphotransfer systems (PTS) of other carbohydrates and prevents translocation of alternative sugars into the cell (termed 'inducer exclusion'). In addition, glycolytic intermediates such as fructose 1,6-bisphosphate and/or 6-phosphogluconate activate the Hpr kinase to phosphorylate a serine residue of Hpr. Coupled with CcpA, serine-phosphorylated Hpr represses the expression of genes involved in the catabolism of alternative sugars by binding to upstream catabolic repression element (*cre*) sites.

While LAB are well known to consume citrate with glucose, lactose or maltose (Kennes *et al.*, 1991; Ramos *et al.*, 1994), simultaneous carbohydrate utilization has been demonstrated in few species. Maltose and fructose co-fermenta-

Abbreviations: CCR, carbon catabolite repression; LAB, lactic acid bacteria; PTS, phosphotransferase system.

Two supplementary tables and three supplementary figures are available with the online version of this paper.

tion has been reported in *Lactobacillus brevis* subsp. *lindneri* CB1 (Gobbetti & Corsetti, 1996).

L. brevis is commonly found on plant materials, although it has been also isolated from various other niches including beverages and animal intestinal tracts (Salminen & Wright, 1993). This species is obligately heterofermentative and employs the phosphoketolase pathway but also possesses inducible glycolytic enzymes (Saier *et al.*, 1996). *L. brevis* has been shown to transport glucose, lactose, xylose and galactose via proton symport systems (Chaillou *et al.*, 1998; Djordjevic *et al.*, 2001; Ye *et al.*, 1994a, b). The regulation of these proton symport systems is mediated through HPr, in which the transport mechanism is reversibly switched between proton symport and facilitated diffusion (Ye & Saier, 1995a, b; Ye *et al.*, 1996). Previous characterization of the *L. brevis* xylose operon (*xyl*) indicated a potential upstream *cre* site, suggesting regulation by CCR (Chaillou *et al.*, 1998). Indeed, when cloned and examined in *Lactobacillus plantarum*, the *L. brevis xyl* operon exhibited a decreased expression pattern in cells grown on glucose (Chaillou *et al.*, 1998).

In this work we demonstrate an unusual phenotype of *L. brevis*, which unlike most LAB possesses a relaxed control of carbohydrate consumption. Numerous independent isolates of *L. brevis* exhibit simultaneous utilization of xylose and glucose. In fact, any carbohydrate that could be consumed by *L. brevis* as a sole carbon source could also be simultaneously consumed with glucose. Further mRNA and proteomic analyses indicate that the *xyl* operon is induced when both glucose and xylose are present in the medium.

METHODS

Bacterial strains and growth conditions. *Lactobacillus brevis* IFO 3960 was obtained from the Institute for Fermentation, Osaka, Japan. *L. brevis* NRRL 1834 and *L. brevis* NRRL 1836 were obtained from the Agricultural Research Service culture collection (Peoria, IL, USA). *L. brevis* ATCC 14869 and *Lactobacillus pentosus* ATCC 8047 were purchased from the American Type Culture Collection (Manassas, VA, USA). For the carbohydrate utilization experiments, 100 ml modified MRS medium (Becton Dickinson), which contained bactopeptone (15 g l⁻¹), yeast extract (5 g l⁻¹), ammonium citrate (2 g l⁻¹), sodium citrate (5 g l⁻¹) and dipotassium phosphate (2 g l⁻¹), was prepared in a 500 ml baffled flask. The carbon sources were prepared and added separately. All experiments were carried out at 30 °C, 100 r.p.m. without pH control. The initial pH of the medium was 6.0.

