ORIGINAL RESEARCH PAPER

Deletion of methylglyoxal synthase gene (*mgsA*) increased sugar co-metabolism in ethanol-producing *Escherichia coli*

L. P. Yomano · S. W. York · K. T. Shanmugam · L. O. Ingram

Abstract The use of lignocellulose as a source of sugars for bioproducts requires the development of biocatalysts that maximize product yields by fermenting mixtures of hexose and pentose sugars to completion. In this study, we implicate *mgsA* encoding methylglyoxal synthase (and methylglyoxal) in the modulation of sugar metabolism. Deletion of this gene (strain LY168) resulted in the co-metabolism of glucose and xylose, and accelerated the metabolism of a 5-sugar mixture (mannose, glucose, arabinose, xylose and galactose) to ethanol.

Keywords Arabinose · Catabolite repression · *E. coli* · Ethanol · Glucose · Methylglyoxal synthase · Xylose

Introduction

Lignocellulosic crops and forest residues represent a potential source of sugars for the production of renewable fuels and chemicals. Lignocellulose is dominated by two carbohydrates: cellulose (a homopolymer of glucose) and hemicellulose, a complex mixture of pentose and hexose sugars in which xylose is most abundant (Hahn-Hagerdal et al. 2006; van Maris et al. 2006; Wyman et al. 2005). Additional sugar constituents include arabinose, mannose, and galactose. Effective metabolism of all sugars in biomass is regarded as essential for commercial ethanol production (Asghari et al. 1996; Ingram et al. 1998; Martinez et al. 2001; Yanase et al. 2007).

Escherichia coli-based ethanol biocatalysts have been developed that have the native ability to metabolize each of these sugars individually (Alterthum and Ingram 1989; Ohta et al. 1991). Mixtures of sugars were co-metabolized to some extent in complex medium although glucose was used preferentially (Moniruzzaman and Ingram 1998; Dien et al. 1999). Diauxic metabolism with the preferential use of glucose was also reported recently for a solvent-tolerant derivative of *Pseudomonas putida* S12 containing *E. coli* genes for xylose metabolism (Meijnen et al. 2008). A similar problem of preferential glucose utilization has been reported in native ethanol-producing microorganisms that have been engineered by adding pathways for pentoses such as *Zymobacter palmae* (Yanase et al. 2007), *Zymomonas mobilis* (Mohagheghi et al. 2002), and *Saccharomyces cerevisiae* (Ho et al. 1998; Karhumaa et al. 2006; Kuyper et al. 2005).

Complex regulatory systems that prioritize the sequential metabolism of sugar mixtures can impede the rapid and complete utilization of sugar mixtures during fermentation. The best understood of these in

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E. coli is carbon catabolite repression (glucose) which involves cyclic AMP, cyclic AMP-binding protein, enzymes of the phosphotransferase system, and other components (Gorke and Stulke 2008; Hernandez-Montalvo et al. 2001; Galinier et al. 1998). With this system, glucose effectively blocks the expression of sugar-specific transporters and key enzymes needed for the metabolism of alternative sugars. Additional regulatory systems, such as Cra (Hardiman et al. 2007), DgsA (Decker et al. 1998; Kimata et al. 1998), CsrA (Babitzke and Romeo 2007) and others, are also involved in regulating sugar metabolism in *E. coli*. Remaining to be discovered are regulatory mechanisms that control the preferential utilization of 5-carbon sugars: arabinose > xylose > ribose (Dien et al. 1999; Kang et al. 1998; Moniruzzaman and Ingram 1998). Ethanologenic strains have been described in which carbon catabolite repression has been partially relieved during growth in complex medium by mutations in the phosphenolpyruvate-dependent phosphotransferase system (Hernandez-Montalvo et al. 2001; Lindsay et al. 1995; Nichols et al. 2001).

