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Adaptation of Sucrose Metabolism in the *Escherichia coli* Wild-Type Strain EC3132†

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Although *Escherichia coli* strain EC3132 possesses a chromosomally encoded sucrose metabolic pathway, its growth on low sucrose concentrations (5 mM) is unusually slow, with a doubling time of 20 h. In this report we describe the subcloning and further characterization of the corresponding *csc* genes and adjacent genes. The *csc* regulon comprises three genes for a sucrose permease, a fructokinase, and a sucrose hydrolase (genes *cscB*, *cscK*, and *cscA*, respectively). The genes are arranged in two operons and are negatively controlled at the transcriptional level by the repressor CscR. Furthermore, *csc* gene expression was found to be cyclic AMP-CrpA dependent. A comparison of the genomic sequences of the *E. coli* strains EC3132, K-12, and O157:H7 in addition to *Salmonella enterica* serovar Typhimurium LT2 revealed that the *csc* genes are located in a hot spot region for chromosomal rearrangements in enteric bacteria. The comparison further indicated that the *csc* genes might have been transferred relatively recently to the *E. coli* wild-type EC3132 at around the time when the different strains of the enteric bacteria diverged. We found evidence that a mobile genetic element, which used the gene *argW* for site-specific integration into the chromosome, was probably involved in this horizontal gene transfer and that the *csc* genes are still in the process of optimal adaptation to the new host. Selection for such adaptational mutants growing faster on low sucrose concentrations gave three different classes of mutants. One class comprised *cscR*(Con) mutations that expressed all *csc* genes constitutively. The second class constituted a *cscKo* operator mutation, which became inducible for *csc* gene expression at low sucrose concentrations. The third class was found to be a mutation in the sucrose permease that caused an increase in transport activity.

The ability to utilize sucrose (Scr) as a sole carbon source is a highly variable phenotype among enteric bacteria. More than 90% of wild-type strains of *Klebsiella* spp. but less than 50% of *Escherichia coli* and less than 10% of *Salmonella* strains are Scr^+ (14). The corresponding genes belong to the set of optional genes, which may be present in an individual genome of a species or may be lacking (37, 43). The characterization of such optional genes supplies valuable information about the mechanisms of horizontal gene transfer and gene adaptation in new hosts (12, 21, 22, 28).

Most Scr^+ enteric bacteria take up and phosphorylate sucrose by a phosphoenolpyruvate (PEP)-dependent sucrose: phosphotransferase system, which generates intracellular sucrose-6-phosphate (reviewed in references 29 and 30). Sucrose-6-phosphate is cleaved by a sucrose-6-phosphate hydrolase (invertase) into D-glucose-6-phosphate and D-fructose, which itself is phosphorylated by an ATP-dependent fructokinase. In *Salmonella* spp. mobile genetic elements, e.g., the conjugative plasmid pUR400 (36) or the conjugative transposon CTn*scr*94 (13), confer the ability to utilize sucrose, whereas in *Klebsiella pneumoniae* a highly similar *scr* regulon is encoded by the chromosome (38). Such optional genes often map in areas on the chromosome which highly diverge among different strains and which are hypervariable. These areas occur in strain-specific clusters of diverse size; the larger ones are called genomic islands or, to describe more precisely their functions, pathogenicity or metabolic islands, etc. (11, 13, 21, 28).

It was previously reported (6) that the *E. coli* wild-type isolate EC3132 in contrast to *E. coli* K-12 is capable of utilizing sucrose. However, growth on 0.2% sucrose was unusually slow, with a doubling time of 20 h. The corresponding *csc* (mnemonic for chromosomally encoded sucrose catabolism) genes were mapped in the equivalent of the 51-min region of K-12. Furthermore, it was shown that uptake and metabolism of sucrose in EC3132 is non-phosphotransferase system dependent (6, 31), and that instead sucrose is transported into the cells by a sucrose:H^+ symporter named CscB (encoded by gene *cscB*). The transporter resembles other well-studied permeases of the cluster 5 of the major facilitator superfamily (MFS) (19), with the lactose permease (LacY) from *E. coli* K-12 being a prominent member.

In this report we describe the subcloning and further characterization of the other *csc* genes: *cscK*, which codes for a fructokinase; *cscA*, which codes for an invertase; and *cscR*, which codes for a *csc*-specific repressor from EC3132. A comparison of the *csc* coding region and adjacent genes with the genomic sequences of *E. coli* K-12 and the recently published sequences of the sucrose-positive, uropathogenic *E. coli* wild-type O157:H7 (12, 28), in addition to *Salmonella enterica* serovar Typhimurium (22), referred to herein as *S. enterica*, revealed a pronounced diversity in this region of the

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† This work is dedicated to the memory of Pieter W. Postma, a highly acclaimed scientist in the field of bacterial physiology and genetics.

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TABLE 1. *E. coli* strains and plasmids used in this study[a]

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains of *E. coli* EC3132 and K-12		
EC3132	F^- csc^+ Csc^+	Bockmann et al. (6)
ECB1	EC3132 *cscB1* Csc^{3+}	Bockmann et al. (6)
ECH1	EC3132 *cscR1* Csc^{3+}	This study
JM109	*thi-1* Δ(*lac-proAB*) *recA1 hsdR1* dsd^+ Δ*csc*/F′ *traD36* $proA^+B^+$ $lacI^qZ$ *M15*	Yanisch-Perron et al. (44)
JC7623	F^- *thi-1 recB21 recC22 sbcB15 sbcC201*	Horii and Clark (15)
LJM1	JC7623 csc^+ *cscB1* Csc^{3+}	This study
LJM2	LJM1 *cscR*::*cat*(Con) Csc^{3+}	This study
KL16-10	csc^+ *cscB1* Csc^{3+}	Bockmann et al. (6)
KL16-212	KL16-10 *cyaA854* zhd::Tn*10*	This study
MC4100-5	MC4100 *cyaA854* zhd::Tn*10*	This study
LJ110	F^- Δ*csc*	Zeppenfeld et al. (45)
LJ200	LJ110 csc^+ *cscB1* Csc^{3+}	This study
LJM115	LJ110 csc^+ *cscB1 cscR*::*cat*(Con) Csc^{3+}	This study
Plasmids		
pACYC184	Cm^r Tc^r	Chang and Cohen (7)
pBluescript SK(+)	Ap^r	Ausubel et al. (3)
pSU18	Cm^r	Martinez et al. (20)
pSU19	Cm^r	Martinez et al. (20)
pTM30	Ap^r	Morrison and Parkinson (24)
pGEM-T	Ap^r	Promega Inc.
pJBL101	Cm^r csc^+	Bockmann et al. (6)
pJBL103	Cm^r csc^+ Δ*cscR* $argW^+$ $yfdC^+$ $vacJ^+$ $b2345^+$	This study
pKJL13	Ap^r *'vacJ b2345'*	This study
pKJL16	Ap^r $argW^+$ $yfdC^+$ *vacJ'*	This study
pKJL101-1	Cm^r csc^+ *cscKo124*	This study
pKJL124	Cm^r csc^+ *cscKo124*	This study
pKJL124-3	Cm^r csc^+	This study
pJBL126	Cm^r csc^+ *cscR126*	This study
pECB1	Ap^r csc^+ *cscB1*	This study

