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Molecular Control of Sucrose Utilization in *Escherichia coli* W, an Efficient Sucrose-Utilizing Strain

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Sucrose is an industrially important carbon source for microbial fermentation. Sucrose utilization in *Escherichia coli*, however, is poorly understood, and most industrial strains cannot utilize sucrose. The roles of the chromosomally encoded sucrose catabolism (*csc*) genes in *E. coli* W were examined by knockout and overexpression experiments. At low sucrose concentrations, the *csc* genes are repressed and cells cannot grow. Removal of either the repressor protein (*cscR*) or the fructokinase (*cscK*) gene facilitated derepression. Furthermore, combinatorial knockout of *cscR* and *cscK* conferred an improved growth rate on low sucrose. The invertase (*cscA*) and sucrose transporter (*cscB*) genes are essential for sucrose catabolism in *E. coli* W, demonstrating that no other genes can provide sucrose transport or inversion activities. However, *cscK* is not essential for sucrose utilization. Fructose is excreted into the medium by the *cscK*-knockout strain in the presence of high sucrose, whereas at low sucrose (when carbon availability is limiting), fructose is utilized by the cell. Overexpression of *cscA*, *cscAK*, or *cscAB* could complement the WΔ*cscRKAB* knockout mutant or confer growth on a K-12 strain which could not naturally utilize sucrose. However, phenotypic stability and relatively good growth rates were observed in the K-12 strain only when overexpressing *cscAB*, and full growth rate complementation in WΔ*cscRKAB* also required *cscAB*. Our understanding of sucrose utilization can be used to improve *E. coli* W and engineer sucrose utilization in strains which do not naturally utilize sucrose, allowing substitution of sucrose for other, less desirable carbon sources in industrial fermentations.

Carbon source is one of the major cost drivers for industrial production of bulk chemicals from microbial fermentation (1, 2). Currently, glucose (typically from corn) is the most common carbon source for industrial fermentation in *Escherichia coli*. Sucrose from sugarcane, however, would be preferable to corn-based glucose as a carbon substrate for *E. coli*-based industrial fermentation. First, it is a cheaper substrate as it can be directly fermented (either as cane juice or as molasses, or it can be easily made into pure sugar by high-temperature crystallization), whereas glucose has to be converted from starch by milling and enzymatic hydrolysis (3, 4). Second, sugarcane sucrose-based bioprocesses are more environmentally friendly and sustainable than glucose-based bioprocesses. This is because bagasse (the fibrous by-product from sugarcane mills) can be utilized to produce energy for the bioprocess, whereas corn glucose-based processes rely on fossil fuels for energy (5). Finally, as a result of these two primary factors, the associated overall bioprocess cost is decreased relative to that with glucose (5–7). In addition, sucrose is highly abundant and readily available.

The ability to metabolize sucrose as a carbon source is a highly variable feature among *E. coli* strains. Sucrose-fermenting strains include the enteropathogenic strains (8), B-62 (9), EC3132 and its mutants (10, 11), and W (6, 12). There are two gene clusters responsible for sucrose catabolism in *E. coli*: the *scr* regulon, encoding a sucrose phosphotransferase system (PTS) (13, 14), and the chromosomally carried sucrose catabolism (*csc*) regulon, encoding a sucrose non-PTS utilization system (11). Sucrose PTS genes may be found either on plasmids (14, 15), on transposons (16, 17), or on the chromosomal DNA (18–20), whereas the *csc* genes have been found only on the chromosome (6, 10, 11).

Escherichia coli W (ATCC 9637) grows particularly quickly on sucrose and is the only safe laboratory or industrial strain that can utilize sucrose (6). Genome sequencing indicated that sucrose is metabolized via the *csc* genes in this strain (6). The *csc* regulon was originally described in *E. coli* EC3132 (11) and consists of four open reading frames which encode a transcriptional repressor (CscR), a sucrose hydrolase or invertase (CscA), a sucrose permease (CscB), and a fructokinase (CscK) (10, 11). The *csc* catabolic genes are negatively controlled by CscR, which presumably represses transcription in the absence of sucrose and at low sucrose concentrations (<2 g/liter) (10, 11). The sucrose permease/proton symporter, CscB, transports sucrose into the cell. Intracellular sucrose is then hydrolyzed to glucose and fructose by CscA. These two sugars are then phosphorylated into glucose-6-phosphate and fructose-6-phosphate by glucokinase (Glk) and CscK, respectively. The phosphorylated sugars are then assimilated into glycolysis.

Although the genetic structure of the *E. coli* W *csc* genes is well characterized, the molecular control of the regulon and the contribution of each gene to sucrose metabolism are not. In order to investigate this in detail, expression and enzyme activity of the *csc* genes were examined by individual and combinatorial gene knockout (KO) approaches and by overexpression in non-sucrose-utilizing strains.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* strains were grown in LB medium (23) for general cloning and maintenance and in the following media for sugar utilization experiments: M9 (23) supplemented

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli strains		
DH5α	*fhuA2* Δ(*argF-lacZ*)*U169 phoA glnV44* ϕ80d*lacZ*ΔM15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Invitrogen
W	Wild type	NCIMB 8666[a]
MG1655	F^- λ^- *ilvG* mutant *rfb-50 rph-1*	Coli Genetic Stock Center
WΔ*cscR*	WΔ*cscR*::FRT	This study
WΔ*cscA*	WΔ*cscA*::FRT	This study
WΔ*cscB*	WΔ*cscB*::FRT	This study
WΔ*cscK-cat*	WΔ*cscK*::FRT-*cat*-FRT	This study
WΔ*cscK*	WΔ*cscK*::FRT	This study
WΔ*cscRK*	WΔ*cscR*::FRT-*cat*-FRT Δ*cscK*::FRT	This study
WΔ*cscRAKB*	WΔ*cscRAKB*::FRT	This study
WΔ*cscK* Δ*mak*	WΔ*cscK*::FRT Δ*mak*::FRT-*cat*-FRT	This study
Plasmids		
pKD46	γ Red recombinase expression plasmid	21
pKD3	Template for amplifying the *cat* gene	21
pCP20	Flp recombinase expression plasmid	21, 22
pCR2.1	Cloning and expression	Invitrogen
pCSCA	pCR2.1 harboring *cscA* under *lac* promoter	This study
pCSCAB	pCR2.1 harboring *cscA* and *cscB* under *csc* promoter	This study
pCSCAK	pCR2.1 harboring *cscA* and *cscK* under *csc* promoter	This study

[a] National Collection of Industrial Bacteria, Aberdeen, Scotland. This strain is also archived as ATCC 9637 in the American Type Culture Collection.

with thiamine (1 mg/liter) and with either 2 g/liter (0.2%) sucrose (M9S2), 20 g/liter (2%) sucrose (M9S20), 20 g/liter (2%) glucose (M9G20), or 20 g/liter (2%) lactose or MacConkey agar base (Difco, BD, North Ryde, NSW, Australia) supplemented with 10 g/liter sucrose (MCS10). Sucrose utilization on MacConkey plates was scored as positive if colonies were pink and negative if colonies were yellow. Ampicillin (100 μg/ml) and/or chloramphenicol (25 μg/ml) was included in media where appropriate.

DNA manipulations and PCR. General recombinant DNA techniques were performed according to standard protocols (23). PCR products were purified using a MinElute PCR purification kit (Qiagen, Doncaster, VIC, Australia). Sequencing of PCR and plasmid DNA was performed by the Australian Genome Research Facility (The University of Queensland, St. Lucia, QLD, Australia) using the Sanger method.

