Peptide transporter CstA imports pyruvate in Escherichia coli K-12

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Running title: CstA is a constitutive pyruvate importer

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ABSTRACT

Pyruvate is an important intermediate of central carbon metabolism and connects a variety of metabolic pathways in Escherichia coli. Although intracellular pyruvate concentration is dynamically altered and tightly balanced during cell growth, the pyruvate transport system remains unclear. Here, we identified a pyruvate transporter in E. coli using high-throughput transposon sequencing. The transposon mutant library (a total of $5 \times 10^5$ mutants) was serially grown with a toxic pyruvate analog (3FP) to enrich transposon mutants lacking pyruvate transport function. A total of 52 candidates were selected based upon the stringent enrichment level of transposon insertion frequency in response to 3FP treatment. Subsequently, their pyruvate transporter function was examined by conventional functional assays, such as growth inhibition by the toxic pyruvate analog and pyruvate uptake activity measurement. The pyruvate transporter system comprises of CstA and YbdD, which is known as peptide transporter and conserved protein functionally related to carbon starvation conditions. In addition to the presence of more than one endogenous pyruvate importer, it has been suggested that the E. coli genome encodes a constitutive and inducible pyruvate transporter. Our results demonstrated that CstA and YbdD comprise of the constitutive pyruvate transporter system in E. coli, which is consistent with the tentative genomic locus previously suggested and functional relationship with the extracellular pyruvate sensing system. This pyruvate transporter system provides valuable genetic information for understanding the complex process of pyruvate metabolism in E. coli.

KEYWORDS

Pyruvate, transporter, transposon sequencing, 3-fluoropyruvate, Escherichia coli
IMPORTANCE

Pyruvate is an important metabolite as a central node in bacterial metabolism, and its intracellular levels are tightly regulated to maintain its functional roles in highly interconnected metabolic pathways. However, our understanding of the mechanism of how bacterial cells excrete and transport pyruvate remains elusive. Using high-throughput transposon sequencing followed by pyruvate uptake activity test of the selected candidate genes, we found that a pyruvate transporter system comprising of CstA and YbdD, currently annotated as a peptide transporter and conserved protein, constitutively transports pyruvate. This physiological role of the pyruvate transporter system provides valuable genetic information for understanding complex pyruvate metabolism in *E. coli.*
INTRODUCTION

Pyruvate is an important branch point intermediate of many metabolic pathways in living organisms. In central carbon metabolism, pyruvate is the final product of glycolysis and enters the TCA cycle after conversion to acetyl-CoA by pyruvate dehydrogenase. Additionally, pyruvate metabolism is highly interconnected with other metabolic pathways including amino acid metabolism, fatty acid metabolism, and gluconeogenesis. When glucose is taken up at a high rate, intracellular pyruvate concentration increases, resulting in a high ratio of pyruvate to phosphoenolpyruvate in E. coli. This increased ratio activates the ArcA/B global two-component system, which participates in regulating a large number of genes in diverse biochemical reactions including repression of tricarboxylic acid cycle genes (1). As a central metabolic node, the intracellular pyruvate concentration is changed dynamically and must be tightly regulated (2). To control intracellular concentrations, pyruvate is also excreted into the media in response to the dynamic cell status (3-5). During the stationary phase, for example, excreted pyruvate can be transported into the cell by carbon scavenging metabolism involving cyclic AMP (cAMP)/cAMP receptor protein regulation (4, 6).

Although metabolic fluxes connected to the pyruvate node have been examined under numerous conditions, the pyruvate transport system in E. coli remains unclear. The presence of at least two uptake systems and one excretion system for pyruvate transport was suggested using the mutant strains that grow with toxic pyruvate analogs (7). Interestingly, one uptake system constitutively imports pyruvate, while the other shows pyruvate uptake activity only when induced by pyruvate. Double mutants lacking both pyruvate-inducible and constitutive pyruvate transporters excreted intracellular pyruvate into the media, indicating the existence of an independent pyruvate exporter. It has also been suggested that membrane protein YhjX function is related to changes in extracellular pyruvate concentration (3). However, its mode of transport
and substrate specificity remain unclear. Recently, BtsT (regulated by BtsS/BtsR pyruvate sensing system) was identified as an inducible pyruvate/H⁺ symporter in *E. coli* (8). Despite these efforts, other pyruvate transporter genes in *E. coli* are yet to be identified.

Identifying microbial transporter genes is challenging for several reasons. Sequence homology-based computational approaches are often used to narrow down the putative transporter genes, which are then investigated by functional assays (9, 10). However, predicting substrate specificity based on sequence homology is often limited because of the flexible and broad substrate specificity of transporters (11, 12). In addition, most transporters are integrated with the cellular membrane, limiting the ability to perform biochemical assays to examine transport activity. To overcome these limitations, transposon sequencing (Tn-seq) is a robust and high-throughput method for genome-scale screening of transporter genes from a large mutant library (13-16). In this study, we employed the Tn-seq approach to identify the pyruvate transporter gene in *E. coli*. We serially cultured the Tn mutant library in the presence and absence of a toxic pyruvate analog to enrich transporter-deficient clones, followed by high-throughput sequencing of Tn insertion genomic positions to determine the insertion frequency. The results identified 52 candidate genes and further functional examination of the corresponding deletion strains revealed a putative pyruvate transporter that constitutively imports pyruvate. Along with the valuable genetic information for understanding complex pyruvate metabolism in *E. coli*, our results provide a versatile pipeline for screening other transporter genes.