[a] The genetic nomenclature is according to Berlyn et al. (4) except for *csc* (mnemonic for chromosomal sucrose genes), which is according to Bockmann et al. (6). Csc^+ corresponds to weak fermentation of sucrose (pink colonies on indicator plates); Csc^{3+} corresponds to strong fermentation (deep-purple colonies on indicator plates).

chromosome. Furthermore, we found evidence that the observed diversity was caused by the presence of the gene *argW*, which codes for an arginine-specific tRNA, which served as an integration site for mobile genetic elements.

Finally, we isolated and characterized mutants with chromosomal and plasmid-borne mutations that grow faster on sucrose than the wild type. Analysis of such adaptational mutations provided evidence that the basal expression level of *cscB* or the transport activity of the sucrose permease in the wild-type strain was not sufficient for an effective induction of the *csc* genes at sucrose concentrations below 5 mM. We speculate that the *csc* genes found in the *E. coli* strain EC3132 have been transferred "recently," in evolutionary terms, and that they are not completely adapted to their host.

MATERIALS AND METHODS

Chemicals. [^{14}C]sucrose (Amersham Biosiences, Freiburg, Germany) was purified from D-glucose and D-fructose by scavenging as described before (6). All other chemicals were of commercial origin.

Bacterial strains, plasmids, and media. The *E. coli* K-12 strains and plasmids used in this study are listed in Table 1. Cells were routinely grown either in standard phosphate minimal medium as described previously (6) supplemented with a 0.2% concentration of the indicated carbon sources, in Lennox broth without glucose and calcium ions, or in 2× TY medium as described in the work of Ausubel et al. (3). Utilization of various carbohydrates was screened on MacConkey plates (Difco) containing 1% of the indicated carbon source. The following antibiotics were used at the indicated concentrations: tetracycline, 10 mg/liter; ampicillin, 50 mg/liter; and chloramphenicol, 25 mg/liter. Transductions were carried out with P1 *kc* essentially as described previously (6).

For testing various *cscR* alleles, a chromosomal *cscR*::*cat* insertion was generated as follows. The *csc* genes from KL16-10 (6) were transferred by P1-transduction into JC7623 (*recBC sbcBC* [15]), giving strain LJM1. The *cscR* gene from pJBL101 was subcloned as an *Eco*RI/*Hin*dIII fragment into the vector pBluescript SK(+). A DNA fragment encoding the *cat* gene from pACYC184 (7) was isolated with *Acc*I and *Bcl*I, blunted, and inserted into the *Stu*I site in *cscR*. The plasmid was digested with *Hin*dIII and *Pst*I, and the linear DNA fragment containing the gene *cat* flanked by *cscR* was used to transform LJM1. Transformants were screened on Luria-Bertani plates with chloramphenicol and tested for constitutive *csc* gene expression. One such CscR(Con) transformant was designated LJM2. Finally, the *csc* gene cluster was transferred from LJM2 to LJ110 by P1 transduction, giving strain LJM115, which likewise exhibited a constitutive *csc* gene expression (Table 2).

Isolation of plasmid DNA, restriction analysis, and cloning procedures. All manipulations with recombinant DNA were carried out using standard procedures (3). Preparation of genomic DNA was done by using the cetyltrimethylammonium bromide method and preparation of plasmid DNA by using standard phenol extraction protocols as described previously (3) or by using the JETstar DNA purification system (Genomed, Bad Oeynhausen, Germany). Restriction enzymes (New England Biolabs, Schwalbach, Germany) were used according to the recommendations of the supplier. Oligonucleotides for sequencing or PCRs were purchased from Interactiva (Ulm, Germany). For testing the cyclic AMP (cAMP)-CrpA dependence of both *cscKop* and *cscAop* promoters, three different *lacZ* operon fusions were generated. For Φ(*cscAop-lacZ*), pJBL101 was digested with *Hin*dIII and the promoterless *lacZ* gene from plasmid pRU869 (40) was inserted. For Φ(*cscKop-lacZ*), pJBL101 was digested with *Eco*RV; for the fusion in *cscB* the plasmid was linearized by a partial digestion with *Hpa*I. Both enzymes generate blunt ends. Therefore, the *lacZ*-carrying *Hin*dIII DNA fragment from pRU869 was isolated, blunt ended with Klenow DNA polymerase, and inserted

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TABLE 2. Phenotype and Csc enzyme activities of strain EC3132 and derivatives

Strain	Relevant genotype	Csc phenotype[a]	Doubling time[b] (min)		Invertase[c]		Sucrose transport[c]		Fructokinase[c]	
			Gly	Scr	ui	i	ui	i	ui	i
EC3132	*csc*$^{+}$	1+	60	1,200	6	30	<3	<3	<1	3.9
ECB1	*cscB1*	3+	60	55	9	375	5	45	<1	7.4
ECH1	*cscR1*	3+	60	50	1,800	1,500	660	500	9.0	7.5
LJ200 (K-12)	*cscB1*	3+	100	85	10	500	5	53	<1	3.0
LJM115 (K-12)	*cscB1 cscR*::*cat*	3+	100	75	1,275	975	260	180	5.0	3.1
JM109/pSU18	—	—	95	NG	<2	<2	<3	<3	<1	<1
JM109/pJBL101	*csc*$^{+}$	1+	95	1,200	4	41	<3	<3	<1	4.0
JM109/pJBL103	*csc*$^{+}\Delta$*csc′R*	3+	160	80	1,560	1,260	150	131	7.9	3.9
JM109/pKJL124	*cscKo124*	3+	95	80	5	466	10	45	<1	7.3
JM109/pJBL126	*cscR126*	3+	160	80	1,550	1,340	151	134	15.5	6.8
JM109/pECB1	*cscB1*	3+	105	100	20	310	33	96	<1	6.7
JM109/pKJL124-3	*csc*$^{+}$	1+	95	1,200	10	40	<3	<3	<1	4.2
JM109/pKJL101-1	*cscKo124*	3+	95	80	10	520	25	119	<1	3.6