Knockout of target genes by homologous recombination. Chromosomal gene knockout (KO) was performed using one-step homologous recombination, as described previously (21), using minor modifications as described by Bruschi et al. (24), with the exception that cultures for electrocompetent cell preparation were grown in 100 ml SOB medium (25) and induced with arabinose for 15 min rather than 1 h. Primers used to target specific genes for homologous recombination are listed in Table 2; PCR was performed using Platinum *Taq* polymerase (Invitrogen, VIC, Australia) according to the manufacturer's instructions. The WΔ*cscR* strain has been described previously (26). The *cscA*, *cscK*, and *cscB* genes were deleted in the wild-type (WT) strain using KOcscA_F and KOcscA_R2, KOcscK_F and KOcscK_R, and KOcscB_F and KOcscB_R primer pairs, respectively. The WΔ*cscRK* strain was made by removal of *cscR* in the WΔ*cscK* strain background using KOcscR_F and KOcscR_R2 primers. The chloramphenicol acetyltransferase (CAT) selection cassette (replacing the *cscR* gene) is still present in this strain. The WΔ*cscRAKB* strain was made by removal of *cscAKB* from the WΔ*cscR* strain as follows: a knockout cassette was generated using KOcscK_F and KOcscB_R primers targeted at *cscK* and *cscB* recombined through the FLP recombination target (FRT) site which remained after removal of *cscR*, thereby resulting in deletion of *cscA*, *cscK*, and *cscB*. The WΔ*cscK* Δ*mak* strain was created in the WΔ*cscK* strain background using the KOmak_F and KOmak_R primers.

Three biological replicates (colonies growing on original transformation plates) were selected for each recombination experiment and sequenced to confirm knockout. The *cat* resistance marker was removed by Flp recombinase as described previously (21, 22). Loss of the resistance gene was confirmed by sequencing. Sucrose utilization phenotypes of each strain were scored on M9S20 and M9S2 agar plates.

Overexpression of *csc* genes and complementation of knockout strains. Individual *csc* genes were cloned for overexpression studies. Genes were amplified using the primers shown in Table 2 and Platinum *Taq* polymerase (Invitrogen, VIC, Australia) according to the manufacturer's instructions. Amplicons were cloned into pCR2.1 (Invitrogen, VIC, Australia) according to the manufacturer's instructions. Ligations were transformed into *E. coli* DH5α by electroporation. Three clones with the insert oriented correctly relative to the *lac* promoter were selected for each plasmid. Sucrose utilization phenotypes were scored on MCS10 and M9S20 media. Plasmids were purified using Qiaprep Spin Miniprep (Qiagen, Doncaster, VIC, Australia). The full sequence of each insert was confirmed by sequencing. Each construct which conferred a suc^+ phenotype in DH5α was also transferred into *E. coli* WΔ*cscRAKB* by electroporation; three clones for each construct were selected, and sucrose utilization phenotypes were confirmed. Each knockout strain made in the W genetic background was also complemented using the appropriate overexpression plasmid.

Growth rate analysis in MTPs. Growth rate analysis of the recombinant *E. coli* strains (with plasmids bearing the *csc* genes) was carried out in 96-well microtiter plates (MTPs) using a FLUOStar Omega multidetection microplate reader (BMG Labtech, Offenburg, Germany) as described previously (24).

Growth in shake flasks. *E. coli* W and the knockout strains were grown overnight on LB plates at 37°C. Three single colonies were transferred into 250-ml baffled Erlenmeyer shake flasks containing 25 ml of M9 (23) with trace metal solutions (mg/liter) as described in the work of Bruinenberg et al. (27) except that the concentration of $Na_2MoO_4 \cdot 2H_2O$ was 10-fold higher: EDTA, 15; $ZnSO_4 \cdot 7H_2O$, 4.5; $CoCl_2 \cdot 6H_2O$, 0.3; $MnCl_2 \cdot 4H_2O$, 1.0; $CuSO_4 \cdot 5H_2O$, 0.3; $CaCl_2 \cdot 2H_2O$, 4.5; $FeSO_4 \cdot 7H_2O$, 3; $Na_2MoO_4 \cdot 2H_2O$, 0.4; H_3BO_3, 1.0; and KI, 0.1, containing sucrose (20 g/liter or 2 g/liter) or glucose (20 g/liter). Flasks were incubated at 37°C and 250 rpm. When the optical density at 600 nm (OD_{600}) was 1, cells were centrifuged at 10,000 × *g* for 2 min at room temperature (RT), washed, and resuspended in 25 ml of the growth medium. The cells were then centrifuged again and resuspended in 5 ml of the growth medium. Cells were then transferred into three 250-ml baffled Erlenmeyer shake flasks containing 25 ml of fresh medium to a final OD_{600} of 0.1. Flasks were incubated as described above. To determine growth rates, growth was monitored using a spectrophotometer at a wavelength of 600 nm. Samples were taken every 40 to 60 min until cells reached stationary phase.

Sugar analyses. Extracellular samples were collected from shake flask fermentations by centrifugation, and the supernatant was stored at −20°C for subsequent chromatographic analyses. Concentrations of sugars (sucrose, glucose, and fructose) were analyzed by high-pressure liquid chromatography (HPLC) using an Agilent 1200 Series chromatograph (Agilent, Forest Hill, Victoria, Australia) as described previously (26).

Enzyme assays. From glycerol stocks, cultures were streaked out on M9S20 agar. After overnight growth, cells from a single colony were inoculated into 5 ml of M9S20 medium and incubated overnight at 37°C and 200 rpm. The overnight culture was transferred to 50 ml M9S20 in a 200-ml baffled shake flask and grown at 37°C and 200 rpm until mid-log phase (OD_{600}, 0.5 to 1). Culture (20 ml) was centrifuged (5,000 × *g*, 10 min, 4°C), and the pellet was resuspended in 2 ml of 100 mM phosphate

TABLE 2 Primers used in this study[a]

