The Trehalose Phosphotransferase System (PTS) in *E. coli* W Can Transport Low Levels of Sucrose that Are Sufficient to Facilitate Induction of the csc Sucrose Catabolism Operon

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**Abstract**

Plasticity in substrate acceptance is a well-characterised phenomenon for disaccharide transporters. Sucrose, a non-reducing disaccharide, is usually metabolised via either the permease-mediated chromosomally-encoded sucrose catabolism (csc) regulon or the sucrose phosphotransferase system (PTS). *E. coli* W is a fast-growing strain which efficiently utilises sucrose at concentrations above 1% via the csc regulon. To examine if sucrose could be metabolised via other routes, a library of transposon mutants was generated and screened on 0.2% sucrose. One mutant identified from this library had an insertion in the repressor for the regulon controlling catabolism of the disaccharide trehalose (treR). A series of mutants was constructed to elucidate the mechanism of sucrose utilization in the treR insertion strain. Analysis of these mutants provided evidence that deletion of treR enables uptake of sucrose via TreB, an enzyme II protein required for PTS-mediated uptake of trehalose. Once inside the cell, this sucrose is not processed by the TreC hydrolase, nor is it sufficient for growth of the strain. QRT-PCR analysis showed that levels of *cscA* (invertase) transcript increased in the WΔtreR mutant relative to the wild-type strain when grown under low sucrose conditions. This result suggests that the intracellular sucrose provided by TreB can facilitate de-repression of the csc regulon, leading to increased gene expression, sucrose uptake and sucrose utilization in the treR mutant.

**Introduction**

Sucrose shows strong potential as an industrial feedstock for *E. coli*-based bioprocesses [1–3]; in addition, sucrose utilisation is prevalent in pathogenic *E. coli* strains [4,5]. Approximately 50% of wild-type *Escherichia coli* strains can metabolize sucrose [6] and in clinical isolates of enteropathogenic *E. coli* (EPEC) this can rise to 90% [4,5]. Two mechanisms for sucrose metabolism have been described in strains of *E. coli*: the sucrose phosphotransferase system (PTS) [7–9] and the chromosomally-located sucrose catabolose (csc) regulon. The csc regulon was originally identified in *E. coli* EC3132 [1,10,11] but it has also been identified in the genomes of enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC) [12–15] and the non-pathogenic strain W [1,16].

In the csc system, sucrose is imported into the cell by the permease, CscB [17]. This gene is essential for sucrose utilisation, and no other gene can provide sufficient levels of sucrose transport for growth on sucrose in a ΔcscB knockout [18]. Once in the cell, the sucrose is hydrolysed into fructose and glucose by the essential invertase, CscA [19]. The cscA gene is also essential for sucrose utilisation [18]. A fructokinase (CscK) phosphorylates the fructose moiety [11]. Sucrose utilization is tightly regulated, requiring both the absence of glucose and the presence of sucrose to allow for full induction of the regulon. In the presence of glucose, the regulon is under catabolite repression [20–22]. Operons under catabolite repression are activated when a complex of cyclic AMP (cAMP) and cAMP receptor protein A (CrpA) bind to the promoter [10]. The formation of this complex only occurs in the absence of glucose. Even in the absence of glucose, often these operons remain poorly expressed until such time as the cognate repressor is de-repressed via the action of an inducer. Repression of the divergently expressed *cscA* and *cscB* operons by the regulator protein CscR is thought to occur via the *cscA* and *cscB* operator binding sites located within the intergenic region between the two operons [10]. Under typical conditions, induction of the regulon occurs when the repressor binds to an inducer, blocking access to the operator binding site. The inducer of the csc regulon has not yet been experimentally determined; however, based on similar systems [23–25] it is likely to be either the sucrose or a product of the metabolism of sucrose (glucose/fructose/other). Unusually, the csc regulon requires relatively high (>1%) sucrose concentrations for de-repression [2,11,16]. Deletion/truncation of the *cscR* gene results in de-repression of the regulon, allowing sucrose utilisation.
at low concentrations [2,11,16]. Mutations in cscB also confer growth on low sucrose concentrations [26], presumably via increased transport rates and subsequent increased in intracellular sucrose resulting in induction of the operon.

Although the csc regulon is highly conserved, notable variation in the sucrose utilisation phenotype has been observed, especially among EPEC strains [5]. For example, strains from serotypes O157:H7 and O55:H7 have been described as “slow” sucrose fermenters as they produce only light pink colonies following growth on MacConkey agar supplemented with 1% sucrose [5]. Furthermore, when laboratory strains are engineered to use sucrose by over-expressing csc genes, widely varying growth rates (from doubling times of many hours to growth rates similar to those observed on glucose) are observed [10,11,27–30]. Large variations in growth rate upon introduction of the same genes imply a strain-specific limitation in either sucrose utilisation or induction, and suggest that genes outside of the csc regulon contribute to the efficiency of sucrose utilisation/induction.