The methylglyoxal pathway diverts carbon from the lower branch of glycolysis, from dihydroxyacetone phosphate to lactate. Methylglyoxal, the product of the first reaction, is believed to function as a general inhibitor of sugar metabolism during metabolic imbalance (Totemeyer et al. 1998; Zhu et al. 2001). Previous studies have shown that deletion of the *mgsA* gene (methylglyoxal synthase) improved the performance of *E. coli* strains engineered for lactic acid production from glucose and eliminated the low-level production of racemic lactate (Grabar et al. 2006). In this study, we provide evidence that methylglyoxal synthese is also involved in increasing the severity of catabolite repression by glucose. Deletion of *mgsA* increased the fermentation rate of ethanologenic *E. coli* by accelerating the co-metabolism of hexose and pentose sugars.

Materials and methods

Organisms, media and growth conditions

Strains and plasmids used in this study are listed in Table 1. Plasmid and strain constructions were made using Luria broth containing antibiotics as appropriate

(Miller 1992). Temperature-conditional plasmids were grown at 30°C. All others were grown at 37°C. Except during constructions, engineered ethanologenic strains were maintained in AM1 mineral salts medium (Martinez et al. 2007) supplemented with 2% (w/v) xylose for solid medium and 5% (w/v) xylose or higher for broth. New strains developed in this study were constructed from strain *E. coli* LY160 (Yomano et al. 2008). Strain LY160 is a derivative of SZ110 (lactic acid production; Zhou et al 2005) that was developed from KO11 (ethanol production). Note that *E. coli* W (ATCC9637) is the prototrophic parent for strain KO11, initially reported to be a derivative of *E. coli* B (Ohta et al. 1991).

Genetic methods

Standard genetic methods were used in this study (Ausubel et al. 1987; Datsenko and Wanner 2000; Martinez-Morales et al. 1999; Miller 1992; Sambrook and Russell 2001). Red recombinase technology (Gene Bridges GmbH, Dresden, Germany) was used to facilitate chromosomal integration. Chromosomal integrations were verified by phenotype and PCR analysis. Primers used to confirm constructions are listed in Table 1. DNA fragments constructed for chromosomal integration are described and deposited in GenBank: Accession No. FJ015154 for deletion of *Klebsiella oxytoca* M5A1 *casAB* (cellobiose phosphotransferase genes) from the *lac* operon; Accession No. FJ15153 for insertion of *P. putida* B-18435 *estZ* (ethyl acetate esterase) with a *Z. mobilis* CP4 promoter into chromosomal *adhE*; and Accession No. FJ032247 for deletion of the chromosomal *mgsA* (methylglyoxal synthase).

Fermentation conditions

Fermentations were carried out in small vessels (500-ml) with automatic pH control (37°C, pH 6.5, and 150 rpm, 350-ml working volume) as described by Beall et al. (1991) using AM1 mineral salts medium. Seed cultures were prepared as described previously (Yomano et al. 2008) except using AM1 medium (5% w/v xylose). Fermentations were inoculated at an initial concentration of 17 mg dcw l^{-1}. All results are an average of two or more fermentations. Values plotted in figures include standard deviations.