[a] The Csc phenotype for sucrose utilization was tested on MacConkey indicator plates with 1% sucrose: —, white colonies; 1+ to 3+, pink to deep-purple colonies.
[b] Doubling times were tested in minimal media with the indicated sole carbon source at 0.2%. NG, no growth.
[c] Exponential cells pregrown on minimal 0.2% glycerol (u [uninduced]) or 0.2% glycerol–0.2% sucrose (i [induced]) media were harvested and tested as described in Materials and Methods. Invertase (CscA) and fructokinase (CscK) activities are given in nanomoles per minute and milligrams of total protein; transport (CscB) activities are given in picomoles per minute and milligrams of total protein. The mean values of at least three measurements are given.

into each *csc* gene, respectively. The appropriate orientations of all *lacZ* insertions were controlled by DNA sequencing. To measure repressor-independent, but cAMP-CrpA-dependent regulatory effects, *cscR* was deleted by digesting all plasmids with *Kpn*I and *Stu*I and recloning the *csc* genes carrying DNA fragments lacking the first 584 bp of *cscR* into the vector pSU19 (20). The obtained plasmids were named pAlacZ with the operon *lacZ* fusion in *cscA*, pKlacZ with the fusion in *cscK*, and pBlacZ with the fusion in *cscB*.

Mutation analysis and DNA sequencing. DNA amplification of the *csc* genes was done according to the method of Saiki et al. (35). For the amplification of the complete *csc* regulon, the forward PCR primer cscB3 (5′-TTTCCGGTTGAGG AATATGG-3′) and the reverse PCR primer cscR2 (5′-GGGCGTTGAG GATATCGTTGGATCTCGCCA-3′), which introduced an *Eco*RV restriction site (underlined), and the Expand Long Template PCR System from Roche Diagnostics, Mannheim, Germany, were used. Starting with 2 min at 93°C, the reaction profile consisted of 30 cycles of denaturing at 93°C for 15 s, followed by annealing at 50°C for 1 min and extension at 68°C for 4 min in a gradient thermocycler from Biometra Inc., Goettingen, Germany. Beginning with the 10th cycle, the elongation time was extended by 5 s to compensate for the loss of DNA polymerase activity.

The amplification of the isolated *csc* genes was performed on an air thermocycler (model 1605) from Idaho Technology Inc., Idaho Falls, Idaho, using the following reaction profile: 32 cycles of denaturing at 94°C for 1 s, annealing at 50°C for 1 s, and extension at 72°C for 45 s. The following PCR primer pairs were used: for *cscB*, cscB3 and cscB4 (5′-GCAGGTAATTCTGCAGCTGAATAT TCCATTC-3′), which contained an artificial *Pst*I site (underlined); for *cscR*, cscR1 (5′-TCGCTCTATGCCTCCCACGGA-3′) and cscR2 (5′-GGGCGTTG AGGATATCGTTGGATCTCCGGT-3′), which contained an artificial *Eco*RV site (underlined); and for the *csc* promoter-operator regions, cscO1 (5′-AG GCAGTAGCCGCCCGTCTG-3′) and cscO2 (5′-CGGCGCTCGTGAAGTTT TGC-3′), respectively. PCR products were directly purified using the Wizard PCR preps DNA purification system (Promega Corp., Mannheim, Germany). All DNA sequencing reactions were performed by the dideoxy chain termination method using the ALFexpress AutoRead or dATP labeling mix sequencing kit from Amersham Biosciences. The nucleotide sequences of both strands were determined after subcloning into the pSU19 (20) or the pGEM-T (Promega Corp.) vector using Cy-5 (5-*N*,*N*-diethyltetramethylindo *di*carbocyanine)-labeled universal and reverse primers or unlabeled internal *csc* sequencing oligonucleotides priming about every 250 bp within the genes. Computer analysis was done with DNASIS sequencing analysis software (Hitachi) and by using the BLAST programs and database services provided by the National Center for Biotechnology Information, Bethesda, Md.

Identification of transcriptional start points. Primer extension experiments for the identification of the transcriptional start points upstream of *cscK* and *cscA* were performed as described previously (3). Total RNA was isolated from the *cscR*::*cat*(Con) derivative LJM115 during the mid-exponential growth phase on Lennox broth without glucose and calcium ions.

Transport and enzyme assays. Transport of [^{14}C]sucrose (15 μM final concentration) and sucrose hydrolase (invertase) and fructokinase activities were measured in exponentially grown cells as described previously (6). Transport activities were calculated from the initial uptake rates. The assay of β-galactosidase was performed according to the method of Pardee and Prestidge (27).

RESULTS

Cloning and sequencing of the *csc* locus and adjacent genes. Previous studies mapped the *cscBKAR* genes of *E. coli* EC3132 close to the *dsd* locus encoding D-serine utilization (6). For subcloning the *csc* genes chromosomal DNA from strain EC3132 was digested with either *Eco*RI or *Pvu*II. Fragments in the range of 5 to 15 kb were ligated into the vector pSU18 (20) and transformed into the *E. coli* derivative JM109, which is unable to use sucrose as a carbon source. Recombinant clones were screened on rich medium plates containing chloramphenicol, IPTG (isopropyl-β-D-thiogalactopyranoside) (0.5 mM), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (0.01%) and further tested for their abilities to utilize sucrose on MacConkey sucrose (1%) indicator plates. Two different Csc-positive clones, one from each subcloning experiment, were further analyzed. A 5.5-kb *Eco*RI and a 9.3-kb *Pvu*II insert in pSU18 were identified, and the plasmids were designated pJBL101 and pJBL103, respectively (Fig. 1). JM109/pJBL101, like EC3132, formed pink (Csc^{+}) colonies on the indicator plates and was unable to grow on sucrose at concentrations below 0.2%, whereas JM109/pJBL103 exhibited a strong Csc^{3+} phenotype (deep purple colonies on the indicator plates). Restriction analysis of the two fragments revealed that they partially overlap. Csc enzyme activities of JM109 harboring pJBL101 or pJBL103 were measured. In contrast to JM109/pJBL101, strain JM109/pJBL103 exhibited constitutive invertase (CscA), transport (CscB), and fructokinase (CscK) activities (Table 2), thus indicating a (partial) deletion of the *cscR* repressor gene in the *Pvu*II subclone (see below).