Nucleic acid isolation. Chromosomal DNA of *L. brevis* was isolated by using the Qiagen DNeasy kit according to the manufacturers' instructions. For the RNA extraction, *L. brevis* was cultivated in MRS medium containing 20 g glucose l⁻¹, 20 g xylose l⁻¹, or a 20 g l⁻¹ glucose/xylose mixture (10 g l⁻¹ each) and cells were collected at mid-exponential phase. Collected cells were immediately suspended in RNAlater (Ambion). Total RNA was extracted with the Qiagen RNeasy kit according to the manufacturer's instructions, with additional treatments with RNase-free DNase (Ambion).

RT-PCR analysis. Reverse transcriptase (RT) reactions were performed by a commercially available reverse transcription system

(Promega) with the recommended protocol. RT reactions for *xylT* and *ccpA* were performed in a final volume of 20 µl, which contained 5 mM MgCl₂, RT buffer (10 mM Tris/HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1 mM (each) deoxynucleoside triphosphates, 1 U recombinant RNasin RNase inhibitor, 15 U avian myeloblastosis virus (AMV) RT, 2 µg substrate RNA and 0.5 µM of the reverse primers, *xylT*-bwd primer (5'-TTTGGTCGTGCTTAGTTCAGCAG-CATC-3') and *ccpA*-b-2 (5'-AAAACGACCACGGTGGGCGT-AATCATCCCT-3'), respectively. After denaturation of RNA at 70 °C for 15 min, reaction mixtures were added and incubated at 42 °C for 15 min. Reactions were terminated by heating the mixtures at 95 °C for 5 min, followed by incubation on ice for 5 min. The cDNA products were PCR amplified in 50 µl mixtures containing 5 µl of the RT reaction mixture as the template. For the amplification of cDNA the primers *xylT*-fwd (5'-ATGCGAAAGTTTCGACCGGA-TTTGTT-3') and *ccpA*-f-2 (5'-CACCGACCAATATCGTACAG-TGGTTGTGT-3') were paired with the *xylT*-bwd and *ccpA*-b-2 primer, respectively.

Sample preparation and protein digestion for shotgun proteomics.

A 10 ml sample of *L. brevis* culture was taken at the exponential phase of growth. The amounts of initial cell mass were normalized to an OD₆₀₀ of 1.0 by dilution or concentration to a final volume of 25 ml. After centrifugation, cell pellets were washed three times with PBS then resuspended in 1 ml lysis buffer containing 100 mM Tris and 8.0 M urea. The initial pH of lysis buffer was 9.0. With addition of 300 mg silica beads (Sigma-Aldrich), cells were disrupted using a bead beater (FastPrep; QBiogen) for six 30 s pulses each with a 30 s interval on ice. Beads and cell debris were removed by centrifugation and the soluble fraction was kept at -80 °C for further analysis. Protein concentration was measured by the Bio-Rad protein assay kit.

For protein reduction, 4 µl 450 mM DTT (Sigma-Aldrich) was added to 25 µl supernatant containing 100 µg protein and incubated for 45 min at 55 °C. Without alkylation, reduced protein was digested by 2.5 µg mass-spectrometry-grade trypsin (Promega) overnight at 37 °C. The tryptic peptides were purified by C18 Ziptip (Millipore) according to the manufacturer's recommendation. The Ziptip was prepared by washing with 50% acetonitrile/H₂O followed by 0.1% (v/v) trifluoroacetic acid (TFA) in H₂O. The tryptic peptide solution was then loaded onto the Ziptip and washed with 0.1% (v/v) TFA in H₂O. The peptides were eluted with 50% acetonitrile in H₂O. The purified sample was dried prior to mass spectrometry analysis.

Protein identification by mass spectrometry. The digested samples were submitted to the Genome Center Proteomics Core at the University of California, Davis. Protein identification was performed using an Eksigent Nano LC 2-D system coupled to an LTQ ion-trap mass spectrometer (Thermo-Fisher) through a Picoview nano-spray source. Peptides were loaded on to an Agilent nanotrap (Zorbax 300SB-C18, Agilent Technologies) at a loading flow rate of 5.0 µl min⁻¹. Peptides were then eluted from the trap and separated by a nano-scale 75 µm × 15 cm New Objectives picofrit column packed in house with Michrom Magic C18 AQ packing material. Peptides were eluted using a 90 min gradient of 2–80% buffer B (buffer A=0.1% formic acid, buffer B=95% acetonitrile/0.1% formic acid). The top 10 ions in each survey scan were subjected to automatic low-energy CID.