Table 1 Strains, plasmids, and primers

Designation	Relevant genotype	Source or citation
Strains		
DH5 alpha	*lacZ*ΔM15 *recA*	Invitrogen
TOP10F′	F′ (*lac*I^q Tn10 *tet*)	Invitrogen
KO11	W) *frd pflB::pdc$_{Zm}$ adhB$_{Zm}$ ldhA$^+$ cat*	Ohta et al. (1991)
LY160	KO11,) *frd::celY$_{Ec}$,) adhE,) ackA, lacA::casAB$_{Ko}$, rrlE::(pdc$_{Zm}$-adhA$_{Zm}$-adhB$_{Zm}$-FRT-rrlE), pflB$^+$ (pflA-FRT-ycaK)*	Yomano et al. (2008)
LY163	LY162 LY160, *lacY′-FRT-lacA*	This study
LY165	LY163, *adhE::Zm* frg-*estZ$_{Pp}$*-FRT::*adhE*	This study
LY168	LY167 Δ*mgsA*::FRT	This study
Plasmids		
pBluescriptIISK$^+$	ColE1, Ap^R	Stratagene
pCR2.1-TOPO	ColE1, TOPO T/A cloning vector, *bla kan*	Invitrogen
pKD46	pSC101^{ts}, *repA101*, Red recombinase, *bla*	Datsenko and Wanner (2000)
pFT-A	pSC101^{ts}, *flp* (FLP recombinase), *bla*	Posfai et al. (1997)
pLOI2511	ColE1, with FRT sites flanking the *kan* gene, Ap^R, Kan^R	Purvis et al. (2005)
pLOI3421	ColE1, with *FRT* sites flanking the *aac* gene, *bla aac*	Wood et al. (2005)
pLOI3470	pBluescript II SK$^+$, *SacI-PacI* linker, *KpnI-PacI* linker, pLOI3181 *EcoRI-NotI* rrn1 terminator in *EcoRI-SmaI*, ColE1, Ap^R	This study
pLOI2228	FRT sites flanking the MCS, pSC101^{ts}, Cm^R	Martinez-Morales et al. (1999)
pLOI3918	Derivative of pLOI2228 with FRT sites and MCS deleted, *PacI* site inserted, pSC101^{ts}, Cm^R	This study
Deletion of *casAB*		
pLOI3924	PCR of *lacY* gene digested at 3′ end with *XhoI*, pLOI2511 *SmaI* FRT-*kan*-FRT, PCR of *lacA* gene in pLOI3470, *lacY*-FRT-*kan*-FRT-*lacA*, Kan^R, Ap^R	This study
estZ plasmid		
pLOI3925C	PCR of 'adhE', Random *Sau3A Z. mobilis* DNA fragment, PCR of 'adhE', FRT flanked *aac* gene from pLOI3421, PCR of 'adhE in pLOI3918, *adhE'::Z. mobilis* frg.-*estZ*-'adhE'-FRT-*aac*-FRT::*adhE*', Cm^{R}Aac^R	This study
mgsA plasmid		
pLOI3946	PCR of *mgsA*, FRT flanked *aac* gene from pLOI3421, *mgsA*, *mgsA*'-FRT-*aac*-FRT-*mgsA*' in pLOI3918, Cm^R, Aac^R	This study

Primers	Sequence
lacA (ORFmer)	N-terminus 5′-TTGCTCTTCCATGCCAATGACCGAAGAATAAGAG
	C-terminus 5′-TTGCTCTTCGTTAAACTGACGATTCAACTTTATA
lacZ	C-terminius 5′-GGTGAAGTGCCTCTGGATGT-3′
cynX	N-terminus 5′-ATGCCTTCGGTGATTAAACG-3′
lacY (ORFmer)	N-terminus 5′-TTGCTCTTCCATGTACTATTTAAAAAACACAAAC
	C-terminus 5′-TTGCTCTTCGTTAAGCGACTTCATTCACCTGAC
adhE (ORFmer)	N-terminus 5′-TTGCTCTTCCATGGCTGTTACTAATGTCGCTGAA
	C-terminus 5′-TTGCTCTTCGTTAAGCGGATTTTTTGCGTTTTTTCT
mgsA	N-terminus 5′-TATTGCGCTGGTGGCACACG-3′
	C-terminus 5-ACGGTCCGCGAGATAACGCT-3′
estZ	N-terminus 5′-AGATCTTCTGGAGAATCGAACGATGTCCCTG-3′ (5′ *Bgl*II site)
	C-terminus 5′-GAATTCATCACCGCCAAGTACAGCTT-3′ (5′ *Eco*RI scite)

Analyses

Cell mass was estimated from the OD_{550} (OD 1 = 333 mg dcw l^{-1}). Organic acids and sugars were determined by HPLC (Grabar et al. 2006). Ethanol was determined by GC (Ohta et al. 2001).

Results

Deletion of *Klebsiella oxytoca casAB* genes to produce LY163

Previous studies have described the construction of *E. coli* strain LY160 from SZ110 (derivative of KO11) for ethanol production in mineral salts medium (Yomano et al. 2008; Zhou et al. 2005). Strains SZ110 and LY160 contain *K. oxytoca casAB* genes for cellobiose utilization integrated into the *lac* operon. During the course of construction, the functionality of these genes declined although *casAB* remained in the chromosome. The *casAB* genes were removed from strain LY160 by double homologous recombination using a DNA fragment from pLOI3924 that restored the *lac* operon (Fig. 1a). The sequence for this fragment has been deposited in GenBank (Accession No. FJ015154). The kanamycin gene used for selection was subsequently removed with FLP recombinase (Martinez-Morales et al. 1999). Loss of *casAB* was confirmed using MacConkey agar plates containing 1% (w/v) cellobiose (filter sterilized) and PCR analysis. The resulting strain was designated LY163.