For a further characterization of the *csc* locus and of the

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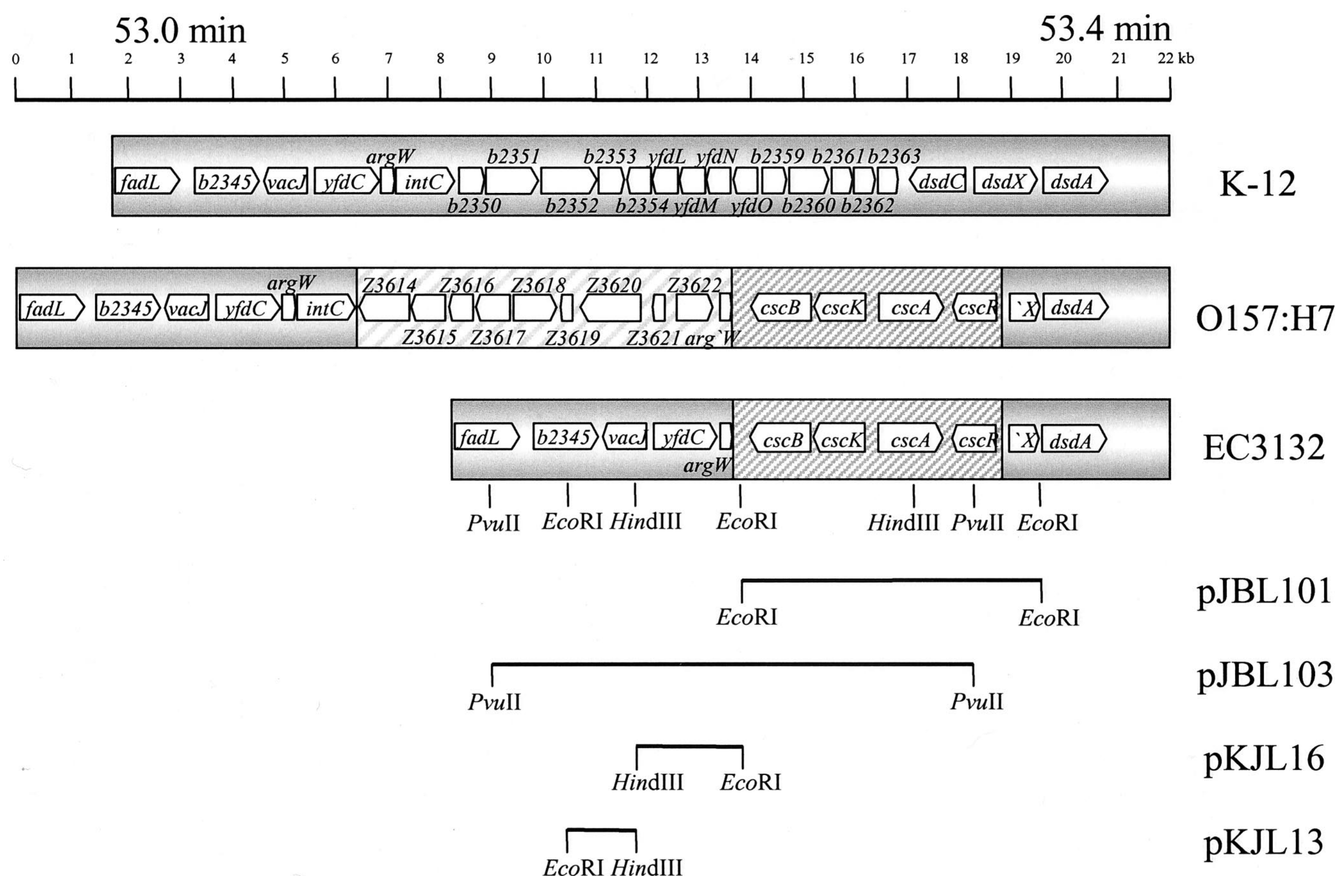

FIG. 1. Detailed comparative map of the *csc-dsd* regions of the *E. coli* strains K-12, O157:H7, and EC3132. Genes are represented by boxes, with their orientations indicated by arrows. Regions of homology between *E. coli* K-12 and the two other strains are shown on a dark grey background; those between O157:H7 and EC3132 are shown on a hatched background. O157:H7-specific DNA is emphasized on a light grey hatched background. The heading numbers refer to the positions of the genes on the *E. coli* K-12 map (in minutes) and to the relative kilobase coordinates.

adjacent genes, the DNA sequences of the *Eco*RI restriction fragment of clone pJBL101, and the two *Eco*RI/*Hin*dIII fragments of pKJL13 and pKJL16 (Fig. 1) were determined and assembled (GenBank accession no. AF473544). A comparison with the previously published DNA sequence (GenBank accession no. gi|608705) revealed several differences. The most important findings were (i) an additional 64 bp in the center of the *csc* promoter-operator region (at position 5514 to 5578 of AF473544) and (ii) a deletion of a base pair (corresponding to position 4533 of AF473544) leading to a frame shift in the 3′coding region of *cscK*, which caused a change in the deduced sequence of the last 17 amino acids.

DNA sequence analysis of the *csc* gene cluster revealed four open reading frames: The previously described *cscB* gene, which codes for a sucrose permease (6), is located immediately downstream of the gene *cscK*. Both were cotranscribed from the promoter *cscKp*. *cscK* encodes a protein of 307 amino acid residues that exhibits high similarities to other fructokinases (e.g., 74% amino acid identity with ScrK from pUR400 [2]). Gene *cscA*, which codes for a sucrose hydrolase (invertase), is divergently oriented in respect to the *cscKB* operon. CscA consists of 477 amino acid residues and exhibits high similarities to other pro- and eucaryotic sucrose- or sucrose-6-phosphate-hydrolyzing enzymes. Downstream of *cscA* an open reading frame was identified for the LacI-GalR-type repressor protein CscR (23% identity with LacI and 26% identity with GalR, respectively). In pJBL103 the coding region for the amino-terminal 112 amino acids of CscR was deleted, explaining the constitutive *csc* gene expression from this plasmid (Table 2).

A further sequence analysis and a comparison with the equivalent DNA sequence of *E. coli* K-12 showed that the *csc* genes of EC3132 partially replaced the *dsd* gene cluster for the utilization of D-serine, thus explaining the Dsd$^-$ phenotype of all *csc*$^+$ strains. The gene *dsdC*, which codes for the activator of the *dsd* regulon, and the 5′-coding region of gene *dsdX* which codes for a putative D-serine transporter (25), were deleted and replaced by the *csc* regulon in EC3132. The gene *dsdA*, which codes for a D-serine-deaminase, is still present but is inactive due to the missing activator DsdC. This particular genotypic exclusion had been observed before (1, 6), but its molecular basis was not clear.