**RESULTS**

**Clonal selection method from Tn insertion mutant library**

To select clones deficient in pyruvate transporter function in the Tn insertion mutant library, we first examined the toxic effect of the pyruvate analog 3-fluoropyruvate (3FP) on cell growth. In
principle, 3FP is imported into cell through the pyruvate transporter and inhibits cell growth by binding to pyruvate dehydrogenase (7, 17-19). As expected, 3FP inhibited wild-type E. coli K-12 cell growth under pyruvate-induced and non-induced conditions (Fig. 1A). Additionally, the formation of wild-type colonies on M9 sorbitol medium was extremely retarded in the presence of 1 mM 3FP (Fig. 1B). Thus, clones deficient in pyruvate transporter function cannot uptake the toxic pyruvate analog and are enriched in the Tn insertion mutant library grown in M9 medium supplemented with the toxic pyruvate analog.

Construction of Tn insertion mutant libraries and Tn-seq
A million viable Tn insertion mutants of E. coli K-12 MG1655, capable of growing on solid LB medium, were constructed and 1 × 10^5 colonies were selected from solid M9 medium supplemented with sorbitol, pyruvate, or sorbitol with 3FP (Fig. 2). Their unique Tn insertion positions and frequency were examined using the Tn-seq approach (See Materials and Methods). A total of 3,623,464 reads were mapped with 18,967 unique Tn insertion sites for sorbitol supplement, 5,898,167 reads were mapped with 57,711 sites for pyruvate supplement, and 3,680,158 reads were mapped with 36,830 sites for sorbitol with 3FP supplemented condition. Thus, on an average, the Tn-seq results yielded a unique Tn insertion per 245 base pairs (bp) for sorbitol, per 80 bp for pyruvate, and per 126 bp for sorbitol with 3FP conditions. To examine the distribution of these unique Tn insertion sites across the genome, distances between two adjacent Tn insertion sites were calculated and their distribution is represented in Fig. 3A–C. Most of the pairs of adjacent unique Tn insertion sites were positioned within less than 50 bp, indicating their nearly uniform distribution across the genome. The instances of two Tn insertions > 500 bp apart accounted for 15%, 4%, and 7% of the total insertion sites for sorbitol, pyruvate, and sorbitol under 3FP conditions, respectively. These results suggest the presence of essential genes (or
enriched clones) under the respective growth conditions. Additionally, some Tn insertion sites have a higher insertion preference over the others, evidencing the existence of enriched clones (Fig. 3D–F). For instance, one essential gene, metK, encoding methionine adenosyltransferase, lacked Tn insertion in the protein-coding sequence while the adjacent non-essential genes contained Tn insertions in a significant portion of their coding regions (Fig. 3G). The GC content of adjacent Tn insertion sites was 52–56%, showing good agreement with the results of a previous study (Fig. S2) (20). Taken together, the Tn-seq results provided sufficient resolution for comparative analysis of differential Tn insertion preferences under the respective growth conditions.

Selection of pyruvate transporter gene candidates
Transposon tends to be incorporated in some essential genes within a significant portion of their 3′ ends. This insertion vulnerability allows for identification of non-essential protein segments (or functional domains) in the essential genes, but may prevent determination of gene essentiality under the conditions of interest. We observed similar insertion trends for essential genes in the Tn insertion libraries obtained under the growth conditions (Fig. 4A). To improve the essential gene calling from the Tn-seq results, the Tn insertion preference of each gene was represented as the sum of insertion counts within 95% region from the 5′ end of the gene normalized by gene length and total mapped reads, under each condition. This enabled comparison of the insertion preference of all genes between the three conditions. Genes with high normalized insertion values indicate enrichment of Tn insertion clones of the corresponding gene. The gene is considered non-essential or beneficial for cell growth under the conditions when functionally deleted. In contrast, a low normalized insertion value for
a gene indicates the elimination of Tn insertion clones of the corresponding gene, whose function is essential for cell growth under the conditions. We identified several genes showing differential normalized insertion values between the growth conditions supplemented by sorbitol, pyruvate, or sorbitol with 3FP as the carbon sources. For example, normalized insertion values of genes related to sorbitol metabolism, which are *srlD* encoding sorbitol-6-phosphate 2-dehydrogenase, *srlA*, *srlB*, and *srlE* encoding sorbitol-specific PTS enzyme components, and *srlM* encoding DNA-binding transcription activator, were higher under the pyruvate condition than under the sorbitol or sorbitol with 3FP conditions (**Fig. 4B** and **Table S1**). As sorbitol was the sole carbon source under the sorbitol or sorbitol with 3FP conditions, sorbitol uptake function is essential for cell growth as well as conversion activity to fructose 6-phosphate. However, the sorbitol metabolism pathway is non-essential under the pyruvate conditions. In the same manner, it was expected that genes with high normalized insertion values under sorbitol with 3FP conditions and low normalized insertion values under sorbitol or pyruvate conditions would demonstrate high potential for pyruvate transporter function.

To identify potential pyruvate transporter genes, fold changes in the Tn insertion of 4498 genes, which are the ratio of normalized insertion values under the sorbitol to the sorbitol with 3FP condition and ratio of normalized insertion values under the pyruvate to 3FP, were calculated (**Table S1**). The fold-changes reflect changes in the frequency of certain Tn insertion mutants according to pyruvate transporter functions and toxic pyruvate analog treatment. To exclude essential genes with low insertions under all three conditions, only genes with more than two unique insertions per 1 kb were selected. A total of 52 genes were selected by the criteria that fold-changes were over 6-fold (**Table S2**). Interestingly, 2 and 14 genes were located between 13–15.12 and 45.17–70 min, respectively, which were previously expected to be genomic regions of genes involved in constitutive and inducible pyruvate transporter functions (**Fig. S3**).
Next, the functions of candidate genes were categorized into three classes as follows: (i) predicted or putative inner membrane transporters such as ybdD, yphA, and yohC; (ii) transporters of other substrates such as cstA (peptide transporter), dsdX (serine transporter), and mtr (tryptophan transporter); (iii) little relevant functions with transporter such as bluR (transcriptional repressor involved in the biofilm formation and acid resistance), nac (nitrogen assimilation regulon transcriptional regulator), and moeB (molybdopterin synthase sulfurylase) (Fig. 4C). Although most potential functional groups include genes related to predicted or putative inner membrane transporter function, genes in group (ii) may also have pyruvate transporter function because of their broad substrate specificity and multifunctional characteristics of transporters (12). Therefore, additional assays using deletion mutants of candidate genes were necessary to identify genes with pyruvate transport activity.