### Insertion of *P. putida estZ* esterase with *Z. mobilis* promoter

Previous studies have reported low levels of ethyl acetate in distillate from KO11 (Hasona et al. 2002), a problem that was previously remedied by functional integration of the *estZ* gene encoding a short chain esterase from *Pseudomonas putida*. This gene was integrated into the *adhE* gene of LY163 together with a strong promoter from *Z. mobilis* (Conway et al. 1987). The DNA fragment constructed for integration (Fig. 1b) has been deposited in GenBank (Accession No. FJ15153). The apramycin gene (*aac*) used for selection was subsequently removed using FLP recombinase. Construction was confirmed using esterase indicator plates (Hasona et al. 2002) and PCR analysis. The resulting strain was designated LY165.

Deletion of the *mgsA* gene

Strain LY160 produced small amounts of lactate during xylose and glucose fermentation (Yomano et al. 2008), reducing ethanol yield. Since this strain contains an *ldhA* deletion, the "methylglyoxal bypass" remained as a likely source of this lactate (Grabar et al. 2006; Totemeyer et al. 1998; Zhu et al. 2001). Plasmid pLOI3946 containing the *mgsA* gene encoding
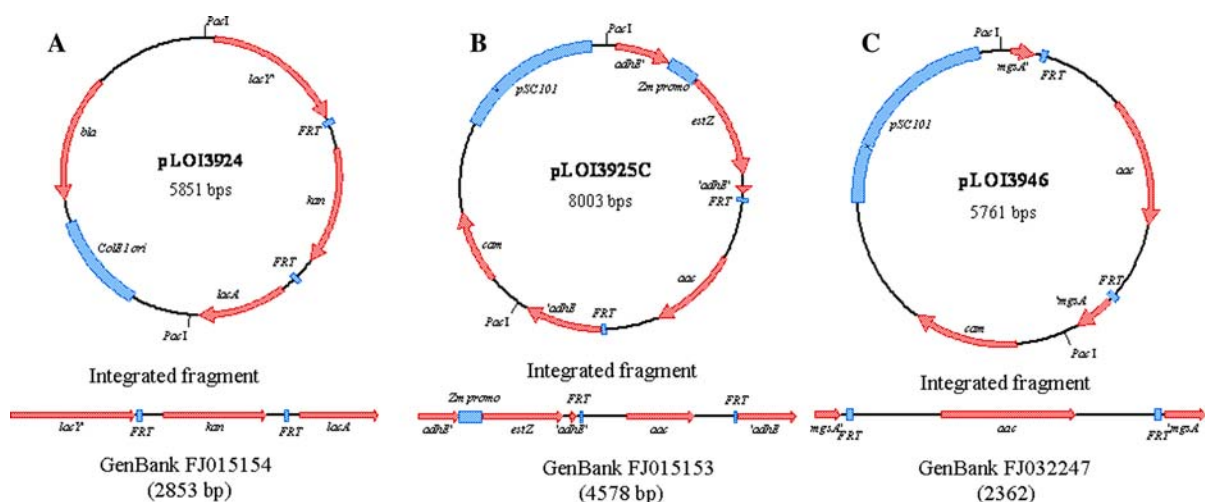