On the opposite end, the *csc* gene cluster is flanked by the *argW* gene which codes for an arginine-specific tRNA. Whereas the upstream region of *argW* in EC3132 is almost identical to the equivalent segments of *E. coli* K-12 (5) and

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TABLE 3. Genes in the *csc* region of EC3132[d]

Gene[a]	Coding region (bp)[b]	No. of amino acids in product[c]	Property and/or putative function of gene product	Comment
'b2345	1-416	(138)	Unknown function	Only 3′ coding region
vacJ	1353-601	251	Putative virulence factor, not classified	
yfdC	1647-2576	310	Putative transport protein, not classified	
argW	2729-2655		Arginine tRNA	Anticodon at bp 2687-2689
cscB	4418-3174	415	Sucrose permease	
cscK	5403-4483	307	Fructokinase	
cscA	5619-7049	477	Invertase	
cscR	8055-7063	331	*csc*-specific repressor	
'dsdX	8300-8512	(71)	Putative D-serine transport protein	Only 3′ coding region

[a] The genetic nomenclature is according to Berlyn et al. (4) and that for *csc* is according to Bockmann et al. (6).
[b] The numbers (start–end) refer to the DNA sequence file with the accession number GB AF473544.
[c] The numbers in parentheses indicate that the genes are incomplete on the cloned fragment.
[d] Summary of the genes in the *csc* region of *E. coli* EC31322.

O157:H7 (12, 28), a comparison of the downstream regions of *argW* genes from the different *E. coli* strains indicated several major rearrangements. The existence of these changes suggests that bacteriophages or related genetic elements, which used *argW* for a site-specific integration into the host chromosome, were responsible for the horizontal gene transfer and stable integration of the *csc* genes (see Discussion). An overview of the genes in the *csc* region of EC3132 is given in Table 3.

Isolation and characterization of mutants with enhanced sucrose utilization. An outstanding characteristic of the *csc*-encoded sucrose metabolism is the unusual slow growth on sucrose compared to other carbon sources; e.g., strain EC3132 exhibited a doubling time of 20 h in minimal medium with 0.2% sucrose compared to 1 h with 0.2% glycerol. Subcloning of the *csc* genes from EC3132 on multicopy plasmids (e.g., pJBL101) and transfer to JM109 did not improve growth properties (Table 2). This might indicate that EC3132 has acquired the *csc* genes relatively recently and that expression of the *csc* genes is not yet optimally adapted. Therefore, we isolated mutants with chromosomal and plasmid-borne mutations that had enhanced sucrose utilization. Cells incubated on 0.2% sucrose on minimal medium segregated at high rates (within 3 days), yielding mutants that were able to grow rapidly under these conditions and were able to form dark purple colonies on sucrose indicator plates (Csc^{3+}). Two rapidly growing derivatives of EC3132, named ECB1 and ECH1, and two plasmid-borne Csc^{3+} derivatives of JM109/pJBL101, named JM109/pKJL124 and JM109/pJBL126, were further analyzed by testing the Csc enzyme activities (Table 2). All mutants were able to grow on 0.2% sucrose as a sole carbon source, and growth on sucrose was significantly faster compared to that on 0.2% glycerol. Whereas the wild-type strain EC3132 exhibited only very low invertase and fructokinase activities and no detectable sucrose transport activities after addition of 0.2% sucrose to cells growing on 0.2% glycerol, ECB1 was clearly inducible under these conditions, showing a 40-fold induction of CscA and a roughly 10-fold increase of both CscB and CscK activities. Mutant ECH1, like the Δ*csc′R* derivative JM109/pJBL103, was fully constitutive for all tested Csc activities (Table 2). Similar effects were observed for the plasmid-encoded systems: JM109 harboring the wild-type *csc* genes on pJBL101 showed only low Csc enzyme activities, whereas cells harboring pKJL124 exhibited a 90-fold induction of the invertase and a 4.5-fold induction of the sucrose transport activity. Interestingly, the basal expression level of the CscB activity was increased more than threefold compared to the wild type. A similar result could not be observed for the fructokinase activity, probably because the true uninduced basal expression levels of cells harboring pJBL101 or pKJL124, respectively, were below the detection limits of the test, as for the wild-type strain. However, the fructokinase activity was clearly induced in JM109/pKJL124 in the presence of 0.2% sucrose. Cells harboring pJBL126 in contrast to pKJL124 exhibited a constitutive *csc* gene expression.

Identification of the mutations leading to enhanced sucrose utilization. Constitutive expression of both *cscKB* and *cscA* operons could be caused by *cscR*(Con) mutations inactivating the repressor CscR. To test this hypothesis, the *cscR* alleles from the wild-type strain EC3132, mutant ECH1, and the mutated plasmid pJBL126 were amplified by PCR and subcloned into the plasmid pGEM-T. The corresponding plasmids were tested for their abilities to complement the *cscR*::*cat* mutation of LJM115. LJM115 carrying the wild-type *cscR* gene on the multicopy plasmid did not show any CscA invertase or sucrose transport activity even in the presence of the inducer sucrose, because of the disproportionately high levels of repressor molecules in these cells. In contrast, both *cscR* alleles from ECH1 and pJBL126 cloned on the same multicopy vector did not alter the constitutive *csc* gene expression of LJM115 (data not shown). DNA sequences of both inactive *cscR* alleles were determined. The *cscR* mutation of strain ECH1 was found to be a deletion of a single adenosine residue at bp 841 of the open reading frame. This altered gene encodes a 290-residue peptide (of 331 amino acids), in which the last 9 amino acids were changed by the frameshift. The *cscR* mutation of pJBL126 was identified as a single adenosine insertion at bp 102 of the *cscR* gene. The altered open reading frame encodes a truncated peptide of 47 residues, in which in addition the last 14 amino acids were altered compared to the wild type.

In contrast to these constitutive mutants lacking an active repressor, the mutants ECB1 and JM109/pKJL124 still exhibited inducible *csc* gene expression. To map the mutation of pKJL124 more precisely two hybrids between the mutant and the wild-type *csc* regulon of pJBL101 were constructed using a single *Nru*I site in *cscA* (Fig. 2). Based on the analysis of the plasmid-encoded phenotypes and the Csc enzyme activities of