Primer	Sequence (5′–3′)	Specific use
cscA_R2	TTAACCCAGTAGCCAGAGTGC	To amplify *cscA*, *cscA* + *cscK*, and *cscA* + *cscB*
cscA_F2	ATGACGCAATCTCGATTGCATG	To amplify *cscA*
cscK_R2	GATAAGAGCGACTTCGCCGTT	To amplify *cscA* + *cscK* and *cscA* + *cscB*
cscB_F2	ATGGCACTGAATATTCCATTCAGA	To amplify *cscB*
cscB_R3	GTTTACGTCTATATTGCTGAAGGTAC	To amplify *cscB*
cscB_R2	CTATATTGCTGAAGGTACAGGCGT	To amplify *cscA* + *cscB*
cscA_qrtF1	GTCCGGACATTCCCACATATAG	QRT-PCR for *cscA*
cscA_qrtR1	AGGCAACACGGGGCAGATCCTG	QRT-PCR for *cscA*
cscB_F1	ATCCGTCTTCAAATACAGCGTGG	QRT-PCR for *cscB*
cscB_R1	CAGCACAATCCCAAGCGAACTGG	QRT-PCR for *cscB*
cscK_F1	GCCGGGTTACTCACAGGTCTG	QRT-PCR for *cscK*
cscK_R1	TTCGCCGTTACTGCAAGCGCT	QRT-PCR for *cscK*
cscR_F1	GTAACGATCGCGCAGCCTTTGTGG	QRT-PCR for *cscR*
cscR_R1	GCTGAATTGTGGTCAGCGGCGGTA	QRT-PCR for *cscR*
fruA_F1	AGAGTATGGAGGCGCTGAAA	QRT-PCR for *fruA*
fruA_R1	CGCCCATGTCAGTACACATC	QRT-PCR for *fruA*
dld_F1	AGCACCCTGCGTCTCGACAAGC	QRT-PCR for *dld*
dld_R1	CACGACGATCCAATCACCGAGTGC	QRT-PCR for *dld*
KOcscR_F	CTCGCCAGTGACGTCTGTTTCTGCTACAGTGCCCGTTTTACGGCAAACGGCTTGGGTGTGTAGGCTGGAGCTGCTTC	To amplify *cscR* KO cassette
KOcscR_R2	CCCACGGAGTGGCTGTGCTGCAACATGGAGCACTCTGGCTACTGGGTTAACATAATACATATGAATATCCTCCTTAG	To amplify *cscR* KO cassette
KOcscA_F	CAATTCACCAAATTTGCTTAACCAGGATGATTAAAATGACGCAATCTCGATTGCATGGTGTAGGCTGGAGCTGCTTC	To amplify *cscA* KO cassette
KOcscA_R2	TATGTTAACCCAGTAGCCAGAGTGCTCCATGTTGCAGCACAGCCACTCCGTGGGACATATGAATATCCTCCTTAG	To amplify *cscA* KO cassette
KOcscK_F	CGATTTTGCGAAAAAGAGGTTTATCACTATGCGTAACTCAGATGAATTTAAGGGAGTGTAGGCTGGAGCTGCTTC	To amplify *cscK* KO cassette
KOcscK_R	CGTTAAAAAATTCACGTCCTATTTAGAGATAAGAGCGACTTCGCCGTTTACTTCTCATATGAATATCCTCCTTAG	To amplify *cscK* KO cassette
KOcscB_F	GAATTTTTTAACGACAGGCAGGTAATTATGGCACTGAATATTCCATTCAGAAATGGTGTAGGCTGGAGCTGCTTC	To amplify *cscB* KO cassette
KOcscB_R	CCGGTTGAGGGATATAGAGCTATCGACAACAACCGGAAAAAGTTTACGTCTATATCATATGAATATCCTCCTTAG	To amplify *cscB* KO cassette
KOmak_F	GTGCGTATAGGTATCGATTTAGGCGGCACCAAAACTGAAGTGATTGCACTGGGCGGTGTAGGCTGGAGCTGCTTC	To amplify *mak* KO cassette
KOmak_R	TACGCCGCTGGAATCACCGTGCTTCGCCTTACGCACCGGCGTTTCACATTCGCCGCATATGAATATCCTCCTTAG	To amplify *mak* KO cassette

[a] Abbreviations: KO, knockout; QRT-PCR, quantitative real-time PCR.

buffer (pH 6.5). Crude extracts of *E. coli* were prepared using a Mini Beadbeater (Biospec, Bartlesville, OK) set at 4,800 rpm for three 1-min agitations. Cellular debris was removed by centrifugation (14,000 × *g*, 10 min, 4°C). Supernatants were transferred to fresh tubes and either used immediately (fructokinase assays) or stored at −20°C for later use.

Invertase activities were quantified by measuring the amount of glucose released after sucrose hydrolysis (28). Cell extracts (in 100 mM phosphate buffer, pH 6.5) were incubated with 50 mM sucrose at 37°C for 1 h. The reaction was stopped by heating at 95°C for 2 min. Glucose concentrations were measured using HPLC. Invertase activities (U) are expressed as nanomoles of glucose per minute per milligram of protein.

Fructokinase activities were assayed spectrophotometrically at 340 nm by coupling the formation of fructose-6-phosphate and glucose-6-phosphate, respectively, to the oxidation of NADH as previously described by Sprenger and Lengeler (29). The fructokinase assay mixture contained the following in 0.2 M Tris-HCl, pH 7.8: 5 mM ATP, 10 mM MgCl, 5 mM NADP, 5 mM fructose, phosphorglucose isomerase (3 U ml^{-1}), glucose-6-phosphate dehydrogenase (2.5 U ml^{-1}), and cell extracts. The assay was performed at 25°C. Under these conditions, the amount of phosphorylated products formed in the assay is proportional to the amount of NADP reduced as measured by the change in absorbance at 340 nm per min per mg protein. Protein concentrations were determined by the Bradford method using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as a protein standard (25).

RNA extraction. *E. coli* W and the knockout strains were grown at 37°C in M9 medium with 20 g/liter sucrose, 2 g/liter sucrose, 20 g/liter glucose, or 20 g/liter lactose to an OD_{600} of 1. The cell suspension (1 ml) was inoculated into 25 ml of fresh medium and incubated at 37°C. When the culture reached an OD_{600} of 0.8 (early exponential phase), cells were collected by centrifugation. RNAprotect bacterial reagent (Qiagen, Doncaster, VIC, Australia) was added to stabilize RNA according to the manufacturer's recommendations. The cell pellets were stored at −20°C. RNA was purified using an RNeasy RNA isolation kit (Qiagen, Doncaster, VIC, Australia), and the samples were treated with RQ1 RNase-free DNase (Promega), as recommended by the manufacturer. DNase-treated RNA was repurified using a second RNeasy column. Purified RNA was eluted with 30 μl of RNase-free water and stored at −80°C. RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent, CA).

RT-PCR. Real-time PCRs (RT-PCRs) were performed using Superscript III Plus RNase H^- reverse transcriptase (Invitrogen, Mulgrave, Australia) with random hexamer primers (Invitrogen, Mulgrave, Australia) according to the manufacturer's instructions. cDNA samples were stored at −20°C. Amplification, detection, and analysis of mRNA were performed using an ABI7900 sequence detection system (Applied Biosystems). An Eppendorf epMotion 5075 robotics system was used to set up the reaction mixtures. Reactions were performed in 10-μl volumes with 0.2 μM concentrations of the appropriate forward and reverse PCR primers (Table 2) and 1 μl of a 20× dilution of cDNA using Platinum SYBR Green qPCR Supermix UDG (Invitrogen, Mulgrave, Australia) according to the manufacturer's instructions. Annealing and extension were performed at 65°C for 30 s. For each set of primers, a standard amplification curve was plotted (cycle threshold [C_T] against log of concentration) to determine the amplification efficiency of the primer pair/gene combination. C_T values were standardized using both amplification efficiency and an internal reference gene. To select an appropriate internal reference gene, two different D-lactate dehydrogenase genes (*dld* and *ldhA*) and the 16S rRNA gene (*rrsA*) were tested. The *dld* gene was selected since transcript levels of *rrsA* were much higher than those of the genes being measured for the experiment, and the amplification efficiency of *ldhA* was found to vary between different strains. In order to perform the inference analysis, log*R* values are used: $\log R = -\log A \cdot C_T + \log A_{ref} \cdot C_{T,ref}$, where A is the amplification efficiency for the target gene, A_{ref} is the amplification efficiency for the internal control, C_T is the cycle threshold time for each target gene, and $C_{T,ref}$ is the cycle threshold time for the internal control. Experiments were performed with three technical replications for each gene/condition and three biological replications for each strain.