**Examination of pyruvate transporter activity of selected candidate genes**

To test the pyruvate uptake activity of the selected candidates, strains lacking the corresponding gene were obtained from the KEIO strain collection (49 deletion strains, 3 strains were not available) (21). Each deletion strain was grown in M9 medium supplemented with sorbitol or sorbitol with 3FP to measure the inhibitory effect of 3FP on cell growth. The specific growth rates of the deletion strains in the functional categories were determined as fold-changes (Fig. 5A and Table S2). Among them, the fold-changes in the specific growth rate of ΔcstA and ΔybdD were much greater than that of the wild-type strain, suggesting the involvement of these two genes in pyruvate transport. In addition to the two candidate genes, other potential genes (Group 4) were also tested. Interestingly, ΔbtsT mutant lacking the inducible pyruvate transporter showed no growth in the medium supplemented with 3FP, thereby clearly indicating the existence of other pyruvate transporters for the uptake of 3FP. To examine the synergetic effect of
these genes on pyruvate uptake, we additionally tested the growth inhibition of two double
knockout strains (ΔcstAΔybdD and ΔcstAΔbtsT) by 3FP. No synergetic effect on the growth rates
was observed in either ΔcstAΔybdD or ΔcstAΔbtsT mutant. Potential candidate genes with
transport-related functions, such as yhaH and yieP, showed less than 0.2-fold change in growth
rates. In addition, transporter genes with potential substrate specificity toward pyruvate, such as
actP and yhjX, were also tested, but cell growth was not observed under sorbitol with 3FP
conditions (Table S2).

The fold-changes in the specific growth rates of the deletion strains were weakly
correlated with normalized insertion under 3FP conditions (Fig. 5B). Notably, ΔcstA and ΔybdD
showed high fold-changes in growth rate and were correlated with normalized insertion under
3FP conditions. The two genes showed clear differences in both Tn insertion frequency and
unique Tn insertion sites under the three growth conditions (Fig. 5C). In addition, we examined
the colony-spotting assays of ΔcstA and ΔybdD strains (Fig. 5D). Three strains were normally
grown on solid M9 medium supplemented with sorbitol, but colonies of ΔcstA and ΔybdD strains
were formed faster than wild-type on medium supplemented with sorbitol and 3FP. This result
confirms that the ΔcstA and ΔybdD strains avoid growth inhibition by 3FP.

The two genes are located at the genomic regions which were previously predicted as the
genomic location of the constitutive pyruvate transporter (7). This transporter may be expressed
constitutively, regardless of the presence of extracellular pyruvate. In contrast, the inducible
pyruvate transporter is expressed only in the presence of extracellular pyruvate, in a different
genomic location (8). To test the induction of inducible pyruvate transporter by pyruvate for 3FP
uptake, three strains were grown under pyruvate-induced conditions, with pre-growth in M9
medium supplemented by 0.2% pyruvate prior to growth in M9 medium supplemented with 0.2%
sorbitol with and without 3FP. Additionally, the cells were grown under non-induced conditions
directly in sorbitol media with and without 3FP. Under non-induced conditions, the fold-changes in specific growth rates between the presence and absence of 3FP were 0.13 for wild-type strain, 0.96 for ΔcstA, and 0.61 for ΔybdD strains, which were consistent with the results of the growth inhibition assay (Fig. 5E). Under pyruvate-induced conditions, however, the fold-changes were 0.02 for wild-type strain and zero for the ΔcstA and ΔybdD strains, which showed complete growth inhibition by 3FP (Fig. 5F). Because the inducible pyruvate transporter is expected to import 3FP under pyruvate-induced conditions, both the ΔcstA and ΔybdD strains showed reduced growth rates. These results confirmed that cstA and ybdD are constitutive pyruvate transporters.

In addition to the growth assay using 3FP, the pyruvate uptake activity of the deletion strains was investigated by measuring extracellular pyruvate concentration under the pyruvate-induced condition (Fig. 5G). Among the eight knockout strains, five strains (ΔcstA, ΔybdD, ΔbtsT, ΔcstAΔybdD, and ΔcstAΔbtsT) imported significantly less pyruvate than wild-type strain (p < 0.01, Welch's t-test). Pyruvate uptake levels of the mutant strains were reduced to 13%, 20%, 36%, 25%, and 5% of wild-type strain, respectively, thereby indicating that both cstA and ybdD are required for constitutive uptake of pyruvate. In addition, the inducible pyruvate transporter BtsT also imports pyruvate in presence of 0.1 – 0.5 mM extracellular pyruvate (8). Notably, double knockout mutant (ΔcstAΔbtsT), which was expected to be deficient in both inducible and constitutive pyruvate transport systems, showed the lowest fold-change in pyruvate uptake (0.05). This result is considered to be a cumulative effect of both the genes on pyruvate transport.