To examine this possibility, we used E. coli W, a non-pathogenic strain that utilises sucrose via the csc regulon and does not have a sucrose PTS [1], as an experimental system. Although E. coli W is capable of growing very quickly on sucrose [31], sucrose metabolism is repressed at concentrations below ~1% [2,16]. A transposon mutagenesis library was developed in this strain and screened on low (0.2%) sucrose.

Several strains with altered sucrose utilisation phenotypes were identified; one of these had an insertion in the trehalose repressor protein, TreR. Using targeted mutagenesis and qRT-PCR, we examined the mechanism of sucrose utilization in the WAtreR mutant. Our data suggest that sucrose can be transported by the trehalose PTS transporter, TreB. The transported sucrose is insufficient for cell growth, but can facilitate induction of the csc operon and subsequent growth via cscB-mediated sucrose uptake.

To our knowledge, this is the first time that TreB has been shown to capable of sucrose transport.

Materials and Methods

Bacterial strains, media and growth conditions

Bacterial strains and plasmids are shown in Table 1. E. coli W (NCIMB 8666) was purchased from the National Collection of Industrial, Food and Marine Bacteria (NCIMB) (Aberdeen, UK). For general cloning and maintenance, E. coli strains were grown in LB medium [32]. For sugar utilization experiments, M9 minimal medium [32] supplemented with thiamine (1 mg/L) was used and supplemented with either 2 g/L sucrose (M9S2), 20 g/L sucrose (M9S20), or 1% w/v glycerol (M9Gly). Ampicillin (100 μg/mL), kanamycin (50 μg/mL), and/or chloramphenicol (25 μg/mL) were included in media where appropriate.

General molecular biology

General molecular biology techniques were performed according to standard protocols [32]. Genomic DNA extraction was performed using either the Ultraclean® Microbial DNA Isolation kit (MO BIO Laboratories, Inc, CA) or the RBC Genomic Extraction kit (RBC bioscience, Taiwan) as per the manufacturers’ instructions. Oligonucleotide primers used in this study are shown in Table 2.

Generation and screening of Tn5 mutants with altered sucrose utilization phenotypes

A library of ~10 000 random E. coli W mutants was generated using the EZ-Tn5™ <KAN-2> Tnp Transposome™ kit (Epigenome Biotechnologies, WI) as per the manufacturer’s instructions to generate transformants containing only one Tn5 insertion. Following transformation, cells were immediately plated onto LB agar containing kanamycin. The library was screened by patching onto M9S2, M9S20 and MacConkey (1% sucrose) plates to identify sucrose utilization mutants. Tn5 insertion sites were identified by direct Sanger sequencing of purified genomic DNA as described previously [33] using primer JSP07 (Table 2) and analyzed by Macromon Sequencing Facility (Clayton, VIC, Australia). To identify nucleotide changes within the csc regulon, overlapping PCR products were generated, purified using MinElute PCR purification kit (QIAGen, Doncaster, VIC, Australia) and subsequently sequenced by the Australian Genome Research Facility (Brisbane, QLD Australia) using the Sanger method.

Site directed chromosomal gene knock-out

Chromosomal gene knock-out was performed using one-step homologous recombination [34], with minor modifications as described previously [18,35,36]. Primers used to amplify gene knock-out constructs using pKD3 [34] as template are shown in Table 2. The cscB gene is in the same operon as, and immediately downstream from, cscK; for this reason, particular care was taken in the primer design to ensure that cscK would not be disrupted. Transformants were selected on chloramphenicol and deletion of the target gene(s) was confirmed by PCR amplification across the knock-out locus and subsequent Sanger sequencing of the PCR product (see Table 2 for details of primers used). Where appropriate, the chloramphenicol resistance marker was removed by Flp recombinase as described previously [34,37]. Precise excision of the chloramphenicol resistance gene was also confirmed by PCR and sequencing. The resulting knock-out strains are listed in Table 1.

Growth rate analysis

Growth rates were analyzed in a 96-well microtitre plate format as described previously [28] except that cultures were plated onto M9S20 agar and then M9S2 agar prior to pre-culture in M9S2. Growth rates of transposon and knock-out mutants were compared with the wild type E. coli W strain and the cscR knock-out (WAcscR) as controls.