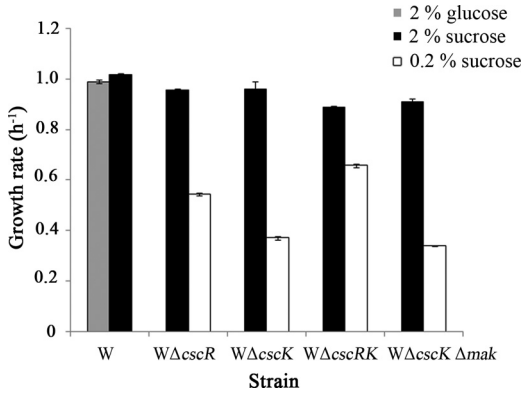

FIG 1 Growth rates of knockout strains grown in a shake flask using minimal medium supplemented with 2% glucose, 2% sucrose, or 0.2% sucrose. Errors are standard deviations ($n = 3$).

Statistical analysis. The enzyme activity, growth rate, and quantitative reverse transcription-PCR (QRT-PCR) data set were analyzed using multiway analysis of variance (ANOVA). Before performing ANOVA, it was confirmed that the data satisfied the appropriate criteria, i.e., that the residuals could be assumed to follow a normal distribution with constant variance (Bartlett's test for homogeneity of variance). All statistical analyses were performed using R software.

RESULTS AND DISCUSSION

E. coli W grows particularly quickly on sucrose, a carbon source of industrial importance. Sucrose is metabolized in *E. coli* W via the *csc* genes; however, relatively little is known about the molecular control of this regulon. The *csc* regulon from *E. coli* W is almost identical to the previously characterized *csc* regulon from EC3132 and to that of other strains containing the *csc* regulon; changes at the nucleotide level are mostly silent or conservative at the protein level. As shown previously for EC3132 (11), *E. coli* W could grow on high concentrations (2%) of glucose or sucrose (Fig. 1) but could not grow on low (0.2%) sucrose (Table 3). The high growth rate of ~1 h^{-1} on 2% sucrose or 2% glucose was similar to that reported previously for *E. coli* W on minimal medium (26). Also as shown for EC3132, the *csc* genes were transcriptionally repressed in the absence of sucrose and derepressed in the presence of 2% sucrose (Fig. 2). The repressor CscR is itself constitutively expressed at low levels under all conditions (Fig. 2A and B). Consistent with *cscR* mutants generated in previous studies (10, 11, 26), knockout of *cscR* enabled growth on low sucrose (Table 3; Fig. 1); repressor gene expression could not be detected (Fig. 2B), and the remaining *csc* genes (*cscA* and *cscKB*) are derepressed (Fig. 2C, D, and E). Invertase and fructokinase activities were also detected in this strain at low sucrose (Fig. 3). The growth rate was not affected by the *cscR* knockout at 2% sucrose; the growth rate in 0.2% sucrose was about 55% of the growth rate in 2% sucrose (Fig. 1). The similarities in sequence and behavior between the *csc* regulon in *E. coli* W and that in *E. coli* EC3132 suggest that information derived from *E. coli* W analysis will be broadly applicable to *csc*-controlled sucrose utilization.

***cscA* and *cscB* are essential for sucrose utilization in the wild-type strain.** In order to characterize the contributions of the different catabolic *csc* genes to sucrose utilization, we performed a detailed chromosomal knockout analysis. The growth of each knockout mutant was examined on minimal agar plates containing either 2% or 0.2% sucrose as a sole carbon source (Table 3). When the entire *csc* regulon was removed, the mutant could not grow on sucrose. Previously, we sequenced the *E. coli* W genome and observed that no other known sucrose uptake and utilization systems were present (6). These data confirm that no other sugar transport/utilization system can compensate for the loss of the *csc* regulon.

Strains with *cscA* or *cscB* knockouts could not grow on sucrose (Table 3), demonstrating that these two genes are essential for sucrose utilization in the wild type. It is well known that disaccharide transporters display plasticity in substrate recognition (30); the essentiality of *cscB* demonstrates that no other transporter in *E. coli* W is capable of transporting sucrose. This result also implies that inversion of sucrose occurs intracellularly in the wild-type *E. coli* W, in contrast to the yeast sucrose utilization system, where

TABLE 3 Map of the *csc* genes in *E. coli* strains and the strain phenotype on M9 agar plates supplemented with 2% or 0.2% sucrose[a]

Strain	*csc* gene map	Phenotype	
		2%	0.2%
Wild type	*dsdX′* *csc* *csc* *cscK* *csc* *torI*	+	-
WΔ*cscRAKB*	*dsdX′*FRT *torI*	-	-
WΔ*cscR*	*dsdX′*FRT *csc* *cscK* *csc* *torI*	+	+
WΔ*cscA*	*dsdX′* *csc* FRT *cscK* *csc* *torI*	-	-
WΔ*cscB*	*dsdX′* *csc* *csc* *cscK* FRT *torI*	-	-
WΔ*cscK*	*dsdX′* *csc* *csc* FRT *csc* *torI*	+	+
WΔ*cscRK*	*dsdX′*FRT *cat* FRT *csc* FRT *csc* *torI*	+	+

[a] A plus sign indicates growth, while a minus sign indicates no growth.