Another knockout strain ΔyhjX, lacking a putative transporter gene, showed slightly reduced fold-change in pyruvate uptake (0.77), indicating YhjX to be potentially involved in the formation of a different pyruvate uptake system. In the 3FP growth inhibition assay, a ΔmhpD mutant strain showed the third highest fold-change in growth rate; however, the strain showed no
significant change in pyruvate uptake \( (p > 0.01, \) Welch’s \( t \)-test) (Fig. 5G). The mhpD gene encodes 2-keto-4-pentenoate hydratase, involved in aromatic compound metabolism (22). The final metabolic products from this pathway are pyruvate and acetyl-CoA, which are eventually converted to succinate through the TCA cycle. Thus, the cellular function of MhpD and the absence of pyruvate uptake activity in its mutant strain suggest that the change in growth rate of \( \Delta mhpD \) mutant could be due to the metabolic perturbation caused by gene deletion. As a negative control, \( \Delta ecnB \), whose knocked out gene encoded entericidin B membrane lipoprotein with no growth under 3FP condition, showed an uptake level closely similar to that in wild-type strain. These results support the impairment of pyruvate uptake activity of \( \Delta cstA \) and \( \Delta ybdD \). The deletion strains incubated without pyruvate treatment showed negligible or no pyruvate uptake, indicating no effect of pyruvate export function on the extracellular measurement of pyruvate level.

Next, we examined the sequence homology of CstA against other related proteins in other bacterial species. CstA showed high similarity (>90%) with carbon starvation protein A, which is highly conserved in other bacterial species. Multiple sequence alignment with pyruvate transporter genes in other species showed no significant sequence homology between CstA and other transporters, or between other transporters themselves. Only BtsT, which is identified as an inducible pyruvate transporter in \( E. \ coli \), showed significant similarity (60%). Taken together, growth inhibition assays using 3FP treatment and the pyruvate uptake activity test verified that the constitutive pyruvate transporter system consisted of \( cstA \) and \( ybdD \) in \( E. \ coli \).

DISCUSSION

In this study, we identified genes encoding pyruvate transport activity in \( E. \ coli \). Pyruvate is a
dynamically regulated metabolite positioned at an important node interconnected with diverse catabolic and biosynthetic pathways. In response to the metabolic states in the cell, pyruvate can be excreted into the extracellular medium, whereas it can also be taken up again into the cell under certain conditions such as carbon starvation (3). This dynamic and reversible regulation of intracellular and extracellular pyruvate concentrations suggests that the pyruvate transport system maintains homeostasis in *E. coli*. Additionally, more than four genes related to pyruvate transport in *E. coli* have been identified: an inducible transporter BtsT, constitutive transporter, putative inducible transporter YhjX, and unknown exporter (3, 7, 8, 17). Despite its importance in bacterial metabolism, only one pyruvate transporter gene, encoding the inducible transporter, has been identified and verified in *E. coli*.

Indeed, Tn mutants lacking pyruvate transporter function were enriched in the presence of 3FP, whereas supplementation with sorbitol or pyruvate provided non-toxic carbon sources for normal cell growth. The fold-change in Tn insertion frequency of the enriched mutant libraries under the three growth conditions revealed 52 candidates for examination of their pyruvate transporter function. These gene identification strategies are valid and effective for three reasons: (i) A number of the transporters have a broad substrate specificity, or the other transporters can replace the transporter function of interest (12). On the other hand, Tn-seq reveals quantitative differences in growth fitness levels between clones with diverse degrees of transporter function. (ii) A gene of interest may be multifunctional or mis-annotated because it exhibits different functions than expected (23, 24). Thus, sequence homology-based target selection for functional assays may result in false-positive calling. In contrast, the Tn-seq library contains entire gene set that can reduce bias. (iii) Our results filled the gap between the genetic information and phenotypic characteristics of the pyruvate transporter gene, which transports an important and abundant metabolite in *E. coli*. The genetic information obtained from this study provides a basis
for understanding the tight regulation of intracellular pyruvate concentrations.

Final candidate genes *cstA* and *ybdD* are known as peptide transporter induced by carbon starvation and conserved protein, respectively. Thus, our study is the first report demonstrating their pyruvate transport function. CstA is an inner membrane protein consisting of 701 amino acids and is regulated by cAMP-cAMP receptor protein complex at the transcriptional level under carbon starvation conditions (25, 26). CstA is a highly conserved protein among several bacterial species, and its peptide transport function has been examined in *E. coli* and *Campylobacter jejuni* (23, 25). In addition, CstA is related to other functions such as biofilm formation, motility, and agglutination (23, 24). It is known that carbon storage regulator CsrA negatively regulates the expression of CstA at translational level (26). Therefore, the pyruvate uptake level of CsrA knockout mutant was expected to be higher than that of wild-type, but the knockout strain was found to be growth-defective instead. YbdD is a small protein consisting of 65 amino acids and whose function is currently unknown. Its genomic location is immediately next to *cstA*, indicating the potential for a protein-protein interaction with CstA to transport pyruvate. For instance, some small membrane protein genes are located in close vicinity to membrane transporters such as *mctB* and *mctC* in *Corynebacterium glutamicum*, as well as *yjcH* and *actP* in *E. coli* (11). In *Bacillus subtilis*, PftAB is the recently identified inducible pyruvate transporter, also comprised of two adjacent genes (27). To demonstrate the role of YbdD in the pyruvate transport system, the direct interaction between CstA and YbdD should be further assessed using a certain assay such as a bacterial two-hybrid system (28, 29).