Database searching. Tandem mass spectra were extracted and the charge states deconvoluted by BioWorks version 3.3. Deisotoping was not performed. All MS/MS samples were analysed using X! Tandem (<http://www.thegpm.org>; version 2006.04.01.2). X! Tandem was set up to search against the *L. brevis* whole proteome. X! Tandem was searched with a fragment ion mass tolerance of 0.60 Da. Oxidation of methionine was specified as a variable modification in X! Tandem.

HPLC analysis. Substrate and end-product concentrations were analysed by a Shimadzu HPLC system using a Bio-Rad HPX-87H column. A 1 ml sample of fermentation broth was centrifuged at 10000 g for 10 min and the supernatant was transferred to a new microcentrifuge tube prior to analysis. For HPLC analysis of the supernatant, the Bio-Rad HPX-87H column was heated at 65 °C and a refractive index (RI) detector was used for identification of substrate(s) and product(s). As a mobile phase, 0.005 M H₂SO₄ was used and the flow rate was 0.6 ml min⁻¹. The standard deviations of the HPLC concentration determinations were within ± 5 mM.

To determine cell density the cell pellet was resuspended in the same volume of deionized water and the optical density (OD₆₀₀) was measured using a Beckman DU 7400 spectrophotometer.

RESULTS

Simultaneous utilization of glucose and xylose by *L. brevis*

L. brevis ATCC 14869 (the type strain) was cultivated in MRS medium containing a mixture of glucose and xylose. As shown in Fig. 1(a), simultaneous utilization of glucose and xylose was observed. During 22 h of fermentation, 11.1 g l⁻¹ of glucose and 10.5 g l⁻¹ of xylose were consumed at the same time, and 12.5 g l⁻¹, 4.5 g l⁻¹ and 0.7 g l⁻¹ of lactate, acetate and ethanol,

Fig. 1. Fermentation profiles of glucose/xylose mixtures by (a) *L. brevis* ATCC 14869 (type strain), (b) *L. brevis* NRRL 1836, (c) *L. brevis* NRRL 1834, (d) *L. brevis* IFO 3960 and (f) *L. pentosus* ATCC 8047. ●, OD₆₀₀; ■, glucose; ▲, xylose; ◇, lactic acid; △, acetic acid; ▽, ethanol. The product yields (mM/mM) based on the mixed sugar are summarized in (e): black, light grey and dark grey bars indicate the yields of lactate, acetate and ethanol, respectively. Fermentations were done in duplicate and a representative dataset is shown. The standard deviation for each chemical measured by HPLC was <0.5 mM.

respectively, were produced. No other significant by-products were observed by HPLC. The product yields of lactate ($Y_{L/(G+X)}$), acetate ($Y_{A/(G+X)}$) and ethanol ($Y_{E/(G+X)}$) from glucose and xylose were 1.01, 0.57, and 0.12, respectively, indicating that both glucose and xylose were metabolized through the heterofermentative pathway simultaneously. While CCR is a common phenomenon in LAB, derepressed strains have been developed via mutagenesis (Chaillou *et al.*, 2001). To determine if the simultaneous utilization pattern witnessed with *L. brevis* ATCC 14869 was a characteristic of the species and not a trait specific to this strain, three more independent *L. brevis* strains were examined (NRRL 1836, NRRL 1834 and IFO3960). As shown in Fig. 1(b–d), all three strains possessed the ability to simultaneously catabolize glucose and xylose through the heterofermentative pathway, although each strain exhibited different carbohydrate consumption rates. The specific cell growth rate for each strain was similar ($0.31\text{--}0.34\text{ h}^{-1}$). The product yields from glucose and xylose are summarized in Fig. 1(e). In contrast, *L. pentosus* ATCC 8047 examined under the same conditions (Fig. 1f) exhibited a typical sequential carbohydrate utilization pattern indicative of overt CCR by glucose.