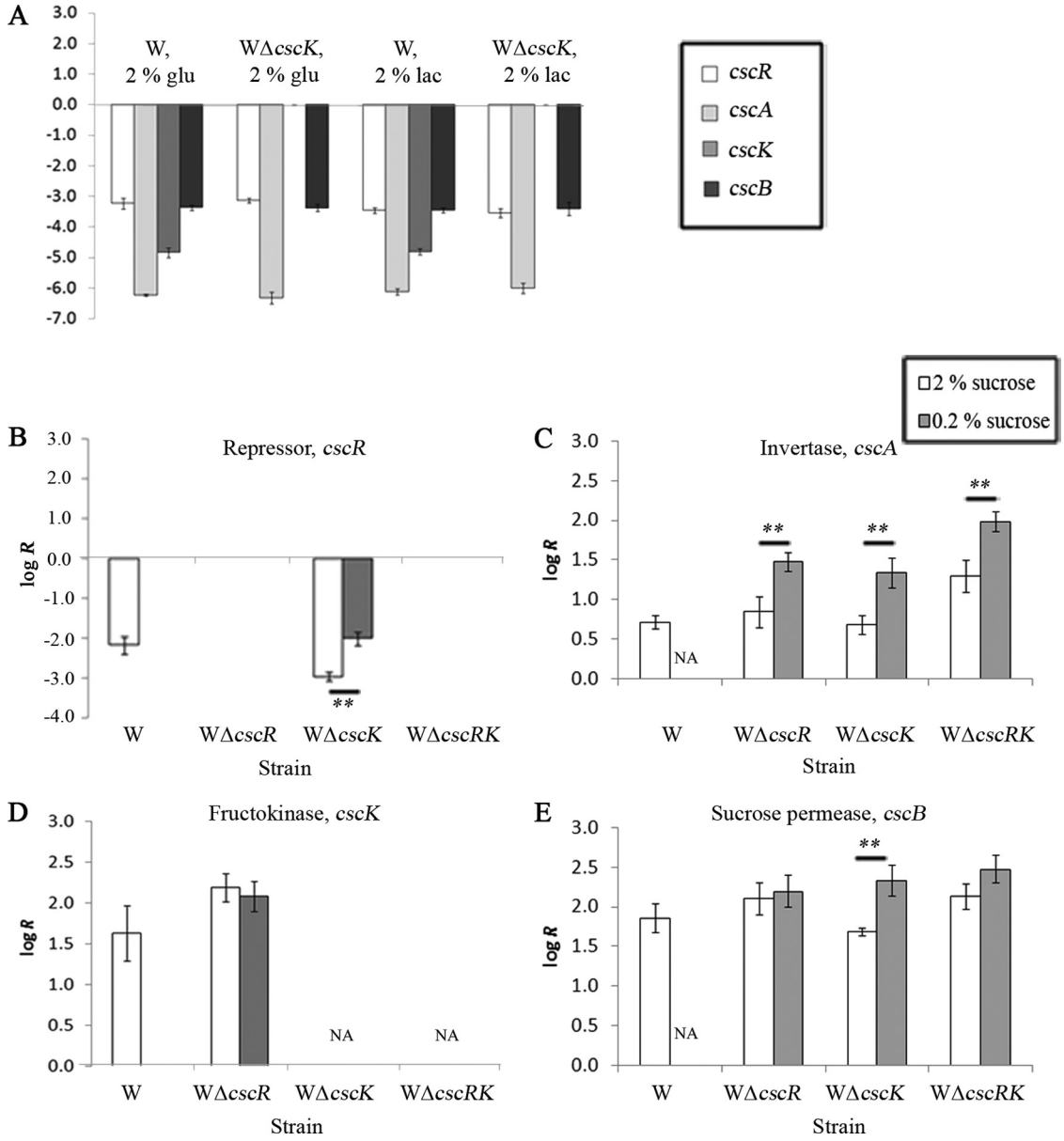

FIG 2 Quantitative real-time PCR analysis. Log*R* values are presented for expression levels of each gene/condition relative to the internal control, *dld*. $n = 3$ biological replications; error bars are standard deviations; **, $P < 0.01$. (A) Relative expression levels of *csc* genes for the control conditions (grown on glucose or lactose). (B to E) Relative expression levels of *cscR* (B), *cscA* (C), *cscK* (D), and *cscB* (E) in strains grown in 2% sucrose (open bars) and 0.2% sucrose (shaded bars).

sucrose can be hydrolyzed outside the cell by extracellular invertase (31, 32). This is consistent with the presence of the *cscB* transporter gene in the *csc* regulon. In addition, the essentiality of *cscA* demonstrates that no other invertase activity is available in *E. coli* W.

***cscK* is not essential for sucrose utilization.** WΔ*cscK* could grow on 2% sucrose, demonstrating that the *cscK* gene is not essential for sucrose utilization (Table 3). Furthermore, the growth rate at 2% sucrose was not compromised by the loss of *cscK* (Fig. 1). Fructokinase activities were in fact relatively low in all strains compared to invertase activities (Fig. 3); the low activity of the fructokinase is curious considering that the sucrose utilization model indicates that fructose must be phosphorylated prior to entry into the glycolytic or pentose phosphate pathways (10, 11, 33). Furthermore, fructokinase activity could be detected in the K-12 and W strains growing on glucose and in the *cscK*-knockout strains growing on high sucrose (Fig. 2), despite transcription of *cscK* being strongly downregulated (when growing on glucose) or completely absent (in the case of the knockout strains and K-12) under these conditions (Fig. 2A). Together, these data suggested the presence of an alternate pathway for fructose utilization (this will be discussed further below).

Knockout of *cscK* relieves repression at low sucrose levels. Surprisingly, knockout of *cscK* (WΔ*cscK*) enabled growth on low sucrose (Table 3; Fig. 1). Initially, we speculated that derepression happened as a result of interference with repressor binding due to the change in sequence context downstream of the binding site in

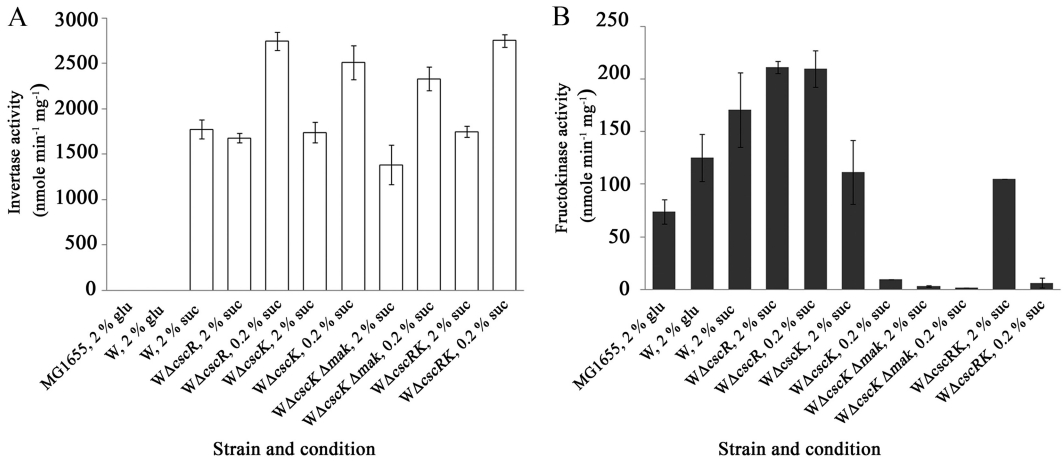

FIG 3 Enzyme activities in chromosomal knockout mutants. Invertase (A) and fructokinase (B) are expressed as nanomoles of product per minute per milligram of protein. Error bars are standard deviations ($n = 2$).

the knockout. This might allow expression of *cscB* in the *cscK* knockout. However, when WΔ*cscK* is grown in the presence of noninducing sugars (glucose or lactose), the transcription of the *csc* catabolic genes is identical to that observed in the wild type under the same conditions (with the exception of the loss of the *cscK* transcript) (Fig. 2A). Together, these data indicate that repressor activity is not affected in the *cscK* knockout.

Since the CscR repressor system is clearly intact and functioning in WΔ*cscK*, derepression in this strain must have been effected by an increase in the concentration of the unknown sensor metabolite. This may be associated with an increase in the intracellular concentration of unphosphorylated fructose as a result of deletion of the fructokinase. Free intracellular fructose (and to a lesser extent, fructose-1-phosphate) is a molecular inducer for the *Scr* sucrose PTS regulon (sucrose-6-phosphate, sucrose, and glucose-6-phosphate are not inducers) (34). This may also be the case for the *csc* regulon. Alternatively, the increased concentration of the sensor metabolite could be a result of increased transport activity by *cscB*. A previous study showed that in addition to truncation or knockout of *cscR*, a gain-of-activity mutation in the *cscB* gene can confer growth on low sucrose concentrations in EC3132 (10, 11). It is known that fewer full-length RNAs are transcribed from genes near the end of an operon than from those near the beginning of an operon (35), and removal of *cscK* places the *cscB* gene in much closer proximity to the promoter than it was in the wild-type strain. Transcript analysis indicated that *cscB* was transcribed at similar levels in WΔ*cscK* and the wild-type (WT) strain at 2% sucrose (Fig. 2E); however, *cscB* transcript levels were significantly higher on 0.2% sucrose than on 2% sucrose ($P = 0.006$). This might explain improved sucrose utilization at 0.2% sucrose; however, if the improvement in *cscB* transcription was solely related to its location relative to the transcription start site, transcription levels would be expected to be similar at both low and high sucrose. These data imply that sucrose concentration may have a direct effect on moderation of transcription levels which is independent of repressor activity, with expression being inversely proportional to sucrose concentration. In support of this, invertase (*cscA*) transcript levels were also higher at 0.2% than at 2% sucrose for all strains (Fig. 2C); this will be further discussed below.