qRT-PCR

Mutant and wild-type strains were cultured in M9 minimal media containing 1% glycerol with either 2% or 0.2% sucrose in shake flasks. At mid-log (OD600~0.4) 10 ml of culture was harvested and immediately resuspended in 1 ml of TRI regent (Invitrogen). Each sample was heated at 70°C for 15 min before storage at ~20°C. TRI extraction, DNaseI treatment (Promega) and RNEasy mini column (QIAGen) clean up were performed as per the manufacturer’s instructions. PCR was used to confirm complete removal of contaminating genomic DNA from the RNA samples. cDNA was amplified from 600 ng total RNA using Superscript III (Invitrogen) as per the manufacturer’s instructions with the following modifications: 50 ng of random hexamer (Invitrogen) was used and the reaction incubated at 50°C for 2.5 hr. An RT- control was performed alongside. RT+ and RT− template was diluted 1/200 with water before use. qRT-PCR was performed in a Corbett Rotor-Gene 3000 using Sybr green UDG mastermix (Invitrogen). Reactions were performed in 15 ul, containing 160 nM each oligo (Table 2) and 2 ul of template. Each assay included (in duplicate): a standard curve generated with genomic DNA (gDNA) for each gene, and triplicate RT+ and duplicate RT− technical replicates for each of three biological replicates. Standard cycling conditions were used and melt curve
Table 1. *E. coli* strains and plasmids used in this study.

| Strain | Relevant genotype or phenotype characteristics | Source |
|--------|-----------------------------------------------|--------|
| W      | Wild-type csc+                                | NCIMB  |
| WΔcscR | WΔcscR::FRT                                  | [2]    |
| Tn2    | W oppC::Tn5 (KanR)                           | This study |
| Tn9    | W cyoA::Tn5 (KanR)                           | This study |
| Tn32   | W mhpR::Tn5 and cscB L363F mutation (KanR)   | This study |
| Tn33   | W mutS::Tn5 (KanR)                           | This study |
| Tn34   | W with unknown Tn5 insertion site and cscB L51F mutation (KanR) | This study |
| Tn51   | W with unknown Tn5 insertion site and cscB L363F mutation (KanR) | This study |
| Tn52   | W with unknown Tn5 insertion site and cscB F371V mutation KanR | This study |
| Tn53   | W with unknown Tn5 insertion site and cscR Q57* mutation (KanR) | This study |
| Tn54   | W pi349::Tn5 (KanR)                          | This study |
| Tn56   | W with unknown Tn5 insertion site and cscB L51F mutation (KanR) | This study |
| Tn57   | W with unknown Tn5 insertion site and cscB C327G mutation (KanR) | This study |
| Tn58   | W with unknown Tn5 insertion site and cscB L51F mutation (KanR) | This study |
| Tn61   | W treR::Tn5 (KanR)                           | This study |
| Tn63   | W gspK::Tn5 (KanR)                           | This study |
| Tn65   | W gltS::Tn5 (KanR)                           | This study |
| Tn68   | W hyp::Tn5 (KanR)                            | This study |
| Tn69   | W with Tn5 insertion mutations and cscB operator binding site mutation (KanR) | This study |
| Tn84   | W truA::Tn5 (KanR)                           | This study |
| Tn86   | W with Tn5 insertion mutations and cscB L363F mutation (KanR) | This study |
| Tn87   | W treR::Tn5 (KanR)                           | This study |
| WΔgltS 1 | WΔgltS::FRT                                 | This study |
| WΔgltS 2 | WΔgltS::FRT                                 | This study |
| WΔgltS 3 | WΔgltS::FRT                                 | This study |
| WΔgspK 1 | WΔgspK::FRT                                 | This study |
| WΔgspK 2 | WΔgspK::FRT                                 | This study |
| WΔgspK 3 | WΔgspK::FRT                                 | This study |
| WΔmphR 1 | WΔmphR::FRT                                 | This study |
| WΔmphR 2 | WΔmphR::FRT                                 | This study |
| WΔmphR 3 | WΔmphR::FRT                                 | This study |
| WΔtreR 1 | WΔtreR::FRT                                 | This study |
| WΔtreR 2 | WΔtreR::FRT                                 | This study |
| WΔtreR 3 | WΔtreR::FRT                                 | This study |
| WΔtreRΔtreB 1 | WΔtreR::FRT ΔtreB::FRT-cat::FRT, Parent: WΔtreR 1 | This study |
| WΔtreRΔtreB 2 | WΔtreR::FRT ΔtreB::FRT-cat::FRT, Parent: WΔtreR 2 | This study |
| WΔtreRΔtreB 3 | WΔtreR::FRT ΔtreB::FRT-cat::FRT, Parent: WΔtreR 3 | This study |
| WΔtreRΔtreC 1 | WΔtreR::FRT ΔtreC::FRT-cat::FRT, Parent: WΔtreR 1 | This study |
| WΔtreRΔtreC 2 | WΔtreR::FRT ΔtreC::FRT-cat::FRT, Parent: WΔtreR 2 | This study |
| WΔtreRΔtreC 3 | WΔtreR::FRT ΔtreC::FRT-cat::FRT, Parent: WΔtreR 3 | This study |
| WΔtreRΔcscA 1 | WΔtreR::FRT Δ cscA::FRT-cat::FRT, Parent: WΔtreR 1 | This study |
| WΔtreRΔcscA 2 | WΔtreR::FRT ΔcscA::FRT-cat::FRT, Parent: WΔtreR 2 | This study |
| WΔtreRΔcscA 3 | WΔtreR::FRT ΔcscA::FRT-cat::FRT, Parent: WΔtreR 3 | This study |
| WΔtreRΔcscB 1 | WΔtreR::FRT ΔcscB::FRT-cat::FRT, Parent: WΔtreR 1 | This study |
| WΔtreRΔcscB 2 | WΔtreR::FRT ΔcscB::FRT-cat::FRT, Parent: WΔtreR 2 | This study |
| WΔtreRΔcscB 3 | WΔtreR::FRT ΔcscB::FRT-cat::FRT, Parent: WΔtreR 3 | This study |