Range of fermentable carbohydrates and co-utilization with glucose

Since CCR is not exclusive to xylose utilization, we examined a range of fermentable carbohydrates, and characterized their consumption with glucose, using several *L. brevis* strains. All these strains rapidly consumed glucose, fructose, galactose, xylose, arabinose, ribose and maltose as a sole carbon source (see Supplementary Table S1, available with the online version of this paper). Mannose was utilized only by NRRL 1834 and NRRL 1836.

We then examined simultaneous carbohydrate utilization of glucose and a second carbohydrate. Monitoring substrate concentration every 6 h, it was confirmed that all sugars that could be fermented by *L. brevis* ATCC 14869 as a sole carbon source (Table S1) were consumed simultaneously with glucose (Fig. 2). The utilization pattern of the second carbohydrate was the same as when it was the sole carbon utilized; however, the end-product profiles were different. As expected, fermentation of pentoses or fructose as a sole carbon source resulted in the production of acetate without ethanol (Supplementary Table S2), probably due to the lesser requirement for cofactor regeneration during fermenta-

Fig. 2. Simultaneous utilization of glucose and (a) xylose, (b) arabinose, (c) ribose, (d) fructose, (e) galactose and (f) maltose by *L. brevis* ATCC 14869. ■, Glucose; ▲, xylose; ▼, arabinose; ○, ribose; ◆, fructose; □, galactose; ▽, maltose. The product yields (mM/mM) from mixed sugar are shown in the bar graphs next to the sugar utilization profiles; L, A and E indicate the yields of lactate, acetate and ethanol, respectively. Fermentations were done in duplicate and a representative dataset is shown. The standard deviation for each chemical measured by HPLC was $<0.5\text{ mM}$.

tion of those carbohydrates (Veiga da Cunha & Foster, 1992). However, a significant amount of ethanol was produced in the co-fermentation of those carbohydrates with glucose (Fig. 2a–d). Consistent with their lack of utilization as a sole carbon source, lactose, sucrose and cellobiose were not consumed in a mixture with glucose (data not shown). The co-utilization profiles of mixed sugars by *L. brevis* ATCC 14869 and the product yields (mM/mM) from the mixed sugars are shown in Fig. 2. Other *L. brevis* strains exhibited similar co-consumption patterns (Supplementary Figs S1–S3). Interestingly, some strains consumed the pentose sugars present faster than glucose; however, consumption was still simultaneous. This appears to be a strain-specific phenomenon (Figs S1–S3).

Proteomic analysis of *L. brevis*

The above results showing utilization of xylose in the presence of glucose suggest expression of xylose-catabolizing enzymes encoded by the *xyl* operon. To investigate this, the proteome of *L. brevis* grown on glucose, xylose and a glucose/xylose mixture was analysed by LC-MS/MS following tryptic digestion of total soluble cellular proteins. After database searching, 90–135 proteins were identified from a single shotgun experiment with high confidence. Spectral counting (Paoletti *et al.*, 2006; Zybailov *et al.*, 2005) was then used to determine the relative expression of proteins from cells grown on xylose or on the glucose/xylose mixture compared to that observed in cells grown in glucose. As shown in Table 1(a, b), the enzymes involved in

Table 1. Relative protein expression levels, determined by LC-MS/MS, of (a) enzymes involved in the heterofermentative pathway of *L. brevis* and (b) proteins in the *xyl* operon and CcpA

The ratio was calculated by a comparison of normalized spectral abundance factor of each protein in cells grown on two different sugars. Experiments were done in duplicate.