In addition to the putative pyruvate-sensing two-component system YpdA/YpdB and its target YhjX, it has been suggested that the histidine kinase/response regulator system consisting of BtsS/BtsR and its target BtsT plays an important role in the cellular response to extracellular pyruvate as well as carbon starvation conditions (30, 31). The BtsS/BtsR system is induced by
high concentrations of casamino acid or pyruvate with high binding affinity (30). Recently, its target BtsT was identified as a specific pyruvate transporter induced by extracellular pyruvate (8). The high sequence similarity between CstA and BtsT (approximately 60%) indicates the possibility that both proteins might have similar functions, namely transport of peptide as well as pyruvate. The additive effect of csta, ybdD, and btsT deletions on pyruvate uptake was also tested. Although YbdD is a small protein with unknown function, its single knockout strain showed similar level of 3FP-induced growth inhibition and pyruvate uptake as that of csta single knockout strain, thus implying that YbdD is essential for pyruvate transport system. Double knockout mutant ΔcstaΔybdD showed no significant difference in either growth rate or pyruvate uptake compared to those of the respective single knockout mutants, indicating that the two proteins constitute a single pyruvate transporter complex. In contrast, double knockout strain of both pyruvate transport systems, ΔcstaΔbtsT, showed an additive effect on pyruvate uptake. A triple knockout mutant ΔcstaΔybdDAbtsT was also constructed, but it did not grow under the growth conditions tested, hence indicating that those pyruvate uptake systems are essential for cell growth. Knockout strains lacking the other candidate genes were also examined. Among those, ΔmhpD, lacking the gene encoding 2-keto-4-pentenoate hydratase, showed the third highest change of growth rate. MhpD is associated with pyruvate metabolism; the final metabolic products are pyruvate and acetyl-CoA, which are eventually converted to succinate through the TCA cycle (22). However, the strain showed no significant change in pyruvate uptake, which could be due to its metabolic perturbation. The ΔyhdX mutant, whose knockout gene is the downstream gene of YpdA/B pyruvate-sensing two-component system, showed no change in growth rate with slightly reduced uptake, hence considered to be possibly related to pyruvate uptake system.

These studies indicate that there are tightly and dynamically regulated systems for
sensing and transporting extracellular pyruvate, peptides, and amino acids according to the growth phases related to overflow metabolism. Because amino acids can be metabolized to pyruvate as a resource for various metabolic pathways, we expect that peptide utilization and pyruvate concentration controlled by transporters are closely related. Thus, our finding that the peptide transporter CstA imports pyruvate may be the missing link in the pyruvate and peptide/amino acid sensing network in *E. coli* (Fig. 6).

Based on the collective analyses of Tn-seq, 3FP growth inhibition assay, and pyruvate uptake assay, we concluded that CstA and YbdD import pyruvate constitutively. However, the reduced growth inhibition by 3FP might have resulted either from metabolic perturbation induced by the knockout or regulatory crosstalk between CstA/YbdD and pyruvate transporters.

Reduced pyruvate uptake could also be due to the indirect feedback effect; for example, peptides imported by CstA may activate the expression of other pyruvate transporters for the uptake of pyruvate. Thus, further investigation of the mode of action and broad substrate specificity of CstA would be needed, along with an understanding of the relationship between carbon starvation and constitutive pyruvate uptake activity. The multifunctional properties of CstA also remain unclear. One limitation of our study was that only the constitutive pyruvate transporter system was identified without identifying the genes encoding inducible pyruvate transporters. This is because the selection conditions for transposon mutant enrichment were pyruvate non-induced conditions. Therefore, the inducible pyruvate transporter gene including *btsT* can be identified using a Tn mutant library constructed from cells lacking ΔcstA under pyruvate-induced conditions. Additionally, we can reconstruct the pyruvate sensing and transport network to understand complex pyruvate metabolism in *E. coli*.

**MATERIALS AND METHODS**
Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table S3. Wild-type *E. coli* K-12 MG1655 cells were grown in 3 mL of Luria-Bertani broth (LB) medium at 37°C. The cells were transferred and grown in M9 medium (0.493 g/L MgSO$_4$$\cdot$7H$_2$O, 0.015 g/L CaCl$_2$$\cdot$2H$_2$O, 12.8 g/L Na$_2$HPO$_4$$\cdot$7H$_2$O, 3 g/L KH$_2$PO$_4$, 0.5 g/L NaCl, and 5 g/L NH$_4$Cl) supplemented with 0.2% of a carbon source, which was D-sorbitol (Sigma-Aldrich, St. Louis, MO, USA) or sodium pyruvate (Sigma-Aldrich). *Escherichia coli* K-12 BW25113 strain and the KEIO knockout collection were purchased from Coli Genetic Stock Center (New Haven, CT, USA) and Dharmacon (Lafayette, CO, USA), respectively, and used in growth and uptake tests (21).

Growth inhibition test by toxic pyruvate analog

Cells were grown in 3 mL of LB medium for 8 h at 37°C. For pyruvate-non-induced, pyruvate-induced, and control growth conditions (7), the cells were washed twice with 1 mL of M9 medium supplemented with 0.2% sorbitol, 0.2% pyruvate, and 0.2% sorbitol, and then inoculated into 30 mL of M9 medium supplemented with 0.2% sorbitol, 0.2% pyruvate, and 0.2% sorbitol to reach optical density (OD) units of 0.05 at 600 nm. For pyruvate-non-induced growth, the cells were grown in M9 medium supplemented with 0.2% sorbitol for one generation. In contrast, for pyruvate-induced growth, the cells were grown overnight in M9 medium supplemented with 0.2% pyruvate, and then transferred to and grown in M9 medium supplemented with 0.2% sorbitol for one generation. After one generation of growth, 400 μL of the culture was transferred to a 48-well plate (SPL Life Sciences Co., Ltd., Pocheon, Korea). Next, β-fluoropyruvic acid sodium salt monohydrate (3FP) (Sigma-Aldrich) was added to the culture at a final concentration of 1 mM and incubated 37°C in a Synergy H1 microwell reader (BioTek, Winooski, VT, USA).
with double orbital continuous shaking at a frequency of 2 mm. Every 15 min, the OD units of each well were measured at 600 nm. For the growth inhibition assay, two deletion strains ($\Delta$cstA and $\Delta$ybdD) were grown under both pyruvate-non-induced and pyruvate-induced conditions as described above and washed twice with 1 mL of M9 medium supplemented with 0.2% sorbitol. The washed cells were inoculated into 50 mL of the same medium and incubated at 37°C in a shaking incubator at 220 rpm.