Fig. 1 Plasmids and DNA fragments used for strain construction. **a** Deletion of *casAB*; **b** integration of *estZ*; **c** deletion of *mgsA*
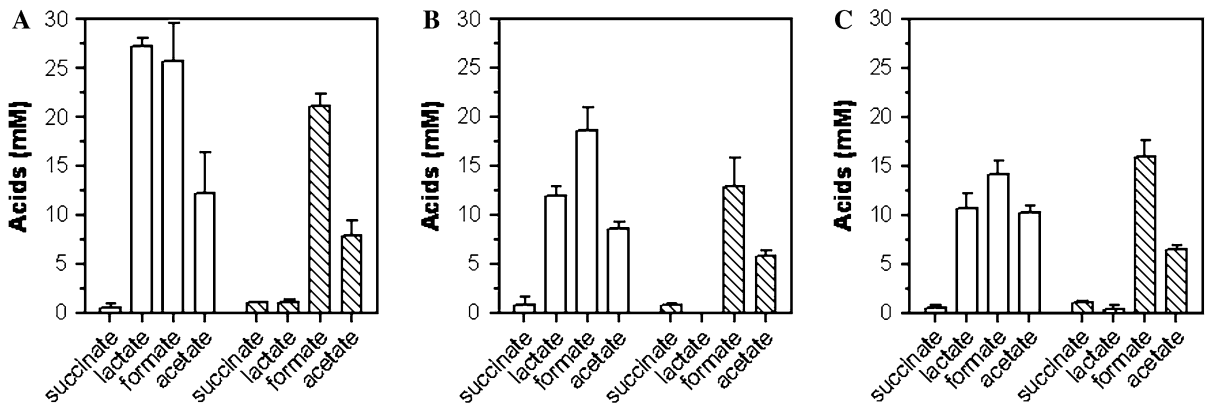
Fig. 2 Effects of Δ*mgsA* on the production of organic acids during the fermentation of glucose (**a**), xylose (**b**), an equal mixture of 5-sugars (2% w/v each: glucose, mannose, arabinose, xylose, and galactose). Organic acids were measured after 96 h with 9% (w/v) glucose or xylose in AM1 mineral salts medium using the parent LY165 (*open bars*) and the mutant LY168 (*hatched bars*)
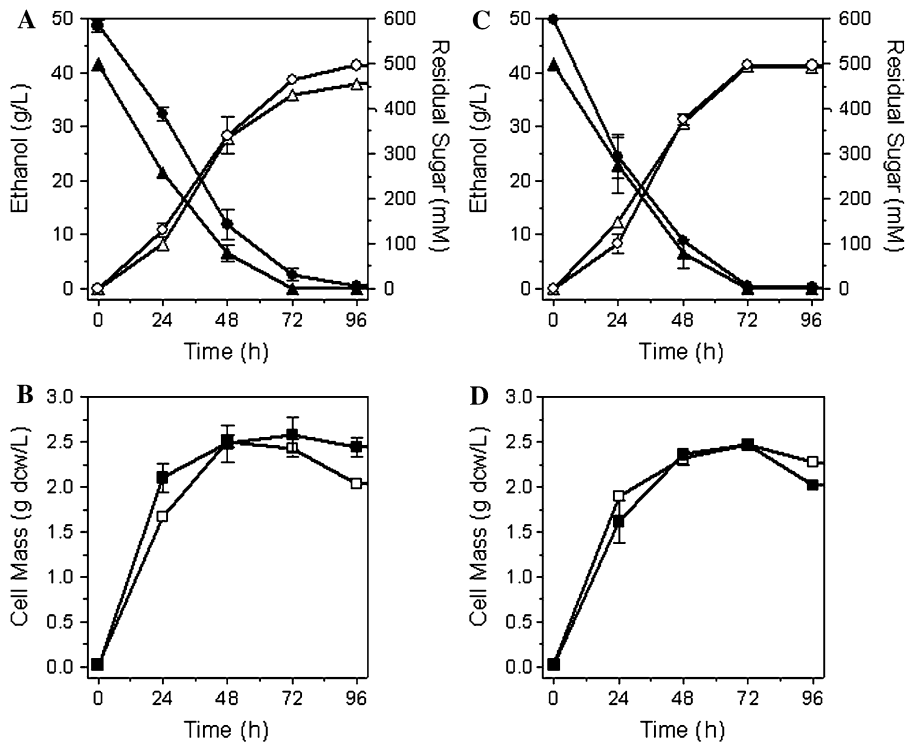
Fig. 3 Fermentation of 9% (w/v) glucose and 9% (w/v) xylose (individually). **a** Sugar fermentation to ethanol by LY165; **b** growth of LY165; **c** sugar fermentation to ethanol by LY168; and **d** growth of LY168. Symbols for all: *filled circle*, xylose; *filled triangle*, glucose; *open circle*, ethanol from xylose; *open triangle*, ethanol from glucose; *open square*, cell mass from glucose; *filled square*, cell mass from xylose

methylglyoxal synthase (first step) was constructed for integration into strain LY165 (Fig. 1c). The sequence of this fragment has been deposited in GenBank (Accession No. FJ032247). After removal of *aac* using FLP recombinase, construction was confirmed by PCR analysis. The resulting strain was designated LY168.