gi	Description*	Expression ratio [†]		
		G	X/G [‡]	(G + X)/G [‡]
(a) Heterofermentative enzymes				
116332772	Glucose-6-phosphate 1-dehydrogenase	1.00 ± 0.00	−1.12 ± 0.42	2.04 ± 0.41
116332769	6-Phosphogluconate dehydrogenase	1.00 ± 0.00	1.14 ± 0.44	1.25 ± 0.25
116332818	6-Phosphogluconate dehydrogenase	1.00 ± 0.00	ND	1.01 ± 0.21
116333142	Phosphoketolase	1.00 ± 0.00	1.69 ± 0.59	1.04 ± 0.22
116333308	Glyceraldehyde-3-phosphate dehydrogenase	1.00 ± 0.00	−1.87 ± 0.57	−1.09 ± 0.21
116334603	Phosphoglycerate mutase family protein§	1.00 ± 0.00	1.03 ± 0.42	1.08 ± 0.22
116333311	Enolase	1.00 ± 0.00	1.03 ± 0.42	1.15 ± 0.23
116333412	Pyruvate kinase	1.00 ± 0.00	1.18 ± 0.45	1.01 ± 0.21
116333957	2-Hydroxyacid dehydrogenasell	1.00 ± 0.00	−1.37 ± 0.45	1.01 ± 0.21
116334182	Acetate kinase	1.00 ± 0.00	ND	ND
116332803	Acetate kinase	ND	ND	ND
116333800	Acetate kinase	1.00 ± 0.00	2.22 ± 0.78	−1.02 ± 0.21
116332794	NAD-dependent alcohol-acetaldehyde dehydrogenase	1.00 ± 0.00	ND	5.11 ± 1.02
116333656	Zn-dependent alcohol dehydrogenase	1.00 ± 0.00	ND	1.77 ± 0.35
(b) <i>xyl</i> operon/CcpA				
		G	X	(G + X)/X¶
116332853	D-Xylose isomerase	ND	1.00 ± 0.00	−2.72 ± 0.84
116332854	Xylulokinase	ND	1.00 ± 0.00	−2.79 ± 0.86
116332856	Xylose proton-symporter	ND	ND	ND
116333813	Carbon catabolite repressor protein A	1.00 ± 0.00	4.74 ± 1.66	1.01 ± 0.21

*Putative annotation in the NCBI genome database.

†Plus and minus indicates an increase and decrease of relative expression level, respectively. A ratio of 1.0 means no change in the expression level between the two samples; ND, not detected.

‡The relative protein expression levels of each protein in the cells grown in xylose or glucose/xylose mixture were calculated by comparison to the cells grown in glucose.

§The expression of isozymes (gi:11633968, 116334035, 116334334, 116334380, 116334724 and 116332815) was not detected.

||The expression of putative lactic acid dehydrogenase (gi:116334008, 116334115 and 116332871) was not detected.

¶For xylose isomerase and xylulokinase the relative protein expression level in the cells grown on the glucose/xylose mixture were compared with proteins from cells grown on xylose (since no expression could be detected in cells grown on glucose). The expression ratio for CcpA was determined by normalizing to the expression level seen during growth on glucose (as in Table 1a).

the heterofermentative pathway and encoded by the *xyl* operon were identified. Among eight proteins annotated as phosphoglycerate mutase (*pgm*) in the *L. brevis* genome, only one (gi:116334603) of them was expressed. Only one (gi:116333957) of four putative *ldh* genes was highly expressed in all conditions. Of three putative acetate kinase genes (*ack*) in the *L. brevis* genome, one (gi:116333800) was expressed in cells grown only on glucose or xylose but not on the glucose/xylose mixture. Among ten genes putatively assigned as alcohol dehydrogenase, the expression of two genes (gi:116332794 and gi:116333656) was identified in cells cultured in glucose and glucose/xylose mixture. The expression of these latter two enzymes was not detected in cells grown on xylose as a single carbon source. There are two 6-phosphogluconate dehydrogenase genes (*6pgdh*) in the *L. brevis* genome. Of these, one (gi:116332769) was expressed constitutively while the other (gi:116332818) was detected only when glucose was present in the medium.