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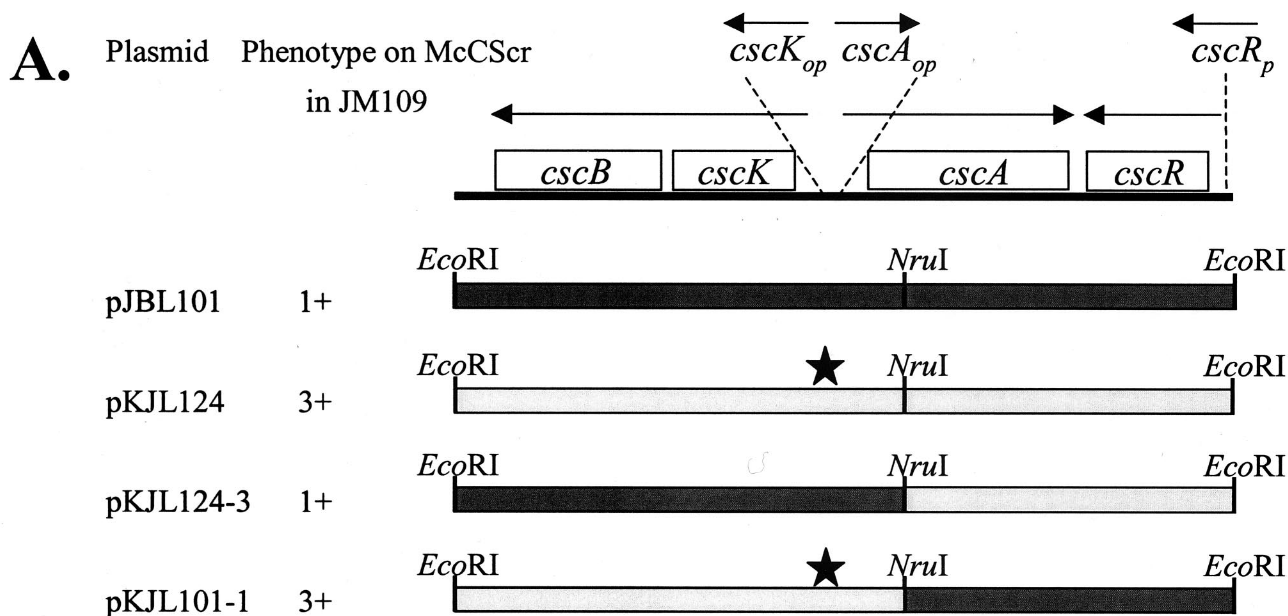

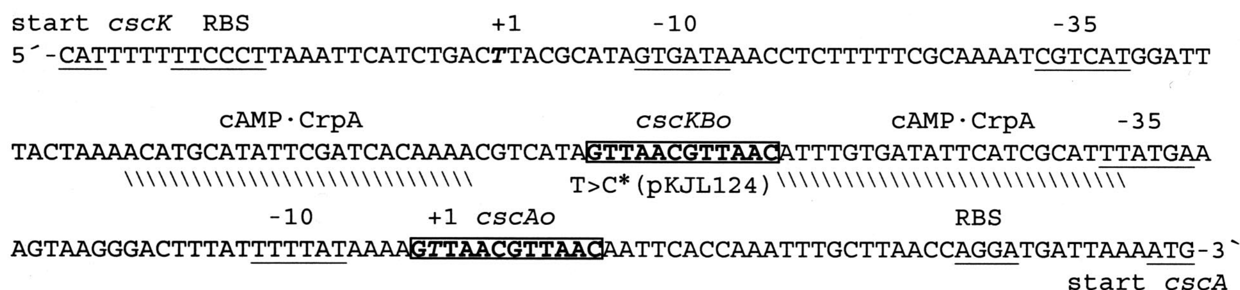

FIG. 2. Schematic illustration of the construction of *csc* hybrid plasmids (A) and nucleotide sequence analysis of the *csc* promoter-operator region (B). (A) The Csc phenotype for sucrose utilization was tested on MacConkey indicator plates with 1% sucrose (scored as 1+ to 3+, pink to deep-purple colonies). The genes are indicated by boxes, with the gene or promoter orientations shown by arrows. DNA of pJBL101 is represented by dark grey boxes, and DNA of pKJL124 is represented by light grey boxes. The position of mutation in pKJL124 is symbolized by an asterisk. (B) The putative −35 and −10 regions are underlined, as well as the putative ribosome-binding sites (RBS) and the start codons of *cscK* and *cscA*. Transcriptional start points (TK) are indicated by letters in boldface italic type, the putative *csc* operators are boxed, and the putative cAMP-CrpA binding sites are marked with diagonal lines. The mutation of pKJL124 is symbolized by an asterisk.

JM109 harboring the different constructs (Table 2) the mutation could be mapped in the DNA segment, which encoded the *cscBK* genes and the *csc* promoter-operator region. The DNA sequence of this fragment was thus determined, and the mutation was identified as a T-to-C transition in the left half of the putative *cscKo* operator (GTTAAC/GTTAAC). As shown in Fig. 2B this particular palindrome motif is present twice between the two operons and most likely corresponds to the operators *cscKo* and *cscAo*.

To map the mutation of ECB1, chromosomal DNA of this mutant was isolated and the *csc* gene cluster was amplified and subcloned into the pGEM-T vector, giving pECB1. JM109 carrying this plasmid also displayed a Csc^{3+} phenotype on sucrose indicator plates and was inducible for *csc* gene expression (Table 2). This indicated that the relevant mutation of ECB1 was located within the *csc* gene cluster and not somewhere else on the chromosome. The DNA sequence of the subcloned fragment of pECB1 was determined, and the mutation was verified as an adenosine-to-cytosine transversion in the CAA triplet for glutamine residue 353 of the sucrose transporter CscB, leading to a Q353H exchange in the putative transmembrane helix XI (6).

Effects of the Q353H substitution in CscB. CscB shares a high degree of similarity (31.2% identical residues over the entire length) to the lactose permease (LacY) from *E. coli* (6). However, as shown earlier the transport activities of the sucrose permease were significantly lower compared to LacY, and transport could be measured only after overexpression of

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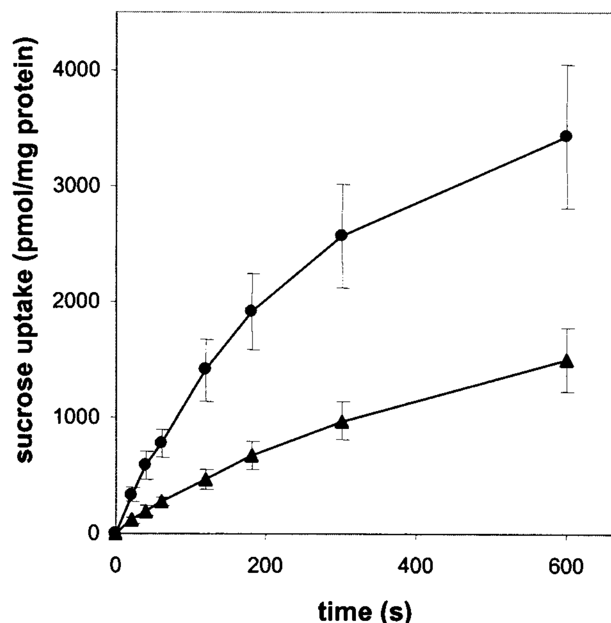