*csc<sup>+</sup>*: positive growth on low sucrose (2 g/l).

Amp<sup>R</sup>, ampicillin resistance (100 μg/ml).

Kan<sup>R</sup>, kanamycin resistance (50 μg/ml).

Ch<sup>R</sup>, chloramphenicol resistance (40 μg/ml).

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| Name       | Sequence (5’ – 3’)                          | Application          |
|------------|--------------------------------------------|----------------------|
| JSP07      | ACCTACAACAAAGCTCTATCAACAGCTGCC            | PCR/Sequencing       |
| cscA F2    | ATGTCAGCAAAAGCTCTATCAACAGCTGCC            | PCR/Sequencing       |
| cscA R2    | TTAACCCAGTACCCACGAGCTGCC                 | PCR/Sequencing       |
| cscA R3    | CAGATCAGCCGGGTATTATGATGCGATC             | PCR/Sequencing       |
| cscB F2    | ATGGCCTCTATGACCTTACGAGCTGCC             | PCR/Sequencing       |
| cscB R2    | CTATATGCGAGTACCCACGAGCTGCC              | PCR/Sequencing       |
| cscB R3    | GACTCCCTTGATAGCTGCCAAGCTGCC             | PCR/Sequencing       |
| cscF1      | GCCCGTATTCTACAGAGCTGCC                   | PCR/Sequencing       |
| cscF R1    | TTCCGTTACTGACAAAGCTGCC                   | PCR/Sequencing       |
| cscR F3    | CAGCTCCATACGTCATATTACGTC                | PCR/Sequencing       |
| cscR F2    | ATGGCACTGAATATTCCATTCAGA                 | PCR/Sequencing       |
| cscR R2    | CTTATTTGCTGAAGGTACAGGCGTC               | PCR/Sequencing       |
| cscR R3    | TGAGTACCGAGTACCCACGAGCTGCC              | PCR/Sequencing       |
| cscR F1    | GCCTTAACTTACTCAGACAGCTGCC              | PCR/Sequencing       |
| csc R2     | CTTACAGCTCCAGACAGCTGCC                   | PCR/Sequencing       |
| csc R3     | ATGGCATATAGCTGCCAAGCTGCC                 | PCR/Sequencing       |
| csc K1     | TTACCAACAAAGCTCTATCAACAGCTGCC           | PCR/Sequencing       |
| csc K2     | TTAACCCAGTACCCACGAGCTGCC                 | PCR/Sequencing       |
| csc K3     | CAGATCAGCCGGGTATTATGATGCGATC             | PCR/Sequencing       |
| csc R4     | CTATATGCGAGTACCCACGAGCTGCC              | PCR/Sequencing       |
| csc R5     | GACTCCCTTGATAGCTGCCAAGCTGCC             | PCR/Sequencing       |
| csc R6     | GCCCGTATTCTACAGAGCTGCC                   | PCR/Sequencing       |
| csc R7     | TTCCGTTACTGACAAAGCTGCC                   | PCR/Sequencing       |
| csc R8     | CAGCTCCATACGTCATATTACGTC                | PCR/Sequencing       |
| csc R9     | ATGGCACTGAATATTCCATTCAGA                 | PCR/Sequencing       |

* Used for the direct sequencing of Tn5 insertion sites from purified genomic DNA.

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analysis was performed, confirming only a single product was produced. A standard curve for each gene was determined and the efficiency of the reaction noted. C_{T} values were determined using the default values on the Rotor Gene-6 software. A correction for gDNA contamination was performed however the levels of gDNA detected were insignificant. The log of the relative abundance value (log R) for each sample was calculated using the equation 
\[ \log R = -\log A \cdot C_{T} + \log A_{R} \cdot C_{T_{R}} \] 
where A is the amplification efficiency and C_{T} is the cycle threshold. The reference gene was dld (D-lactate dehydrogenase). To determine statistical significance between log R values for each gene and each mutant, a one-way ANOVA was performed, followed by a Tukey’s Honest Significant Difference post hoc test.