As expected, the expression levels of the proteins in the heterofermentative pathway were similar regardless of the carbon source in the medium. The expression levels of 6-phosphogluconate dehydrogenase, phosphoglycerate mutase, enolase, pyruvate kinase and lactate dehydrogenase were the same when *L. brevis* was cultivated on glucose, xylose or the glucose/xylose mixture. The expression of glucose-6-phosphate 1-dehydrogenase showed a slight increase in cells grown on the glucose/xylose mixture, and phosphoketolase and glyceraldehyde-3-phosphate dehydrogenase exhibited a slight decrease in expression in cells grown on xylose as a single carbon source.

Expression of the *xyl* operon and *ccpA* in the presence of xylose and/or glucose

The apparent relaxed control of sugar utilization in *L. brevis* suggests that xylose utilization genes are expressed

when xylose is available regardless of the presence or absence of glucose. As shown in Table 1(b), XylA (xylose isomerase) and XylB (xylulokinase) proteins were observed when *L. brevis* was cultivated on xylose but not when glucose was the sole carbon source in the medium. Expression of these two proteins was observed in cells grown on the glucose/xylose mixture; however, the expression level of both XylA and XylB decreased by 2.8-fold compared to that seen when xylose was used as a sole carbon source. This suggests that the presence of glucose suppresses, but does not completely inhibit, the expression of *xylA* and *xylB*.

CcpA is known to bind upstream of, and regulate expression of, the xylose operon in *L. pentosus* (Chaillou *et al.*, 2001; Mahr *et al.*, 2000; Posthuma *et al.*, 2002). In *L. brevis*, CcpA was expressed consistently regardless of the carbon source; however, the expression level increased fourfold when xylose was used as a single carbon source. Expression of *ccpA* was also verified by RT-PCR, with a 700 bp *ccpA* amplicon observed from *L. brevis* cultured in glucose, xylose or the glucose/xylose mixture (Fig. 3a, b).

The xylose transporter (XylT) was not detected in the proteome analysis. RT-PCR indicated that *xylT* was expressed in *L. brevis* cultures grown on xylose and glucose/xylose mixture but not in cultures grown on the glucose as sole carbon source (Fig. 3c). This suggests that *xylT* expression is induced by xylose, as previously proposed (Chaillou *et al.*, 1998), and the presence of glucose does not fully inhibit its expression. Thus, the expression of xylose utilization genes in the presence of mixed sugars correlates with the observed co-utilization phenotype.

DISCUSSION

Hierarchical carbohydrate utilization is a common phenotype for micro-organisms whereby a more desired sugar,

Fig. 3. Transcriptional analysis of *xylT* and *ccpA*. (a) Schematic representation of the PCR amplicon, and PCR primer locations. (b, c) Agarose gel electrophoresis of RT-PCR products of (b) *ccpA* and (c) *xylT*. Total RNA was extracted from cells cultured in MRS medium containing the indicated carbon source. Reactions performed in the absence of RNA template did not generate an amplicon (data not shown). Lanes: 1, DNA ladder; 2, cells grown on glucose; 3, cells grown on xylose; 4, cells grown on glucose/xylose mixture.

cre box

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xylT: ttgattgctcagaaagaaaacggtttcacaataactcacggaagtgttatgatgaactcatggttggttg
xylA: ttgaaattttctgcgaaaacggttgcaattcaaaataaatcctgtataacttgattttgaagtgtggtt
ccpA: ggtattctttgattcgaaaacggtttcaatgttgacgaatcggcgaggttctgtataactgggtcaag ..... aacaaggaggaatggctacgctatggaaaa