FIG. 3. Sucrose transport by JM109 harboring *cscB* expression plasmids. Time courses of active sucrose transport by JM109 expressing wild-type sucrose permease from pTM3132 (triangles) or the Q353H derivative of CscB from pTMB1 (circles) are indicated. Cells were grown aerobically at 37°C until mid-log exponential growth phase, harvested, washed with minimal medium, and immediately tested. The final sucrose concentration in the test was 15 μM. Mean values and standard deviations (error bars) from at least three different experiments are given.

the protein, e.g., by using a plasmid-encoded system (31). To test whether the Q353H exchange alone caused the increased transport activity, alleles both from the wild type and from mutant ECB1 were amplified by PCR and subcloned as *Pst*I/*Eco*RV fragments into the expression vector pTM30 (24). This expression vector provides a regulated *tac* promoter-operator, a $lacI^q$ gene, and a ribosome binding site in an optimal position with respect to the start codon. The expression levels of *cscB* from these plasmid constructs were sufficient for measuring specific transport activities in JM109, even without induction by IPTG. Addition of IPTG led to an immediate growth inhibition and inactivation of CscB (data not shown). JM109 cells expressing the Q353H derivative of CscB from pTMB1 accumulated sucrose under identical conditions approximately twofold higher (537 ± 17 pmol per min and mg of protein) than cells expressing the wild-type protein from pTM3132 (206 ± 40 pmol per min and mg of protein) (means ± standard deviations) (Fig. 3).

cAMP-CrpA dependence of *csc* gene expression. The analysis of the *csc* promoter-operator region revealed two segments (Fig. 2b) that share high similarities with the 22-bp palindromic consensus sequence for a cAMP-CrpA binding site (8). One region, which overlaps with the −35 region of the *cscA* promoter, exhibited 18 identical base pairs out of 22; the other region upstream of the *cscK* promoter showed 12 identical base pairs out of 22. Both regions are separated by 19 bp. Therefore, expression of the *csc* gene was tested in the adenylate cyclase negative ($CyaA^-$) mutant KL16-212 and compared to the isogenic $cyaA^+$ strain KL16-10. In contrast to KL16-10, mutant KL16-212 was not able to utilize sucrose, but addition of 0.5 mM cAMP restored a Csc^+ phenotype, providing first evidence that the *csc* gene expression is under the global control of cAMP-CrpA. To address the question of whether both divergently oriented *cscK* and *cscA* promoters were subject to the control of cAMP-CrpA, *lacZ* operon fusions were generated in *cscK*, *cscB*, and *cscA*. To test only effects caused by cAMP-CrpA the CscR-specific regulation was eliminated by a partial deletion of the repressor gene in all constructs. The β-galactosidase activities from these fusions were determined in the Δ*lac* $CyaA^-$ strain MC4100-5 (Table 4). The results confirmed the cAMP-CrpA dependence for both promoters. The low basal activities were enhanced for all four constructs by the addition of 0.5 mM cAMP by a factor of 6 to 8. To ensure that the enhanced β-galactosidase activities were not caused by an increase of the plasmid copy numbers in the presence of cAMP, the intracellular plasmid DNA concentrations for each culture were simultaneously determined. The semiquantitative analysis revealed no detectable differences (data not shown) that could account for the observed differences in the β-galactosidase activities.

TABLE 4. cAMP-CrpA dependence of *csc* gene expression[a]

Strain	Fusion	Mean activity ± SD (nmol/min/mg of protein) for strain grown:	
		Without cAMP	With cAMP
MC4100-5/pSU19	—	0	0
MC4100-5/pAlacZ	Φ(*cscAop-lacZ*)	128 ± 20	766 ± 30
MC4100-5/pKlacZ	Φ(*cscKop-lacZ*)	92 ± 20	750 ± 30
MC4100-5/pBlacZ	Φ(*cscBop-lacZ*)	107 ± 20	72 ± 30

[a] Cells of mutant MC4100-5 (Δ*lac* Cya^-) harboring the plasmid indicated were grown in LB_0 supplemented with chloramphenicol and with or without 0.5 mM cAMP as indicated. Cells were harvested during exponential growth and tested for β-galactosidase activities. The mean values of at least three measurements are given.

DISCUSSION

In this study we have described the cloning, sequencing, and further analysis of the *csc* gene locus from *E. coli* wild-type strain EC3132 that enables the cells to use sucrose as a carbon source. A comparison of the *argW-csc-dsd* regions of *E. coli* strains K-12, O157:H7, and EC3132 indicated a surprising level of diversity even in this small part of the chromosomes. Several findings argue that bacteriophages or other mobile genetic elements which used *argW* as a specific integration-site in the host chromosome were responsible for these chromosomal rearrangements. The most important hint for this hypothesis is a 21-bp sequence repeat at the 3′ end of *argW* that we found between Z3622 and *cscB* in O157:H7 (Fig. 1) but that has not been annotated in the previous analysis of the O157:H7 sequence (12, 28). The repeat is a typical indicator for a site-specific integration of phage-like elements via tRNA-encoding genes, for which an increasing number of reports now exist (reviewed in reference 41). These genetic elements comprise bacteriophages, insertional elements, conjugative transposons, and genomic islands, including pathogenicity islands (reviewed

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in reference 11), and encode virulence or pathogenicity factors or complete metabolic pathways. In agreement with this hypothesis, several of the potential open reading frames downstream of *argW* in *E. coli* K-12 and O157:H7 are known phage proteins, e.g., the putative integrase IntC of the lambdoid phage family. Several subsequent integration and deletion events must have led to the current situation in which *E. coli* K-12 possesses a complete *dsd* operon for D-serine utilization (23) while EC3132 and O157:H7 do not. For both wild-type *E. coli* derivatives EC3132 (6; this study) and O157:H7 (12, 28) it was shown that the *csc* genes were integrated into the *dsd* gene cluster (Fig. 1). As a result of this insertion the *dsdC* gene was completely and the *dsdX* gene was partially deleted, causing a Dsd^- phenotype. This led to a genotypic exclusion between *csc* and *dsd* genes as noted first by Alaeddinoglu and Charles (1). This gene arrangement could also account for the observation that within the ECOR collection (26), 23 of the 72 *E. coli* wild-type strains failed to use D-serine but could utilize the trisaccharide D-raffinose (Raf) instead (18). The Raf^+ phenotype is coupled with the presence of the *csc* genes and is caused by the unusual properties of the CscA invertase, which seems to be partially released into the periplasm (32; our unpublished results).