Results and Discussion

Sucrose is an important industrial feedstock for bioprocesses [1,2] and sucrose utilisation is widespread in pathogenic E. coli strains [5]. However, significant variation in growth rates on sucrose have been observed between wild-type strains [5] and laboratory strains modified to express the csc regulon from EC3132 typically grow slowly when compared to the WT EC3132 strain [11,27,28]. These observations suggest that non-csc genes might contribute to the sucrose utilisation and/or induction phenotype in E. coli. To investigate this, we used E. coli W, a non-pathogenic wilt type (WT) strain which uses a csc regulon to confer sucrose utilisation [1]. Although E. coli W grows as quickly on sucrose, induction of the csc regulon requires concentrations ≥1% of sucrose [2,16].

Generation and screening of the mutant library

A Tn5 transposon mutagenesis library was constructed and screened for growth on minimal medium supplemented with either 2% or 0.2% sucrose. From the library of 10,000 kanamycin resistant mutants, 32 mutants with altered sucrose fermentation patterns (improved utilisation at low sucrose, or lack of sucrose utilisation) were identified. All except one of the strains that could not utilize sucrose had insertions in the csc operon (four in cscB and three in cscA). This supports our earlier studies showing that these two genes are essential for sucrose utilisation in wild type E. coli [16]. Previous research has demonstrated that truncation or deletion of the cscR gene can confer de-repression at low sucrose [2,11,16]; consistent with this, four mutants that could utilize sucrose at 0.2% had insertions in cscR. These csc mutants were excluded from further studies.

Improved sucrose fermentation can also be attributed to spontaneous mutation events within the csc regulon [10,11]. Frequently, these mutations occur within the repressor or the repressor binding site [10], and lead to de-repression of the regulon, allowing growth on low concentrations of sucrose. To exclude this as a possible reason for improved sucrose utilization, the csc regulons of the remaining strains were sequenced. A further ten Tn5 strains were found to contain spontaneous point mutations in the csc regulon. Eight of these were in cscB, one in a putative operator binding site within the csc promoter region (cscKDBP), and one in cscR (Q132*) that resulted in truncation of the repressor protein. The mutation in the cscKDBP has been observed previously and was shown to confer growth on low sucrose [10,11]. Interestingly, nearly all of the mutations identified in this study were located within cscB despite the fact that only one CscB mutation (Q353H) that allows for growth on low sucrose has been characterized previously [10]. Mutations in cscB which improve sucrose transport [10,11,38] can also relieve repression, presumably as a consequence of increased concentrations of intracellular sucrose (or fructose and/or glucose) which can then facilitate de-repression. All of these csc gene mutants were excluded from further study.

Deletion of the trehalose repressor gene treR permits growth on low sucrose

Once the csc mutants were removed from the study, the Tn5 insertion sites within the 11 remaining Tn5 mutants were identified using direct Sanger sequencing of purified genomic DNA (Table 3). The single mutant that was unable to utilize sucrose contained a Tn5 insertion within cysA (adenylate cyclase), a gene which is essential for cyclic AMP (cAMP) production. Two cAMP-CrpA binding sites are found in the bi-directional csc promoter [18], and regulation of both the csa and cscB transcription units by cAMP has been demonstrated previously [10]. In the absence of cAMP, activation of the csc regulon by cAMP-CrpA cannot occur; this most likely provides an explanation for why this strain cannot grow on sucrose. Of the remaining mutants, Tn5 insertions in four mutants were found to disrupt genes associated with DNA replication, recombination and repair. Disruption of these genes may lead to an increased potential for accumulation of mutations elsewhere in the chromosome that we could not identify; for this reason, these strains were not examined further.

Transposon insertions were also found within two transcriptional regulators (two isolates with insertions in treR and one with an insertion in mhpR) as well as the membrane bound transporters gbuS and gbkA. To investigate the role of these genes, three independently isolated site-directed deletion mutants of each of the four targets were constructed in the wild type W strain using homologous recombination. Of the genes targeted, only the treR mutants were able to grow on 0.2% sucrose. The growth rate of the WΔtreR mutant on 0.2% sucrose was not statistically different from that of the original mutant strains (Tn61 and Tn67; p>0.05; Table 4), suggesting that deletion of treR is responsible for the phenotype. The lack of growth in the other deletion mutants suggests that the original phenotype observed was not due to the transposon insertion. It is possible that those mutants carry additional mutations which confer the ability to grow on 0.2% sucrose; the frequency of spontaneous mutations observed in the csc regulon even in the absence of selection pressure supports this hypothesis.