Although differences in transcript levels at exponential growth might give some clues as to how sucrose is metabolized in these strains, ultimately it is not possible to infer from these measurements whether reduced conversion or increased transport caused increased accumulation of the sensor metabolite and derepression in the early stage of cultivation. Once derepression has been effected, sufficient transcription of catabolic genes will ensure that the available sucrose is utilized.

In the presence of high sucrose, the fructose moiety is not used by the *cscK*-knockout strain. To examine what happens to fructose in the absence of *cscK*, we first analyzed extracellular sugars to determine if the fructose was excreted from the cell or was metabolized inside the cell (Fig. 4). At high sucrose concentrations, sucrose is gradually consumed with little/no glucose or fructose accumulating in fermentations using the wild-type or repressor knockout strains (Fig. 4A and B). However, in the *cscK*-knockout strains, fructose accumulated in the growth medium at approximately stoichiometric levels (20 mM fructose at the end of the cultures where 20 mM sucrose had been metabolized) (Fig. 4D, F, and H). This demonstrated that the fructose moiety is not utilized under these conditions. The fact that the growth rate is not compromised at high sucrose concentrations (Fig. 1) and that fructose is exported from the cell and not reimported suggests that the glucose moiety is sufficient for maximal growth under these conditions. Despite this, the absence of fructose in the medium in wild-type and *cscR*-knockout strain fermentations indicates that CscK is responsible for incorporation of fructose from inversion into the glycolytic pathway in these strains. However, clearly the fructose moiety is not a major determinant of growth rate.

At low sucrose concentrations, an alternative fructose utilization pathway is available. At low sucrose concentrations (0.2%), no fructose accumulation was observed in the fructokinase-knockout strains (Fig. 4E, G, and I). Furthermore, the growth rate was decreased in the *cscK* knockout relative to the *cscR* knockout (Fig. 1). Together, these observations suggest that carbon availability becomes limiting for the *cscK* knockout at 0.2% sucrose and that other methods to utilize the available fructose moiety are engaged.

As noted previously, our fructose phosphorylation experiment indicated that an alternative fructose→fructose-6-phosphate ac-

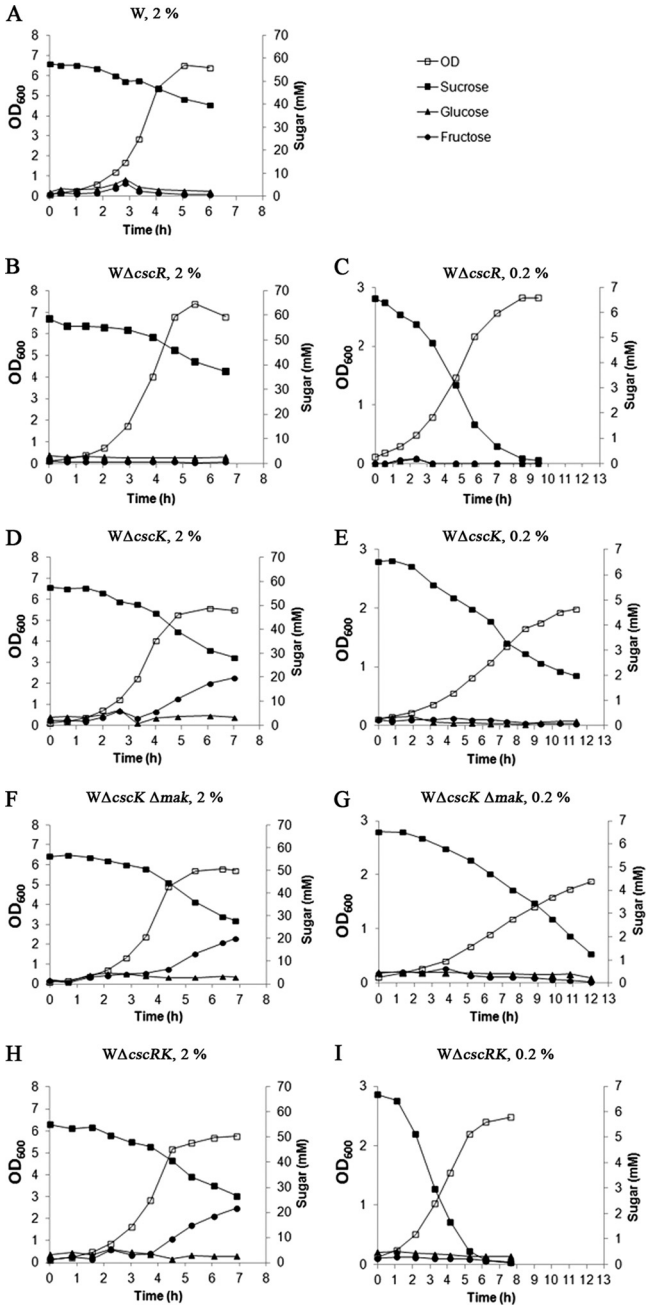

FIG 4 Culture growth and sugar utilization of *E. coli* strains grown in shake flasks. OD_{600} was used to measure growth (□). Sugars were measured in the extracellular medium by HPLC: sucrose (■), glucose (▲), and fructose (●). The strains and growth conditions are as follows: W in 2% sucrose (A), WΔ*cscR* in 2% sucrose (B), WΔ*cscR* in 0.2% sucrose (C), WΔ*cscK* in 2% sucrose (D), WΔ*cscK* in 0.2% sucrose (E), WΔ*cscK* Δ*mak* in 2% sucrose (F), WΔ*cscK* Δ*mak* in 0.2% sucrose (G), WΔ*cscRK* in 2% sucrose (H), and WΔ*cscRK* in 0.2% sucrose (I).

tivity was available in both W and MG1655 strains (Fig. 3B). For the sucrose PTS, inactivation of the fructokinase component (ScrK) also does not affect growth on sucrose (14). This is due to phosphorylation of intracellular fructose by manno(fructo)kinase (*mak*), a gene which is cryptic in wild-type K-12 but has broad substrate specificity for D-riboses and can be decryptified by mutation of various kinases (15, 36–38). A search in the *E. coli* W genome sequence (6) identified a *mak* gene. To examine whether Mak was contributing to fructose phosphorylation and sucrose utilization, we deleted the *mak* gene in the WΔ*cscK* strain background (WΔ*cscK* Δ*mak*). Fructose still accumulated in the growth medium at stoichiometric levels at 2% sucrose (Fig. 4F). This strain could also grow on low sucrose (Fig. 1); however, fructokinase activities were undetectable (Fig. 3). Though these experiments demonstrate that the Mak gene encodes an alternative fructokinase activity, there is no evidence that Mak has any functional relevance as the growth rate relative to that of the *cscK*-knockout strain was not compromised by loss of *mak* at either 2% or 0.2% sucrose (Fig. 1). Curiously, *mak*-mediated fructokinase activity is much higher at 2% sucrose than at 0.2% sucrose (Fig. 3; compare in *cscK*- and *cscRK*-knockout strains); it is therefore unlikely that *mak* accounts for fructose utilization at low sucrose levels.