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Fig. 4. Comparison of upstream sequences of the xylose:proton symporter (*xylT*), xylose isomerase (*xylA*) and carbon catabolite repressor protein A (*ccpA*) genes in *L. brevis*. The potential *cre* sequence of each gene is indicated in the box. Grey shading denotes putative -35 and -10 consensus sequence in the promoter region. A potential ribosome-binding site is indicated by asterisks and the start codon for *ccpA* is marked in italics and underlined.

typically glucose, is consumed first followed by secondary sugar utilization. Repression of secondary carbohydrate utilization is achieved through several mechanisms that are collectively termed carbon catabolite repression (CCR) (Görke & Stülke, 2008; Stülke & Hillen, 1999). Relatively few bacteria have been reported to consume different sugars simultaneously. *Clostridium thermohydrosulfuricum* has been reported to co-metabolize mixtures of glucose and xylose as well as combinations of cellobiose, xylose and xylobiose (Cook *et al.*, 1993; Slaff & Humphrey, 1986). Simultaneous utilization of glucose with xylose or sucrose has been observed in yeast (Kastner & Roberts, 1990; Kastner *et al.*, 1998) and thermophilic fungi (Maheshwari & Balasubramanyam, 1988); however, it remains to be determined if this phenotype is a common trait of the species or a particular trait of the strain. Fibrolytic ruminal bacteria are able to degrade the hemicellulose of plant materials and utilize pentose sugars with glucose. *Butyrivibrio fibrisolvens* was reported to simultaneously consume glucose and xylose, although this effect was strain specific (Marounek & Kopečný, 1994; Strobel & Dawson, 1993). *Ruminococcus albus* consumed xylose and glucose simultaneously but the dominant repressor was cellobiose (Thurston *et al.*, 1994).

The results presented here suggest that *L. brevis* does not possess a rigorous hierarchical control of carbohydrate utilization and can metabolize a range of fermentable carbon sources simultaneously with glucose. Simultaneous fermentation of mixed carbohydrates by *L. brevis* exhibited utilization rates and fermentation behaviours similar to those seen when a sole carbon source was fermented although, as expected, different end-products were observed with some sugars. The mass balance between substrates (mixed sugars) and products indicates that the xylose was metabolized via the heterofermentative pathway. Relaxed CCR was observed in several different *L. brevis* isolates, including the type strain, suggesting that this is a common trait for the species. In contrast, other facultatively heterofermentative LAB exhibited the classical biphasic carbohydrate consumption expected where CCR is dominant (Fig. 1f).

CCR has been previously observed in various lactobacilli including *L. pentosus*, *L. casei*, *L. delbrueckii*, *L. plantarum*

and *L. sakei* (Mahr *et al.*, 2000; Marasco *et al.*, 1998; Morel *et al.*, 1999; Schick *et al.*, 1999; Veyrat *et al.*, 1994; Viana *et al.*, 2000; Zuniga *et al.*, 1998). Such control is achieved by two independent mechanisms: inducer exclusion and genetic repression by the catabolite control protein (CcpA) and repressor protein. As shown in Fig. 3, *xylT* expression requires the presence of xylose. In the standard model of CCR in LAB the primary sugar, often glucose, triggers the inhibition of the inducer uptake (inducer exclusion), mediated through the action of a serine-phosphorylated Hpr on secondary sugar transporters (Saier, 1998; Ye & Saier, 1995a; Ye *et al.*, 1996). Our results (Fig. 1a) indicate that in *L. brevis*, xylose can readily enter the cell even in the presence of glucose, suggesting that inducer exclusion is relaxed in this species. The carbohydrate transport systems identified in *L. brevis* are proton symport systems (Chaillou *et al.*, 1998; Djordjevic *et al.*, 2001; Ye *et al.*, 1994a, b). Through detailed biochemical studies, *L. brevis* has been shown to exhibit PTS-mediated control of non-PTS permease activities for glucose, lactose and galactose (Chaillou *et al.*, 1998; Djordjevic *et al.*, 2001; Ye & Saier, 1995a, b). For example, when bound by a serine-phosphorylated Hpr, the *L. brevis* galactose: H^+ symporter changes from an active symporter to a facilitated diffusion uniporter (bidirectional), prohibiting uptake of galactose against a concentration gradient (inducer exclusion) and enabling passive expulsion of galactose (inducer expulsion) (Djordjevic *et al.*, 2001). In spite of this biochemical evidence, our results clearly suggest that secondary sugars, such as galactose, simultaneously enter, and are metabolized by, *L. brevis* cells in the presence of glucose. An explanation may be that galactose is not excluded but is instead metabolized, resulting in little internal buildup of galactose to drive expulsion, as would be seen with a non-metabolizable galactose analogue. While it is not known if XylT, a xylose: H^+ symporter (Chaillou *et al.*, 1998), operates in a similar fashion, it is clear from expression of the xylose operon seen in Fig. 3(c) that enough xylose enters the cell to derepress the *xylT* operon.