The deletion of *mgsA* in LY168 greatly reduced the production of lactate and presumably methylglyoxal (precursor) in comparison to LY165 (Fig. 2). Both glucose and xylose were metabolized to completion individually by LY165 and LY168, with a small increase in ethanol yield for LY168 (Fig. 3). These results are consistent with the proposed role of methylglyoxal as a general inhibitor of sugar

metabolism (Grabar et al. 2006; Totemeyer et al. 1998; Zhu et al. 2001).

Co-metabolism of sugar mixtures

Surprisingly, deletion of *mgsA* also improved the co-metabolism of glucose and xylose (Fig. 4). Inocula for medium with sugar mixtures were grown in
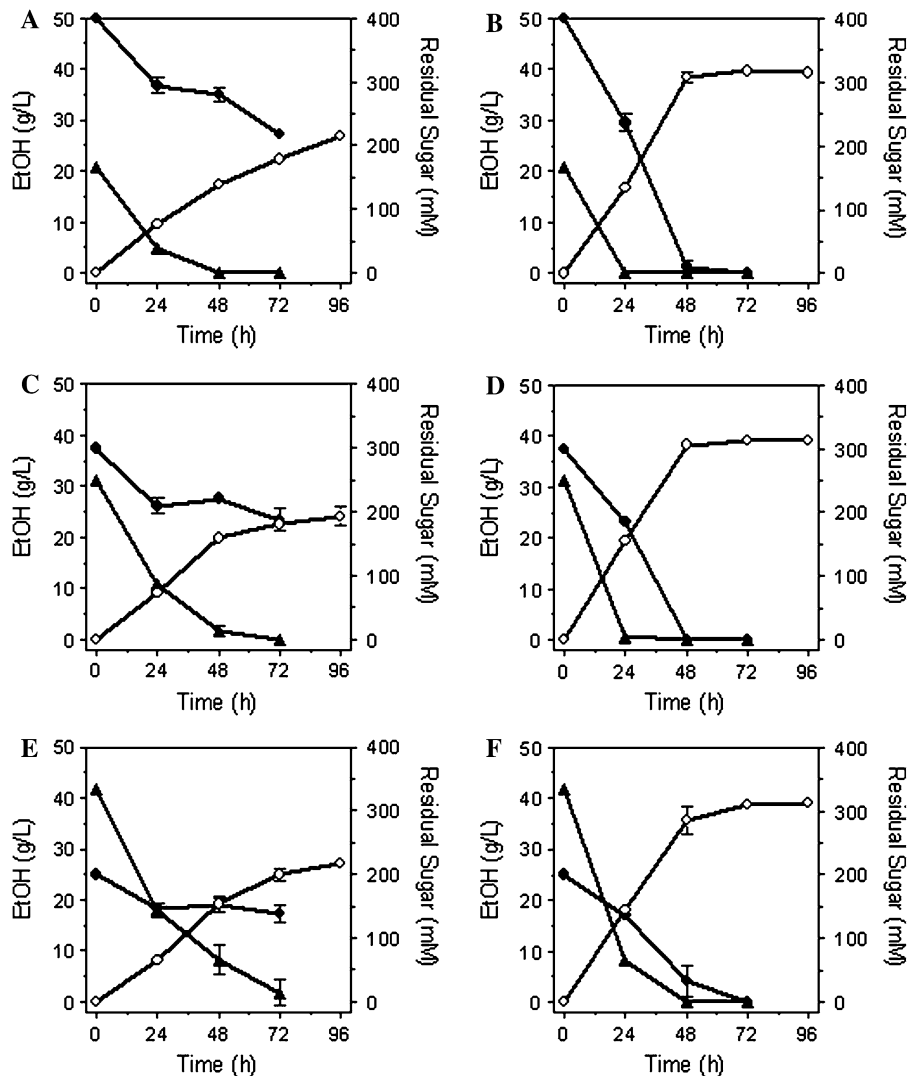
Fig. 4 Fermentation of xylose and glucose mixtures (total of 9% w/v) by LY165 and LY168. Inocula were grown with xylose. Cultures were sampled daily. Residual sugars were measured after 72 h. Cell mass and ethanol