A fructose PTS (the *fru* regulon) is encoded on the *E. coli* W genome, and *E. coli* W can grow on fructose as a sole carbon source (6). This is an obvious alternative route for fructose utilization in the *cscK*-knockout strains. However, under normal conditions the fructose must be extracellular for the PTS to efficiently phosphorylate it, since phosphorylation is coupled with transport (39). This clearly does not happen in the case of the high sucrose fermentation, as sucrose accumulates in the extracellular medium (Fig. 4D, F, and H). In the case of low-sucrose conditions, there are two possible mechanisms for PTS-mediated fructose utilization. First, fructose which is excreted into the medium may be imported and phosphorylated using the normal PTS mechanism. However, there is no evidence that fructose is ever excreted at low sucrose concentrations since we could not detect fructose in the extracellular medium (Fig. 4C, E, G, and I). It is also theoretically possible that intracellular fructose can be phosphorylated by both fructose and mannose PTS fructokinase functions (reviewed in reference 39). The transport and carbohydrate phosphorylation functions of EIIs are coupled under normal physiological conditions but are mechanistically separate processes. Some EIIs can carry out facilitated diffusion at low rates when they are unphosphorylated (i.e., in the presence of high glucose; phosphorylation when glucose is low/absent significantly increases the transport rate). Unphosphorylated carbohydrates can dissociate after transport and then rebind and be phosphorylated by EII at low efficiency. This mechanism may provide an alternative route for phosphorylation of intracellular fructose at low concentrations, thus explaining the absence of fructose in the extracellular medium at low sucrose levels.

In a separate transcriptomics study, we observed that the fructose PTS (but not the mannose PTS) was upregulated during growth on sucrose relative to glucose in the wild-type strain (Y. Arifin, unpublished results). This is not surprising given the general derepression of sugar catabolic genes observed in the absence of glucose-mediated carbon catabolite repression (39). We also observed that transcript levels of *fruA* (encoding the fructose PTS permease) were significantly higher in the *cscK*-knockout strain than in the *cscR*-knockout strain (Fig. 5). This suggests that the fructose PTS kinase function may be available for phosphorylation of the fructose moiety and compensate for loss of *cscK*. Levels of *fruA* transcript in the *cscK* knockout are higher than those in the *cscR* knockout at both 0.2% and 2% sucrose; however, obviously at 2% sucrose the PTS is not functioning for uptake of extracellular fructose. Therefore, at low sucrose concentrations, the fructose

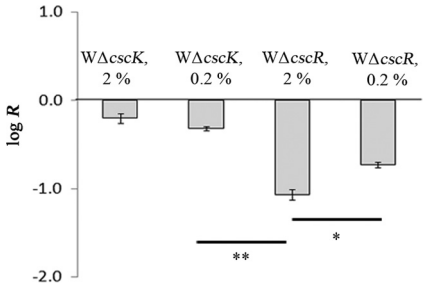

FIG 5 Quantitative real-time PCR analysis of *fruA* gene in WΔ*cscK* and WΔ*cscR* grown in high (2%) and low (0.2%) sucrose concentrations. Log*R* values are presented for expression levels of the *fruA* gene relative to the internal control, *dld*. $n = 3$ biological replications; error bars are standard deviations; **, $P < 0.01$; *, $P < 0.05$.

TABLE 4 Growth rateS of DH5α and WΔ*cscRAKB*::FRT harboring various different *csc* genes overexpressed from a high-copy-number plasmid using the *lac* promoter (*cscA*) or the native *csc* promoter (*cscAK* and *cscAB*)[a]

Strain	Growth rate (h^{-1})	
	2%	0.2%
DH5α/pCSCA	NM	NM
DH5α/pCSCAK	NM	NM
DH5α/pCSCAB	0.54 ± 0.09	NM
WΔ*cscRAKB*/pCSCA	0.26 ± 0.02	0.20 ± 0.02
WΔ*cscRAKB*/pCSCAK	0.82 ± 0.04	0.38 ± 0.02
WΔ*cscRAKB*/pCSCAB	1.15 ± 0.1	0.76 ± 0.02

[a] All strains were able to grow on minimal agar plates with 2% or 0.2% sucrose. Growth rates were determined using MTP cultures in the same medium (liquid). NM, growth rate was not measurable in MTPs. Errors are standard deviations ($n = 3$).

PTS may provide an alternative route for utilization of the fructose moiety; however, this pathway is not available at high sucrose concentrations. There is no obvious mechanism by which the PTS might be inhibited at high sucrose but not at low sucrose; however, the intracellular concentration of unphosphorylated fructose is presumably higher at 2% than at 0.2%, and this may be important.

Regardless of the mechanisms by which fructose is utilized at low sucrose and by which utilization is repressed at high sucrose, it is clearly unfavorable to utilize fructose at high sucrose concentrations in the absence of CscK. If carbon is not limiting at high sucrose (as we presume from the growth rate data), it would be energetically unfavorable to spend phosphoenolpyruvate (PEP) for phosphorylation of the fructose moiety, whereas at low sucrose (under carbon-limiting conditions), it becomes favorable to phosphorylate and utilize the available fructose. Whatever mechanism is at play for fructose utilization in the absence of CscK, it clearly functions only when carbon is limiting (at low sucrose). Presumably, the cell has less control over the activity of CscK and cannot prevent fructose phosphorylation (and the associated ATP cost) when it is present. Acquisition of the *csc* genes is thought to be a relatively recent evolutionary event in *E. coli* (11); possibly, such control mechanisms have yet to evolve. Alternatively, ATP-based phosphorylation by CscK is energetically preferred over substrate-level phosphorylation by the PTS, as fructose does not accumulate in the wild-type strain harboring CscK.

Low sucrose levels are associated with increased invertase transcription and enzyme activity. On 2% sucrose, all mutant strains containing the invertase gene had levels of invertase activity similar to that of the wild-type strain (two-way ANOVA, $P = 0.1169$) (Fig. 3A). However, on 0.2% sucrose, the invertase activity levels were ~60% higher than that seen on 2% sucrose (two-way ANOVA, $P = 1.935 \times 10^{-8}$). This increase in activity was matched by an 80 to 100% increase in *cscA* transcript levels (two-way ANOVA, $P = 0.002$) (Fig. 2C). Conversely, fructokinase transcript levels and enzyme activities were similar on both 2% and 0.2% sucrose in WΔ*cscR*, the only strain containing *cscK* that grew on 0.2% sucrose (other discrepancies in fructokinase activities have been discussed above). For permease transcripts, the pattern was more complicated. No significant difference was observed between transcript levels on low and high sucrose where the repressor was knocked out; when the repressor was present (in the *cscK* knockout), transcription was again higher on low sucrose. These data provide further circumstantial evidence that sucrose concentration (on top of the presence/absence of sucrose) affects transcription of the *csc* genes, with a particularly strong effect on the *cscA* transcription unit.