The trehalose-specific enzyme II (TreB) is required for growth of WΔtreR on low sucrose

In E. coli, uptake of trehalose occurs via the phosphoenolpyruvate (PEP)carbohydrate phosphotransferase system (PTS) [39,40]. In this system the trehalose-specific enzyme EIICBTreB (TreB) transports trehalose into the cell with concomitant phosphorylation to deliver trehalose-6-phosphate [41] (see Figure 1A). EIICBTreB does not have a covalently-bound EIIA domain for phosphorylation of the transported sugar; like other non-glucose PTS transporters in the EIIBC domain type, it relies on kinase activity provided by EIIA^Acr, a component of the primary glucose transport system [41–43]. The transported trehalose-6-phosphate is hydrolyzed by TreC to produce glucose and glucose-6-phosphate [39,44] which are directed into central carbon metabolism. Expression of treB and treC is activated in the absence of glucose and repressed by TreR in the absence of trehalose-6-phosphate [40].

Substrate plasticity is common in disaccharide transporters [45–50]. Functional analysis of the trehalose PTS has shown that maltose can enter the cell via the TreB transporter [50] (see
Figure 1B: Trehalose is a glucose α(1→1) disaccharide, and maltose is a glucose α(1→4) disaccharide. Interestingly, maltose is transported by facilitated diffusion, and enters the cell in an unphosphorylated state [50]. The substrate flexibility of TreB led us to hypothesize that it might also be able to transport sucrose, a glucose-fructose disaccharide, and that deletion of the treR repressor might allow production of TreB at sufficient levels to transport sucrose into the cell to allow for cell growth.

To determine if deletion of the treR gene allows for the uptake of sucrose via TreB, a double WΔtreRΔtreC deletion mutant was constructed and tested for growth on 0.2% sucrose. In support of our hypothesis, this mutant was unable to grow on sucrose (Table 4), suggesting that sucrose uptake via TreB is essential for the growth of WΔtreR on 0.2% sucrose.

The trehalose hydrolase TreC is not required for growth of WΔtreR on low sucrose
Maltose is thought to be transported by TreB in an unphosphorylated form via facilitated diffusion [50]. Maltose is not recognised by the TreC trehalose hydrolase [44], however, transport via TreB allows induction of the maltose ABC transporter [50] (see Figure 1B). To determine if hydrolysis by TreC is required for low sucrose utilisation in WΔtreR, a double WΔtreRΔtreC deletion mutant was also constructed. Growth of WΔtreRΔtreC on 0.2% sucrose (Table 4) was indistinguishable from the WΔtreR strain (p≥0.05), indicating that, like for maltose, processing of the incoming sucrose by TreC is not required for growth on low concentrations of sucrose. This is consistent with transport studies showing that TreC does not recognise unphosphorylated sucrose [44]; however, there is no information

### Table 3. Tn5 insertion sites in selected non-csc insertion/mutation strains.

| Strain | Genotype | Suc | Comment | COG group description | COG |
|--------|----------|-----|---------|-----------------------|-----|
| Tn2    | W appC::Tn5 | +   | Cytochrome bd-II terminal oxidase subunit | Energy production and conversion | C  |
| Tn9    | W cyoA::Tn5 | –   | Adenylate cyclase, family 3 | Signal transduction | T  |
| Tn32   | W mhpR::Tn5, cscB L363F | +   | Mhp operon transcriptional activator | Transcription | K  |
| Tn33   | W mutS::Tn5 | +   | Methyl-directed DNA mismatch repair protein | DNA replication, recombination and repair | L  |
| Tn54   | W pi349::Tn5 | +   | Phage recombinase | DNA replication, recombination and repair | L  |
| Tn61   | W treB::Tn5 | +   | HTH-type transcriptional repressor, trehalose PTS uptake operon | Transcription | K  |
| Tn63   | W gspK::Tn5 | +   | General secretion pathway protein K | Cell motility and secretion | N  |
| Tn65   | W glyE::Tn5 | +   | Sodium/glutamate symport carrier protein | Amino acid transport and metabolism | E  |
| Tn68   | W hyp::Tn5 | +   | Conserved hypothetical protein | Unknown | S  |
| Tn84   | W truA::Tn5 | +   | Trna pseudouridine synthase | Translation, ribosomal structure and biogenesis | J  |
| Tn87   | W treC::Tn5 | +   | HTH-type transcriptional regulator, trehalose PTS uptake operon | Transcription | K  |

Strains were grown on M9S2 and M9S20. Sucrose phenotype (Suc) is listed as positive if the strain can grow on low sucrose (0.2%) and negative if it cannot grow on sucrose at all. COG (Clusters of Orthologous Genes) groupings are listed.