**Colony spotting assay**

Cells were grown in 3 mL of LB media at 37°C overnight and serially diluted in M9 medium containing 0.2% sorbitol from $1 \times 10^9$ to $1 \times 10^7$ cells as duplicates. Next, 10 μL of the five most diluted cultures were spotted onto solid M9 medium with 0.2% sorbitol with and without 1 mM 3FP. The culture plates were imaged in the Gel-Doc XR+ (Bio-Rad, Hercules, CA, USA) every 1 h. The images were obtained using ImageLab™ software.

**Clonal selection from Tn mutant library**

*Escherichia coli* K-12 MG1655 cells were treated with EZ-Tn5™ <KAN-2>Tnp Transposome™ (Epicentre, Madison, WI, USA) to construct the Tn mutant library (32). After overnight growth in LB medium, three samples of $2 \times 10^9$ cells were collected, washed twice with M9 salt solution, and spread onto solid M9 medium supplemented with 0.2% sorbitol, 0.2% pyruvate, and 0.2% sorbitol with 1 mM 3FP. Although pyruvate supplement results in the highest induction of pyruvate uptake activity, pyruvate transporter-deficient clones are either unable to grow or show slow growth in M9 medium supplemented with pyruvate as the sole source of carbon (7). In contrast, pyruvate transporter-deficient clones grow normally under sorbitol or sorbitol with 3FP supplemented conditions. Sorbitol partially induces pyruvate uptake activity with a relatively low
level of catabolite repression compared to that by glucose (7). For clones with pyruvate
transporter function intact, normal growth can be expected under both pyruvate and sorbitol
conditions. Thus, after three serial passages under the given growth conditions, we expected that
pyruvate transporter-deficient clones would be outcompeted and rather enriched under sorbitol
with 3FP conditions compared to that under sorbitol or pyruvate conditions. Therefore, after 36 h
incubation, approximately $5 \times 10^6$ colonies were collected from each plate. To enrich
outcompeted clones under each condition, we repeated the clonal selection step for three
passages. The colonies were observed with a phase-contrast microscope to detect contamination
during incubation.

**Tn-seq**
The collected cells were divided into 100 aliquots to extract their genomic DNA and generate the
Tn-seq library by the modified 3-step PCR method (33). Primers used in this study are listed in
**Table S4.** The cell pellet was lysed by adding 600 μL of Nuclei Lysis Solution (Promega,
Madison, WI, USA) followed by incubation at 80°C for 10 min. Next, 12 μg of RNase A (Qiagen,
Hilden, Germany) was added and incubated at 37°C for 30 min to degrade the RNA. Next, 200
μL of Protein Precipitation Solution (Promega) was added to precipitate the proteins. The sample
was centrifuged at 17,000 $\times g$ for 3 min, and 600 μL of supernatant was mixed with same volume
of isopropanol to precipitate genomic DNA. The genomic DNA was collected as a pellet by
centrifugation and then washed twice with 600 μL of 70% ethanol to remove remaining salts.
After drying, the genomic DNA was dissolved in 50 μL of nuclease-free water. Ten of the
extracted genomic DNA samples were mixed at the same concentration (100 ng/μL) and used as
a template for subsequent PCR (**Fig. S1**). In the first PCR step, one primer specifically anneals to
the Tn sequence, while the other primer anneals to a random genomic region next to the Tn
junction. The random primer contains 8N and one of four 5-bp sequences (GCTGG, CCAGC, CTGGC, and TGGCG). These 3′ end 5-bp sequences of the random primer appear approximately once per 51 bp in the whole genome. In the second and third PCR steps, sequencing adapters were added to both ends for Illumina sequencing. Each library representing different selection conditions contained different sequencing indexes of 3′ end adapter. The first PCR mixture (10 μL) contained 2× HotstarTaq master mix (Qiagen), 0.1 μL of pFU-X (Solgent, Daejeon, Korea), 1 μL of the genomic DNA sample, and 0.5 μM of each primer (Fig. S1). The PCR mixtures were cycled at 95°C for 5 min (one cycle), 94°C for 20 s, 63°C for 45 s, and 72°C for 3 min (total 9 cycles), 94°C for 20 s, 63°C for 45 s, and 72°C for 3 min (total 12 cycles), 94°C for 20 s, 44°C for 45 s, and 72°C for 3 min, and 72°C for 7 min on a LightCycler (Bio-Rad). The first PCR products were diluted by 100-fold and used as templates for the second PCR with primers (Table S4). The PCR mixtures were cycled at 95°C for 5 min (one cycle), 94°C for 20 s, 63°C for 45 s, 72°C for 5 min, 94°C for 20 s, 63°C for 45 s, 72°C for 5 min, 94°C for 20 s, 53°C for 45 s, and 72°C for 5 min (9 cycles), 94°C for 20 s, 63°C for 45 s, 72°C for 5 min, 94°C for 20 s, 53°C for 45 s, and 72°C for 5 min (8 cycles), and 72°C for 7 min. The second PCR products were diluted by 100-fold and used as templates for the third PCR with primers (Table S4). The PCR mixtures were cycled under same condition as the second PCR. The third PCR products were pooled and loaded onto a 1% LE agarose gel. After running at 100 V for 50 min, 300–700 bp bands were excised from the gel and extracted with the MinElute Gel Extraction Kit (Qiagen). The concentration of each library was measured with the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The quality of each library was checked by 2% SYBR gold gel and KAPA library Quantification Kit (KAPA Biosystems, Wilmington, MA, USA). Tn-seq libraries were then sequenced using an Illumina MiSeq v2 instrument with MiSeq Reagent Kits v3 (150 cycled single-end read) according to the manufacturer’s protocol (Illumina, San Diego, CA, USA).
Sequencing data analysis