Combinatorial KO of *cscR* and *cscK* confers improved growth rates on low sucrose. Deletion of *cscR* in the *cscK* deletion strain (double *cscRK* deletion) resulted in a higher growth rate on 0.2% sucrose than those for the individual knockout strains (WΔ*cscR* and WΔ*cscK*) ($P < 10^{-5}$ for both) (Fig. 1). An increase in invertase transcripts at both sucrose concentrations relative to the individual knockout strains and to the WT at 2% sucrose was observed (Fig. 2C). However, all three strains had similar invertase activities of ~1,700 nmol min^{-1} mg^{-1} at 2% sucrose and ~2,500 nmol min^{-1} mg^{-1} at 0.2% sucrose, so the increase in relative transcript levels clearly does not affect final enzyme activity for CscA. Transcript levels for *cscB* were not significantly different for any of the knockout strains at 0.2% sucrose (Fig. 2E). These data suggest that neither CscB nor CscA is responsible for the improved growth rates in the double knockout. Since either there is no difference in transcript (in the case of *cscB*) or the difference in transcript does not carry through to the protein level (in the case of *cscA*), it is not clear why the growth rate is improved in the double deletion strain. Indeed, there is no obvious reason why deletion of *cscR* should improve growth rates in an already derepressed strain. We are currently investigating this phenomenon further. Regardless of the mechanism, the WΔ*cscRK* strain may have a further improved performance for production of industrial compounds in fed-batch fermentation given that the WΔ*cscR* strain has an improved performance relative to the wild type (26).

Overexpression of *cscAB* confers reasonable plasmid-mediated sucrose utilization in *E. coli* K-12. The ability to easily confer a sucrose utilization phenotype on non-sucrose-utilizing strains is desirable for industrial applications. However, we and others have previously observed plasmid instability (and, consequently, phenotypic instability) when plasmids are used to confer sucrose catabolic properties on non-sucrose-utilizing strains of *E. coli* (9, 24, 40). Here, individual genes, as well as gene combinations, were tested by overexpression in both K-12 (DH5α) and WΔ*cscRAKB* to examine the effect of the genetic background of different strains on sucrose utilization. Overexpression of *cscA* alone has previously been shown to confer sucrose utilization in different *E. coli* strains, albeit with low growth rates (9, 33, 41, 42). We also observed this for K-12 (DH5α) and WΔ*cscRAKB* (Table 4). The

growth rate of WΔ*cscRAKB* carrying the pCSCA plasmid was quite low on both 2% and 0.2% sucrose (0.26 h^{-1} and 0.20 h^{-1}, respectively); in the case of K-12, the growth rate was extremely low, and accurate growth rate analysis could not be performed due to phenotypic instability. Sahin-Tóth et al. (41) originally suggested that overexpression of the invertase might result in leakage of the excess invertase across the cell membrane; however, this could not be shown conclusively. Lee et al. (33) later demonstrated that CscA leakage did indeed happen, as they could identify invertase activity in the extracellular medium. This provides a mechanistic explanation for *cscA*-mediated sucrose utilization in plasmid-overexpressing strains. This mechanism does not occur in the wild-type strain, which utilizes sucrose intracellularly exclusively (see above). Lee et al. (33) also proposed that resulting extracellular fructose was imported via the fructose PTS; our data (discussed above) suggest that this may be possible at low sucrose concentrations but not at high sucrose concentrations.

Combinatorial overexpression of *cscK* or *cscB* improved growth rates relative to expression of *cscA* only (Table 4). This demonstrates that, where growth rate is limited (in the case of the *cscA* overexpression strain), the presence of CscK can improve sucrose utilization despite the fact that it is not essential for sucrose utilization. Consistent with our knockout data showing that CscB is more important for sucrose utilization than is CscK, co-overexpression of *cscB* with *cscA* resulted in a higher growth rate than that of co-overexpression of *cscK* with *cscA*. Previously, we have also examined the effect of overexpression of *cscAKB* in the WΔ*cscRAKB* strain (24). The growth rate of this strain on 2% sucrose was the same as the growth rate that we observed for WΔ*cscRAKB* harboring the *cscAB* construct. This demonstrates that the maximum growth rate on 2% sucrose in the WΔ*cscRAKB* strain can be achieved by overexpression of just two genes, *cscA* and *cscB*. This is also consistent with our knockout data, which showed that removal of *cscK* did not affect the growth rate on 2% sucrose (Fig. 1). However, the growth rate of K-12 on 2% glucose is 0.7 h^{-1} (24), significantly higher than that of the K-12 *cscAB* overexpression strain on 2% sucrose (0.54 h^{-1}; Table 4). Our previous experiments demonstrated that when *cscAKB* was overexpressed from a plasmid, the growth rate was only 0.2 h^{-1} on sucrose, and severe phenotypic instability was observed (24). It is not immediately apparent why this is the case; clearly, however, transfer of *cscAB* without *cscK* is the best method to achieve the most efficient sucrose utilization phenotype when using a plasmid-mediated overexpression system in K-12. Chromosomal integration of *cscAB* may also be sufficient to confer reasonably good sucrose utilization on non-sucrose-utilizing strains; integration of smaller DNA sequences is technically less challenging, so decreasing the number of genes required to confer a good phenotype is desirable for strain construction.

In the K-12 strain, only the *cscAB* plasmid conferred stable growth and only on 2% sucrose (Table 4). However, the growth rate was half of that of the W-derived strain harboring the same construct. Interestingly, no phenotypic instability was observed in the WΔ*cscRAKB* strain for any of the plasmids. Together, these observations suggest that W has an adapted advantage over K-12 for sucrose utilization. This may be due to a contribution of genes other than *csc* genes to sucrose utilization in W. The W genome is significantly larger than the K-12 genome and carries 271 more genes (6). Genes found only in W might be contributing to the improved phenotypic stability. Alternatively, differences in expression patterns of specific genes may be responsible. With regard to the relative growth rates, it is known that K-12 strains do not grow as fast as W, even on the preferred carbon source, glucose (24, 26). It is therefore likely that the slower growth of K-12 on sucrose is related to a general physiological difference between the two strains and is not a sucrose-specific phenomenon.

Conclusions. The data presented here and previously (10, 11) indicate that the *csc* regulon shares several regulatory similarities with the *lac* operon. However, unlike the *lac* operon, the *csc* regulon includes two distinct transcription units. Several lines of evidence presented here suggest that sucrose affects transcription of the two units (and, consequently, sucrose catabolism) differently at high and low sucrose concentrations (at least, in the absence of CscK). It is possible that this is mediated mechanistically through the fructose moiety. This is consistent with recent speculation around the importance of fructose as a carbohydrate source for bacteria. There is strong circumstantial evidence that fructose catabolism was a driver for evolutionary development of carbohydrate metabolic pathways in bacteria (reviewed in reference 43), and it is thought that glycolysis originally evolved as a fructose catabolic pathway (44–46). The catabolite repressor/activator (Cra) protein (originally characterized as the repressor for the fructose regulon and known as FruR) is a pleiotropic global transcription factor which regulates a wide variety of genes in both positive and negative fashion to determine whether metabolism will be fermentative or oxidative/gluconeogenic (43, 47). Micromolar concentrations of fructose-1-phosphate or millimolar concentrations of fructose-1,6-diphosphate bind to Cra, displacing it from its binding site and presumably derepressing negatively regulated transcripts and preventing activation of positively regulated transcripts. Interestingly, in the *csc* promoter, a sequence very similar to the consensus for the Cra binding site (RSTGAAW CSNTHHW, where R represents A/G, S represents C/G, W represents A/T, H represents A/C/T, and N represents any) can be found; it overlaps the transcriptional start site and the putative CscR binding site for *cscA* and is 160 bp upstream of *cscKB*. Future work will be directed at determining if Cra is involved in regulation of the *csc* genes and at examining exactly how the fructose moiety affects sucrose metabolism. This may provide a mechanism explaining the sucrose concentration-dependent responses that we have observed here.

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