### Table 4. Summary of the growth characteristics of the WΔtreR double mutants generated in this study.

| glucose 1% | sucrose 2% | sucrose 0.2% |
|------------|------------|--------------|
| phenotype  | phenotype  | \(\mu (h^{-1})\) ± SD | \(p\) value | phenotype  | \(\mu (h^{-1})\) ± SD | \(p\) value |
| W          | +++        | 1.36±0.12    | -           | -          | -           | n/a          |
| WΔcscB     | +++        | 1.42±0.06    | Ns          | ++         | 0.65±0.01   | **           |
| Tn61       | +++        | 1.01±0.04    | Ns          | ++         | 0.19±0.03   | ns           |
| Tn87       | +++        | 1.05±0.13    | Ns          | ++         | 0.28±0.03   | ns           |
| WΔtreR     | +++        | 0.99±0.16    | Ns          | ++         | 0.35±0.02   | -            |
| WΔtreRΔtreB| +++        | 1.18±0.33    | Ns          | -          | -           | n/a          |
| WΔtreRΔtreC| +++        | 1.29±0.21    | Ns          | ++         | 0.42±0.04   | ns           |
| WΔtreRΔcscB| +++        | -            | n/a         | ND         | -           | n/a          |
| WΔtreRΔcscA| +++        | -            | n/a         | ND         | -           | n/a          |

Strains plated on M9 minimal media supplemented with various carbon sources as indicated in the table. Phenotype, +++ fast growth (sizable colonies in 15 h); ++, growth (small colonies after 15 h); –, no growth after 24 h; ND, not determined. Statistical significance was determined using the Kruskal-Wallis test combined with Dunn’s Multiple Comparison test. \(p\) value, comparison between each sample and W; \(p\) value, comparison between each sample and WΔtreR.

**NS, p value<0.01;
ns, not significant; n/a, not applicable.

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available in the literature for transport or otherwise of phosphor-
ylated sucrose (see comments below).

cscB is required for the growth of W
D
WtreR on low sucrose
Based on the results from the W
D
DtreR mutant, growth on
low concentrations of sucrose in the presence of a
treR mutation relies on uptake of sucrose
via TreB. To determine if sucrose
uptake via TreB is sufficient to support growth in a W
D
treR background, a double W
D
treR DcscB deletion mutant was con-
structed and tested for growth on 0.2% and 2% sucrose.
W
D
treR DcscB was unable to grow on either concentration
(Table 4), demonstrating that
cscB is also required for growth in
W
D
treR mutants, and that the uptake of sucrose
via TreB alone is insufficient for growth.

Sucrose uptake via TreB de-represses the csc operon
allowing for growth on low concentrations of sucrose
Based on the results from the mutagenesis studies, growth of the
W
D
DtreR mutant on low sucrose relies on the uptake of sucrose via
TreB to induce the csc regulon. We hypothesised that sucrose
transported by TreB, while insufficient to support growth, can
trigger de-repression of the csc operon, allowing for uptake of sucrose via CscB and subsequent processing of the sucrose into
fructose and glucose by CscA. To confirm this hypothesis, QRT-
PCR was performed on W, W
D
DtreR, W
D
DtreR DcscB and
W
D
DtreR DacscB cultured on minimal media containing 1% glycerol
supplemented with 0.2% sucrose (Figure 2). Glycerol was included
to support the growth of strains that would otherwise not grow on

Figure 1. Models for disaccharide transport and utilisation by
EIICTre. In phosphoenolpyruvate:carbohydrate transport systems,
sugars (in this case, trehalose) are transported with concomitant
phosphorylation via a PTS-associated phosphorylation cascade (A). For
the trehalose PTS, the transporter protein EIIBC Tre consists of a
permease (EIIC Tre) and a kinase (EIIB Tre) domain. EIIB Tre accepts a
phosphate group from EIAGlc in the presence of trehalose transport.
EIIC Tre accepts a phosphate from HPr, which in turn accepts a
phosphate from the PEP-dependent histidine-protein kinase EI Glc,
which accepts its phosphate group from phosphoenolpyruvate.
Phosphorylated trehalose is cleaved by TreC to yield 1 × phosphory-
lated (G6P) and 1 × unphosphorylated glucose; both of these feed into
central carbon metabolism (CCM) (the unphosphorylated glucose is
phosphorylated by glucokinase, Glk). In the presence of PTS-mediated
glycogen transport, the concentration of unphosphorylated EIAGlc
increases; unphosphorylated EIAGlc transcriptionally inhibits non-PTS
permeases and catabolic enzymes that generate internal inducers of the
various catabolic regulons (a mechanism known as inducer exclusion)
via a variety of transcription factors [51]. The trehalose PTS has also
been shown to transport maltose [50] (B). In this case, transport is
thought to be achieved through facilitated diffusion by EIIC Tre and the
maltooligosaccharide not phosphorylated by EIIC Tre. In the absence of PTS-mediated
phosphorylation, carbon catabolite repression is released: the concen-
tration of unphosphorylated EIAGlc remains high; this activates adenylate
cyclase (AC), resulting in an increase in intracellular cAMP concen-
tration; cAMP complexes with the transcription factor CrpA; and cAMP-
mediated activation activates a wide variety of non-PTS transport
systems, including the ABC transporter for sucrose. Sucrose transport
by TreB may occur with or without concomitant phosphorylation,
but most likely occurs without phosphorylation (see text). Transport
results in sufficient intracellular sucrose to facilitate induction of the csc
regulon; phosphorylated EIAGlc remains high, and the csc regulon is
activated through cAMP-CrpA. Transferred unphosphorylated sucrose
may be metabolised via csc gene products; phosphorylated sucrose is
most likely not metabolised. Once the csc genes are induced, sucrose
can be imported through the CscB permease and cleaved by the CscA
invertase into fructose and sucrose. Fructose is phosphorylated by the
CscK fructokinase, and glucose is phosphorylated by Glk; both
phosphorylated sugars feed into CCM.
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Sucrose transport by TreB: phosphorylated or unphosphorylated?