were measured for 96 h. **a** LY165 fermenting a mixture of 6% (w/v) xylose and 3% (w/v) glucose; **b** LY168 fermenting a mixture of 6% xylose and 3% glucose; **c** LY165 fermenting a mixture of 4.5% (w/v) xylose and 4.5% (w/v) glucose; **d** LY168 fermenting a mixture of 4.5% (w/v) xylose and 4.5% (w/v) glucose; **e** LY165 fermenting a mixture of 3% (w/v) xylose and 6% (w/v) glucose; **f** LY168 fermenting a mixture of 3% (w/v) xylose and 6% (w/v) glucose. Symbols for all: *filled circle*, xylose; *filled triangle*, glucose; *open circle*, ethanol
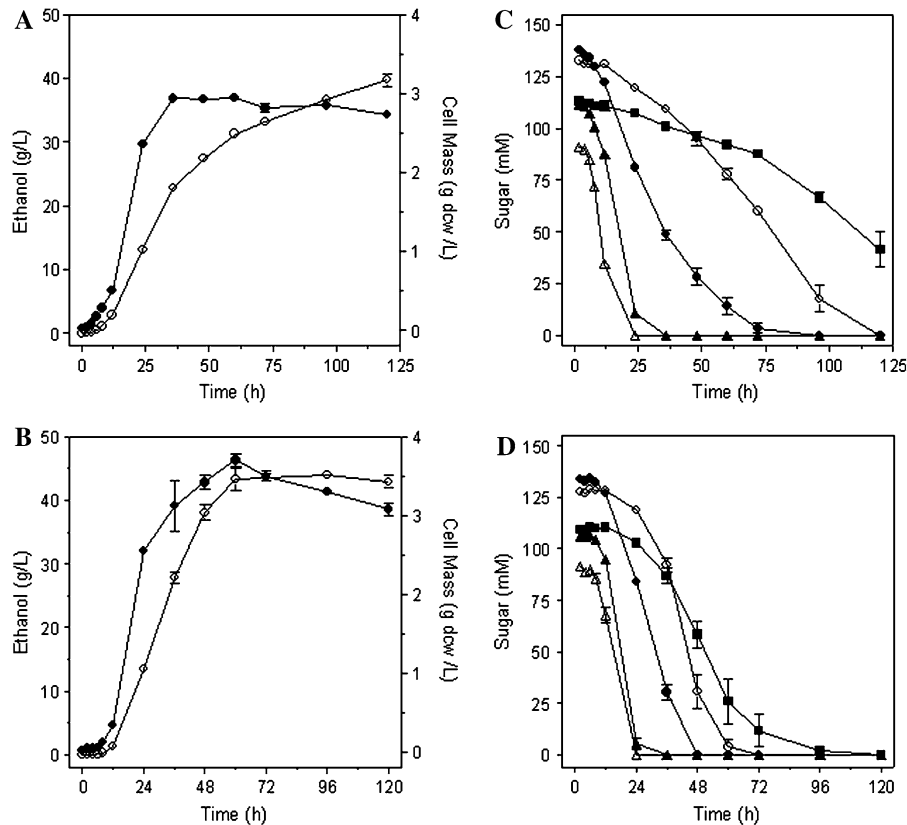
Fig. 5 Fermentation of 5-sugar mixture (2% w/v each: glucose, mannose, arabinose, xylose, and galactose. Inocula were grown with xylose. **a** Growth and ethanol production by LY165; **b** growth and ethanol production by LY168. Symbols for **a** and **b**: *filled circle*, cell mass; and *open circle*, ethanol.