Finally, in *S. enterica* LT2 (gi16763390 [22]) downstream of *argW*, both the *dsd* and *csc* gene clusters are replaced by a cluster of five genes for the transport and utilization of phosphoglycerate not found in strains of *E. coli*. The sequence homology of the DNAs of the different enteric bacteria starts again close to the *glk* gene for a glucokinase, which is located about 10 kb downstream of *argW* in *Salmonella* and about 42 kb downstream of *argW* in both *E. coli* K-12 and O157:H7 strains, respectively. Interestingly, *S. enterica* LT2 lacks the *csc* genes, while the *dsd* gene cluster is located in the equivalent of the 83-min region of *E. coli* K-12. The *dsd* and the *csc* genes thus do not belong to the set of housekeeping or backbone genes but rather belong to the group of optional genes that are highly mobile among the enteric bacteria.

We speculate that the very low efficiency of the *csc* genes and the corresponding permease CscB and metabolic enzymes indicate a relatively recent acquisition at around the time when the different strains of the enteric bacteria diverged. Therefore, the characterization of mutations which optimize these genes and enzymes in the new host should reveal mechanisms involved in its evolution. The analysis of suppressor mutants with improved growth rates corroborates the hypothesis that the sucrose transport in EC3132 could be the rate-limiting step for the induction of the *csc* genes. This means that the intracellular inducer concentration in the presence of 0.2% sucrose never reaches a threshold value, which would be necessary for the effective induction of the *csc* genes. Support for this idea comes from the fact that (as for ECH1 or pJBL126) the majority of adaptational mutations we have analyzed thus far were found in *cscR* (more than 60% [our unpublished results]). The two other classes of adaptational mutations seem to increase either the basal expression level of *cscB* (caused by an operator mutation in pKJL124) or the CscB activity (in ECB1), which should lead to a situation in which even at low sucrose concentrations in the medium the threshold value for induction could already be achieved.

Direct proof for the *csc* operators is still missing. However, several findings argue that the perfect palindrome sequences highlighted in Fig. 2 constitute the *csc* operators. CscR, like other repressors of the LacI-GalR family (42), possesses an *N*-terminal helix-turn-helix motif that is responsible for the interaction with the DNA. Essential for the specific recognition of the cognate operator is the second or "recognition" helix that crosses the major groove of the DNA almost parallel to the base pairs (9). By direct modifications of the operator sequence or direct amino acid exchange in the recognition helix of the *lac* repressor, rules for the interaction of specific amino acid residues with individual bases of the operator were defined (17). According to these rules arginine 20 in the recognition helix of CscR would be specific for a guanine residue in position 6 of the cognate operator, whereas methionine 16 could interact only with a thymine residue in position 4 of the operator. Both nucleotides are present in the putative *csc* operator sequences ($G_6T_5T_4A_3A_2C_1/G'_1T'_2T'_3A'_4A'_5C'_6$; numbering of the bases according to the *lac* operator). These findings confirm the rules of the DNA-protein interaction set up for this regulator family. Furthermore, the mutation found in pKJL124 is exactly located in the palindrome in front of the *cscBK* operon. This should cause a reduction in the affinity of CscR for the operator and therefore an increase of the basal expression level of *cscB*, which would result in an increase in the concentration of the intracellular molecular inducer. Sahin-Tòth et al. (32) reported the cloning and sequencing of *cscA* and the *cscA*/*cscKB* promoter-operator region from the sucrose-positive *E. coli* wild-type strain B-62. The published sequence (gb|AF084030) is almost identical to the sequence of EC3132 except that the equivalent *cscKB* operator exhibits a C'_6-to-T'_6 transition in the right half of the palindrome. A doubling time of 48 min on 0.2% sucrose was measured for this strain (39). Therefore, it is tempting to speculate that B-62 has already acquired an adaptation mutation that enables the cells to grow fast on relatively low sucrose concentrations. Finally, the fact that this particular palindrome was found twice in the *csc* promoter region might indicate a cooperative regulatory effect antagonistically to the two cAMP-CrpA activator binding sites located between the two operons. Further experiments are currently under way to characterize the interaction between CscR and its operators.

An increase of the transport activity of the sucrose permease (as in ECB1) should also lead to an improvement of the induction characteristics under relatively low sucrose concentrations. CscB belongs to cluster 5 of the MFS (19). In many aspects the sucrose permease shares properties with the permeases of the MFS, in particular with LacY. There are, however, some characteristic differences between CscB and the lactose permease. The transport activity of the wild-type sucrose permease, for example, is for unknown reasons about 10-fold lower than that of LacY (6, 31). A further difference between CscB and other permeases of this family is CscB's unusual substrate specificity. Only sucrose (31) and palatinose (6-*O*-α-D-glucopyranosyl-D-fructofuranose) (our unpublished results) were identified as substrates, as no detectable transport of lactose, melibiose, or other galactosides was observed even at high substrate concentrations (≥10 mM). For LacY a detailed secondary structure model has been proposed (16). On the basis of primary sequence alignments of CscB and LacY, a topological model for the sucrose permease with 12

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transmembrane helices was proposed and further tested (6, 33, 34; our unpublished data). CscB possesses five out of six highly conserved intramembrane charged residues, which seem to be involved in ligand binding and transport, and three out of four putative intramembrane charge pairs. This indicates that the relative positions of the transmembrane helices and therefore the overall topologies of these permeases are identical. The missing charge pair in respect to LacY (Asp237 in helix VII and Lys358 in helix XI of LacY) was introduced into the wild-type sucrose permease by site-directed mutagenesis of Asn234 (helix VII) and Ser356 (helix XI) (10). Whereas individual replacements of these residues abolished active sucrose transport, simultaneous replacement of Asn234 with Asp or Glu and replacement of Ser356 with Arg or Lys resulted in high activity. Interestingly, when expressed at a low rate, the CscB double mutant Asn234Asp-Ser356Arg was present in the membrane in a significantly greater amount than the wild type (10), which indicates that the import of CscB into the membrane and therefore its activity can be improved. For this reason it is tempting to speculate that the replacement of the Gln353 residue in helix XI by a His residue, which is in close proximity to Ser356, in the fast-growing derivative ECB1 might also cause an enhanced protein import into the membrane and/or an improved protein stability, which would explain the observed increased transport activity. Alternatively, one could speculate that the His353 residue might directly improve sucrose binding properties in the mutant protein of strain ECB1. Further experiments are necessary to test these hypotheses. Interestingly, the G+C content of the *cscB* gene was only 43.6%, whereas the G+C content for the other *csc* genes was 52%, which is in the range typical for *E. coli*. This could mean that *cscB* might have been the last gene that has become part of the *csc* regulon and that especially the sucrose transport is still in the process of adaptation.

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