A question that remains is whether sucrose is transported in a phosphorylated form, like trehalose [41,42] (Figure 1A), or unphosphorylated, as maltose is thought to be [50] (Figure 1B). One possible explanation for the lack of growth in the WΔtreRΔcscB mutant is that the sucrose is phosphorylated and cannot be metabolized (Figure 1C); this is plausible given that the W genome does not encode a sucrose-6-phosphate hydrolase [1]. The phosphorylated sucrose might still act as an inducer of the csc regulon, allowing transport of sucrose via CscB. Under this scenario, phosphorylated EIA^{Glc} remains at relatively high concentrations, since transport levels are low. Phosphorylated EIA^{Glc} activates adenylate cyclase, increasing cellular cAMP concentrations. An increase in cellular cAMP would allow release of catabolite repression via the cAMP-CrpA transcriptional activator, activating the csc regulon (which responds to cAMP [10]; see Figure 1C). Thus, cellular conditions would be permissive for transcription of the csc regulon. However, it is known that transport rates of EII enzymes that can carry out facilitated diffusion increase significantly when they are phosphorylated [51]. EII enzyme phosphorylation is linked to carbohydrate phosphorylation; consequently, one would expect transport rates to increase, and potentially be sufficient to support growth. This situation occurs in PTS-mediated sensing of carbon sources for PTs that can transport unphosphorylated carbohydrates, and provides a mechanism for rapid upregulation of transport in the presence of preferred substrates. Feedback of this cycle results in increased transport rates, more phosphorylation of EIB^{Tre}, and decreased concentrations phosphorylated EIA^{Glc}. In this case, this would ultimately result in repression of the csc regulon (see Figure 1A and 1C). Furthermore, the increase in unphosphorylated EIA^{Glc} is likely to result in transcriptional inhibition of the csc regulon (‘inducer exclusion’; [51]) (see Figure 1A). Clearly, however, this does not happen: the csc regulon is instead induced, and the requirement of CscB demonstrates that transport of sucrose by EIB^{Tre} remains at very low levels. Transport of phosphorylated sucrose is therefore only a likely mechanism if phosphorylation of EIB^{Tre} does not significantly enhance transport of sucrose.

Alternatively, sucrose might be transported in an unphosphorylated state (Figure 1C). Under this scenario, the unphosphorylated sucrose facilitates induction of the csc regulon and EIA^{Glc} remains high, allowing cAMP-CrpA mediated de-repression in concert with induction (Figure 1C). The sucrose is cleaved by CscA, and the resulting hexoses are phosphorylated by CscK/ Glk before feeding into central carbon metabolism. Transport of sucrose by EIB^{Tre} remains at very low rates and, in the absence of CscB, is insufficient to support growth. This scenario most easily explains the observed experimental data and is consistent with currently-understood models of PTS-mediated transport.

Conclusion

Direct demonstration of sucrose transport by TreB using available methods is technically challenging due to the very low levels of sucrose transported. However, the mutant analysis presented here provides compelling evidence that TreB can transport sucrose, albeit at very low concentrations. We also showed that the transported sucrose is sufficient to facilitate induction of the csc operon. Interestingly, a very similar association is shown between the trehalose transporter and maltose catabolism, controlled in this case by an ABC transporter [50]. TreB is also highly homologous to the EII^{Glc} of the sucrose-specific PTS encoded on the pUR400 plasmid of enteric bacteria [42], which is...
can also transport maltose and lactose [52]. Maltose porins can also transport sucrose and trehalose efficiently [53]. Furthermore, CscB in fact transports maltose at a higher affinity than sucrose [45]. These observations suggest that the substrate plasticity shared by the enzymes might be related to their structural similarity, and that the sucrose/trehalose/maltose might share common biochemical properties from a transport point of view. As is the case with direct demonstration of transport in the first instance, direct demonstration of what form the sucrose is transported in is technically challenging due to the low levels transported, and this question remains unanswered. Regardless, to our knowledge, this is the first time that TreB has been shown to transport sucrose.

**Author Contributions**

Conceived and designed the experiments: JAS CEV LKN. Performed the experiments: JAS NB. Analyzed the data: JAS CEV LKN. Wrote the paper: JAS CEV LKN.

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