c Sugar utilization by LY165; **d** sugar utilization by LY168. Symbols for **c** and **d**: *filled triangle*, glucose; *open triangle*, mannose; *filled circle*, arabinose; *open circle*, xylose; and *filled square*, galactose

xylose and should initially contain functional levels of enzymes for xylose uptake and metabolism. However, the parent LY165 was unable to complete the fermentation of xylose when glucose was also present (Fig. 4a, c, e). Although both sugars were initially co-fermented by LY165, xylose metabolism stalled after 24 h and further fermentation of xylose was minimal consistent with glucose repression. In contrast, all mixtures of glucose and xylose (2:1, 1:1, and 1:2) were rapidly co-fermented to completion by LY168 containing the *mgsA* deletion. For individual sugars and mixtures (xylose and glucose), volumetric rates of sugar metabolism were higher for LY168 than for LY165 (Fig. 4). These results indicate that the deletion of *mgsA* substantially relieved the repression of xylose metabolism by glucose.

The importance of *mgsA* for glucose repression does not appear to be limited to xylose (Fig. 5). LY165 required over 120 h to ferment a 10% (w/v) mixture of five sugars (mannose, glucose, arabinose, xylose and galactose) (2% w/v each) while a similar fermentation by LY168 was near completion in 72 h. Although all sugars were co-metabolized by both strains without abrupt transitional delays in growth or ethanol production, each sugar was metabolized more rapidly by LY168. With both strains, the period of rapid utilization of individual sugars followed a defined order (mannose > glucose > arabinose > xylose > galactose). Galactose utilization lagged behind all other sugars tested. Together, these results implicate *mgsA* and methylglyoxal as important regulatory agents of mixed sugar metabolism in *E. coli*.

Discussion

The carbohydrate of cellulosic biomass is dominated by polymers of glucose (cellulose) and xylose (hemicellulose). Hemicellulose also includes lesser amounts of other sugars such as glucose, arabinose, and galactose. In softwoods such as pine, mannose is the most abundant hemicellulose sugar. For fuel ethanol production, it is important to maximize product yield by developing biocatalysts that effectively metabolize mixtures of all of these sugars to completion.

Previous studies have focused on the sequential addition of heterologous sugar pathways to *S. cerevisiae* (Karhumaa et al. 2006; Kuyper et al. 2005), *Z. palmae* (Yanase et al. 2007), and *Z. mobilis* (Mohagheghi et al. 2002) with partial success. These organisms contain native pathways only for hexose metabolism. Since ethanologenic *E. coli* contains native pathways for all sugars present in cellulosic biomass, further efforts have been directed at minimizing carbon-catabolite repression and improving the co-metabolism of sugar mixtures. Mutations in *ptsG* have been investigated and decreased the time required to complete the fermentation of sugar mixtures in rich medium (Lindsay et al. 1995; Nichols et al. 2001) but have not been shown to be effective in mineral salts medium.

Simple mineral salts medium is particularly desirable for commercial processes (Zhang and Greasham 1999). Little work has been previously reported for the fermentation of high concentrations of mixed sugars to ethanol in mineral salts medium. Though not generally regarded as part of the carbon-catabolite repression system, methylglyoxal synthase (*mgsA*), and methylglyoxal appear to be directly involved in regulating the co-metabolism of sugars in ethanologenic *E. coli*. Upon deletion of *mgsA* in LY165, the resulting strain (LY168) was able to concurrently metabolize a complex combination of the five principal sugars present in cellulosic biomass (glucose, xylose, arabinose, galactose, and mannose). These results suggest that the *mgsA* gene and the methylglyoxal pathway play a critical role in the controlled expression of sugar specific transporters and other catabolic genes in native strains of *E. coli*. Apparently, the absence of the overflow pathway through methylglyoxal, increased the metabolic flux through the lower part of the glycolysis pathway in mineral salts medium as seen by an increase in volumetric productivity of ethanol (Figs. 3, 4). Whether this increase is a physiological and/or genetic response to the absence of methylglyoxal pathway and methylglyoxal is yet to be deciphered.

A blast search (0.85% similarity) of GenBank indicated that *mgsA* is widely distributed among both Gram positive and Gram negative bacteria. Considering the similarities of carbon catabolite repression among these bacteria, it is likely that *mgsA* and methylglyoxal may also function in the regulation of carbon metabolism in other organisms.

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