Sequencing data were processed using CLC Genomics Workbench 6.5.1 software (CLC Bio, Aarhus, Denmark). Low-quality reads with more than two ambiguous nucleotides and sequencing reads to the PhiX genome (NC_001422) were discarded. To obtain only Tn-gDNA junction sequence, the 5' end Tn sequence was trimmed. Trimmed reads were mapped to the reference genome (NC_000913.3). Detailed parameters are shown in Figure S1. Mapped read information was exported as a BAM file format, which was further converted to a GFF file format containing Tn insertion counts for each genomic position. The GFF file was visualized on SignalMap (v2.0.0.5, Roche NimbleGen, Basel, Switzerland). To reduce selection errors, only genes with 2 or more Tn insertion sites were analyzed. Tn insertion frequency of remained genes was calculated by the sum of insertion counts within the 95% region of the gene from the 5' end. This value was normalized by gene length and total mapped reads under each condition.

Growth inhibition assay of KEIO strains

KEIO strain cells were inoculated into 3 mL of LB media supplemented with 25 μg/mL kanamycin. After overnight growth, the grown cells were washed twice with 1 mL of M9 medium supplemented with 0.2% sorbitol and inoculated into 50 mL of M9 medium supplemented with 0.2% sorbitol to an OD of 0.05 at 600 nm. After one generation of growth, 100 μL of the culture was transferred to a 96-well plate (SPL Life Sciences Co., Ltd.). Next, 3FP was added to the culture at a final concentration of 1 mM and incubated 37°C in Synergy H1 microwell reader with double orbital continuous shaking at 2 mm frequency. Every 30 min, the OD of each well was measured at 600 nm.
Construction of knockout strains

Two double knockout strains ($\Delta$cstA$\Delta$ybdD and $\Delta$cstA$\Delta$btsT) and one triple knockout strain ($\Delta$cstA$\Delta$ybdD$\Delta$btsT) were constructed by lambda recombination (34). Primer sequences used in knockout are listed in Table S4. Briefly, genomic copy of 5’ coding region of cstA to 3’ coding region of ybdD (cstA_KO_F and ybdD_KO_R) or coding region of btsT (yjiY_KO_F and yjiY_KO_R) were deleted with kanamycin resistant DNA cassette amplified from pKD13 in pKD46 carried BW25113, $\Delta$cstA, and $\Delta$btsT, respectively. For knockout from $\Delta$cstA and $\Delta$btsT, pCP20 (34) was introduced into the strain before the recombination to remove of kanamycin cassette flanked by FLP recognition sites. pKD46 and pCP20 were cured by overnight incubation at 42°C.

Measurement of pyruvate uptake activity

We measured extracellular pyruvate concentration after pyruvate treatment (7, 17). The cells were grown in 3 mL of LB medium for 5 hr at 37 ºC in a shaking incubator at 220 rpm. The cells were washed with 1 mL of M9 medium supplemented with 0.2% sorbitol twice and inoculated into 50 mL of M9 medium supplemented with 0.2% sorbitol in biological triplicates. A total of 10 mL of cultures at exponential growth phase were collected and washed twice with 10 mL of ice-cold 1% NaCl. The cells were then resuspended by 10 mL of M9 medium without any carbon sources. To examine pyruvate uptake activity, 300 μM of sodium pyruvate was added to each sample. After 10 min incubation at room temperature, the cells were centrifuged at 3134 ×g for 5 min at 4°C. The supernatant was collected and stored at −20 ºC until further analysis.

Extracellular pyruvate concentration was determined with the Cedex Bio HT Analyzer (Roche, CustomBiotech) according to the manufacturer’s instructions.
Sequence analysis

Transmembrane domains and conserved domains were predicted by TMHMM and Pfams, respectively. Multiple alignment of related transporter sequences was conducted by geneious software (Auckland, New Zealand) with global alignment and blosom62 matrix (mid-range). Phylogenetic trees were drawn by MEGA 7 software with ClustalW, blosom62 matrix, and maximum likelihood statistical methods.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

S.C. and B.-K.C. conceived and supervised the study. S.H., S.C., and B.-K.C. designed the
experiments. S.H., D.C., M.Y., S.C., and S.C. performed the experiments. S.H., S.C., and B.-K.C. analyzed the data. S.H., S.C.K., S.C., and B.-K.C. wrote the manuscript. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1. Growth inhibition tested with toxic pyruvate analog. (A) Growth inhibition profile using toxic pyruvate analog 3FP (3-fluoropyruvate), under pyruvate-non-induced and induced conditions. A final concentration of 1 mM 3FP was added at OD<sub>600nm</sub> = 0.1. PDH: Pyruvate dehydrogenase, PyrTp: Pyruvate transporter. (B) Colony spotting assay for E. coli K-12 MG1655 under growth inhibition by 3FP. The spot image of plate was taken 32 h after spotting and incubation. 3FP-: M9 + 0.2% sorbitol solid medium without adding 3FP. 3FP+: M9 + 0.2% sorbitol solid medium with 1 mM 3FP added.