CCR is also mediated by a global transcriptional regulator, the catabolite control protein, CcpA. In other LAB, CcpA-based regulation occurs by binding of *cre* sites upstream of, or within, target genes (Mahr *et al.*, 2000; Marasco *et al.*,

1998; Saier, 1998; Schick *et al.*, 1999; Stülke & Hillen, 1999). Two *cre* sites have been identified in the *xyl* operon, suggesting possible regulation by CcpA (Chaillou *et al.*, 1998); however, the simultaneous utilization of xylose and glucose, along with induction of the *xyl* operon, belies any rigorous repression by CcpA.

Proteomic analysis clearly showed that CcpA was expressed regardless of the carbon source in the medium. However, expression of CcpA in xylose-grown cells was 4.7-fold higher than in cells grown on glucose or glucose/xylose mixture. Interestingly, there is a putative *cre* site upstream of *ccpA*, suggesting possible autoregulation of the *ccpA* operon by CcpA (Fig. 4).

At present the role of CcpA in the relaxed nature of *L. brevis* sugar prioritization is unclear. Given the putative *cre* sites in the upstream regions of *xylA* and *xylB*, CcpA-mediated regulation may drive reduction of *xyl* operon expression in cells grown on a xylose/glucose mixture by comparison to growth on xylose alone (XylA and XylB expression was reduced 2.8-fold; Table 1b). A similar result was reported previously in which the activities of xylose isomerase and xylulokinase were reduced fourfold in *L. brevis* grown on a glucose/xylose mixture (Chaillou *et al.*, 1998). The same authors did not detect these activities in cells grown on glucose alone, a result that parallels the lack of detectable *xyl* operon mRNA or proteins shown here (Fig. 3c, Table 1b).

Our results clearly demonstrate that *L. brevis* can co-metabolize xylose, or other fermentable carbon sources, simultaneously with the glucose. Given its obligately heterofermentative mode of fermentation one might postulate that *L. brevis* has evolved a relaxed hierarchical control of glucose or xylose utilization because both carbohydrates are present in the environmental niches where this bacterium predominates, and both feed into the same phosphoketolase pathway. While the molecular underpinnings of this relaxed control of mixed carbohydrate utilization have yet to be elucidated, the phenotype itself is attractive for fermentation of biomass-derived substrates that possess mixtures of five and six carbon sugars. Simultaneous utilization of mixed carbohydrates is desired in order to completely ferment all available sugars, thereby maximizing potential yields and purification efficiency. Additional studies have shown that *L. brevis* is quite tolerant of inhibitors commonly derived from lignocellulosic biomass (data not shown). The ability to readily consume mixed sugars, combined with the available genomic sequence, suggests that *L. brevis* is an attractive candidate for future metabolic engineering of various bio-products.

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