Figure 2. Transposon sequencing (Tn-seq) to identify pyruvate transporter genes in E. coli K-12 MG1655. Tn-seq strategy for the selection of differential clones. To construct the Tn mutant library, a transposon was inserted into a random site in the wild-type E. coli K-12 MG1655 genome. The library was serially grown on solid minimal medium with three different carbon sources: sorbitol, pyruvate, and sorbitol + 1 mM 3FP. Through selection, clones with a detrimental insertion under each condition decreased in frequency. Blue circle indicates clones with the functional pyruvate transporter gene, while red circle indicates clones with transposon insertion within the pyruvate transporter gene. Reads obtained from high-throughput parallel sequencing were mapped to the reference genome. Candidate genes were selected considering the ratios of insertion frequency between sorbitol + 3FP condition and the other two conditions.

Figure 3. Distribution of unique transposon insertion sites across the genome. (A–C) The distribution of distances between transposon insertion sites across the genome in (A) sorbitol, (B) pyruvate, and (C) sorbitol + 3FP conditions, respectively. (D–F) The distribution of insertion
numbers at each insertion site in (D) sorbitol, (E) pyruvate, and (F) sorbitol + 3FP. (G) An example of unique transposon insertion profile of an essential gene, metK.

**Figure 4. Candidate selection from Tn-seq results.** (A) Distribution of normalized insertions over the relative position within a gene. Red bar indicates insertions within PEC essential genes (n = 302), and grey bar indicates insertions within total genes (n = 4498). Insertions in the 5% region from 3’ end of the gene were excluded from normalization for all genes. (B) Normalized insertion of genes involved in sorbitol metabolism. The pathway for sorbitol utilization and their normalized insertions are depicted as a heat map. Grey rectangle represents sorbitol PTS transporter, red circle represents a transcriptional activator (SrlM), and green circle represents a transcriptional repressor (SrlR) of the sorbitol metabolism operon. Sor: Sorbitol, Pyr: Pyruvate, 3FP: Sorbitol + 3FP condition. Nor.Ins.: Normalized insertion. (C) Three functional categories of the 52 selected candidate genes.

**Figure 5. Final candidates, cstA and ybdD, selected from additional assays.** (A) Specific growth rates of single gene knocked-out strains (from KEIO collection, detailed in Materials and Methods) in sorbitol (3FP-) and sorbitol + 3FP (3FP+) minimal medium. A total of 58 strains were tested, whose knockout genes were selected from Tn-seq. Group 1: genes belong to ‘predicted or putative function’ category, Group 2: genes belong to ‘transporters of other substrates’ category, Group 3: genes belong to ‘other function’ category, and Group 4: additional genes without candidate groups from Tn-seq. (B) Correlation between normalized insertion value in 3FP condition from Tn-seq and specific fold-change of growth rate from 3FP growth assays. cstA and ybdD showed strikingly high values in both experiments. (C) Unique transposon insertion profile of cstA and ybdD. Sor: Sorbitol, Pyr: Pyruvate, 3FP: Sorbitol + 3FP. (D) Colony
spotting assay of *E. coli* K-12 MG1655, ΔcstA, and ΔybdD under growth inhibition by 3FP. The spot images of plate were taken 32 h after spotting and incubation. 3FP-: M9 + 0.2% sorbitol (solid) medium without adding 3FP. 3FP+: M9 + 0.2% sorbitol (solid) medium with 1 mM 3FP added. (E–F) Growth inhibition of the three strains by 3FP under (E) pyruvate-non-induced and (F) pyruvate-induced conditions. 3FP-: Minimal medium with sorbitol, 3FP+: Minimal medium with sorbitol and 3FP. (G) Pyruvate uptake activity test by measuring the extracellular pyruvate concentration 10 min after the pyruvate treatment. WT: *E. coli* K-12 BW25113, and Δ*gene_name*: *E. coli* K-12 BW25113 Δ*gene_name*. Statistical significance was calculated by Welch’s t-test from python, and stars indicate significance. (***: *p* < 0.001, **: *p* < 0.01).

**Figure 6. Schematic diagram of CstA constitutive pyruvate transporter system and two pyruvate-sensing systems.** Orange color subjects are related to CstA, blue color subjects are related to YbdD, grey color subjects are related to CRP or CsrA regulators, and green color subjects are related to previously studied BtsT and YhjX transporter systems. Black characters are the protein names, and italicized black characters are the gene names. Black lined boxes represent substrates for corresponding proteins. Black solid arrows represent expression of the target. Black dotted arrows mean activation while black dotted arrows with line end mean repression of the process. Red arrows indicate the transport of substrates, and light blue arrows indicate putative interactions between constitutive and inducible pyruvate transport systems.
PEC essential gens
Total genes
B
C
A
Position within a gene
Normalized insertions
0 0.2 0.4 0.6 0.8 1
0
0.2
0.4
0.6
0.8
1
1.2

Transporters of other substrates
Predicted or putative function
Others
28 genes
(53.8%)
4 genes
(7.7%)

Sorbitol
Sorbitol-6P
Fructose-6P
Glycolysis
NADH
NAD+
H+

SrlA
SrlB
SrlD
SrlM
SrlR
SrlQ

Log$_{10}$ (Nor.Ins. + 1)

srlA
srlB
srlD
srlM
srlR
srlQ

srlQ

Periplasm
Inner membrane
Cytoplasm

srlA
srlB
srlD
srlM
srlR

srlQ

Fructose-6P → Glycolysis

srlA srlE srlB srlD srlM srlR srlQ
