Differential Selectivity of the *Escherichia coli* Cell Membrane Shifts the Equilibrium for the Enzyme-Catalyzed Isomerization of Galactose to Tagatose

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An *Escherichia coli* galactose kinase gene knockout (ΔgalK) strain, which contains the L-arabinose isomerase gene (*araC1*) to isomerize L-galactose to L-tagatose, showed a high conversion yield of tagatose compared with the original *galK* strain because galactose was not metabolized by endogenous galactose kinase. In whole cells of the ΔgalK strain, the isomerase-catalyzed reaction exhibited an equilibrium shift toward tagatose, producing a tagatose fraction of 68% at 37°C, whereas the purified L-arabinose isomerase gave a tagatose equilibrium fraction of 36%. These equilibrium fractions are close to those predicted from the measured equilibrium constants of the isomerization reaction catalyzed in whole cells and by the purified enzyme. The equilibrium shift in these cells resulted from the higher uptake and lower release rates for galactose, which is a common sugar substrate, than for tagatose, which is a rare sugar product. A ΔmglB mutant had decreased uptake rates for galactose and tagatose, indicating that a methylgalactoside transport system, MglABC, is the primary contributing transporter for the sugars. In the present study, whole-cell conversion using differential selectivity of the cell membrane was proposed as a method for shifting the equilibrium in sugar isomerization reactions.

Recently, the biotransformation of galactose to tagatose, a valuable sweetener (20), has been intensively investigated using many bacterial L-arabinose isomerases, including those from *Alicyclobacillus acidocaldarius* (19), *B. halodurans* (17), *B. stearothermophilus* (26), *Escherichia coli* (24, 27, 30), *Geobacillus stearothermophilus* (11, 13, 15, 28), *G. thermudenitrificans* (2, 14, 25), *Thermus* sp. (16), *Thermoanaerobacter mathranii* (10), *Thermotoga neapolitana* (12), and *Thermotoga maritima* (18). The reported equilibrium fraction of tagatose from galactose is 32% at 30°C for L-arabinose isomerase purified from *E. coli* (27), 58% at 65°C for that from *G. thermodenitrificans* (25), and 68% at 85°C for that from *T. neapolitana* (12). The equilibrium of the isomerization reaction shifts toward the tagatose product as the temperature increases. The equilibrium fraction of product for an enzyme-catalyzed isomerization reaction, i.e., the equilibrium between substrate and product, cannot be changed simply by protein engineering of the enzyme, since it is controlled by the reaction temperature (4). Compared to conversion using hyperthermophilic enzyme, whole-cell conversion has advantages such as greater resistance to environmental perturbations and a lower effective enzyme cost, while eliminating the enzyme purification and extraction steps. Also, it may not be easy to find a hyperthermophilic enzyme to carry out the reaction at high temperatures.

In the conversion of galactose to tagatose by *E. coli* expressing L-arabinose isomerase, transport systems of galactose and tagatose through the cell membrane are important because they may affect the conversion rate and yield. Three separate transport systems for galactose are reported for *E. coli*: (i) a methylgalactoside transport system, which is designated MglABC because it is a member of the ATP-binding cassette (ABC) superfamily of transporters and transports β-methylgalactoside in addition to its natural galactose substrate (29); (ii) a galactose-specific transport system (GalP) (5, 22); and (iii) sugar efflux transporters (SETs, including SetA, -B, and -C) for sugar release, which are believed to have broad substrate specificity to various sugars including galactosides (21). However, an *E. coli* transport system for tagatose has not previously been reported.

Instead of using temperature to control the enzymatic process in isomerization reactions, whole-cell conversion is proposed as a new alternative method to shift equilibrium. The method uses the cell membrane to separate the substrate and product (extracellular region) from the enzyme reaction (intracellular region). Membrane selectivity can cause differences in the concentrations of substrate and product inside and outside of the cells, resulting in an equilibrium shift. However, direct or kinetic evidence for the equilibrium shift in whole-cell conversions has never been documented. We measure here the equilibrium shift of whole-cell conversions and describe it quantitatively based on enzyme kinetic studies, which unambiguously show that membrane selectivity for the substrates results in an equilibrium shift. Moreover, the uptake and release rates of galactose and tagatose were determined to explain the equilibrium shift, and the related transport systems for the sugars were investigated.
by colony-based PCR, and the resulting knockout mutants were designated in the cells were harvested by centrifugation at 15,000 g. When the absorbance of the culture reached 1.0 at 600 nm, the cells were harvested by centrifugation at 15,000 × g for 20 min at 4°C, washed with saline solution, and then resuspended in 50 mM Tris-HCl buffer (pH 7.0) for use in the conversion of galactose to tagatose.

DNA cloning, purification, and quantification of L-arabinose isomerase. Genomic E. coli DNA was prepared using a Genomic DNA buffer set (Qiagen, Hilden, Germany). The araC gene encoding the enzyme L-arabinose isomerase was isolated from the genomic DNA by PCR using forward (araA-F, 5'-TGGCGATTTTTGATAATTATGAAGTG-3') and reverse (araA-R, 5'-CTCT ATTAGGCAGCAAAACCGGTAATC-3') primers designed to introduce NcoI and XbaI restriction sites (underlined). PCR was carried out using Phu DNA polymerase (Invitrogen, Carlsbad, CA) according to a standard PCR protocol. The E. coli araC gene was inserted into the NcoI and XbaI sites of pTrc99A, resulting in pTaraA.

Cells were harvested from the culture broth by centrifugation at 15,000 × g for 20 min at 4°C, washed with saline solution, and then resuspended in 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride. The resulting pellets were disrupted by passing them twice through a French press at 15,000 lb/in². The cell debris was removed by centrifugation at 15,000 × g for 20 min at 4°C, and the resulting supernatant fraction was filtered through a 0.45-μm-pore-size filter and applied to a HiTrap Q ion-exchange chromatography column (Amersham Biosciences Uppsala, Sweden) equilibrated with 50 mM Tris-HCl buffer (pH 7.0). All chromatographic purification steps were carried out in a cold room using a fast protein liquid chromatography system (Amersham Biosciences, Electronic, Berlin, Germany). The lysate was centrifuged at 15,000 × g for 20 min at 4°C, and the supernatant fraction was loaded onto the chromatography column and eluted with a gradient from 0.15 to 0.4 M NaCl in the same buffer. The active fractions were pooled and further purified on a Resource Q ion-exchange chromatography column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl buffer (pH 7.0). The purified enzyme were calculated using the Haldane equation (7).

Determination of equilibrium fractions. To determine the extracellular concentrations of galactose and tagatose, 10 ml of a 19 mg/ml cell suspension was incubated at 37°C for 22 h in a 10 mM concentration of sugars at three different initial ratios of galactose to tagatose (0.100, 50.50, and 100.00), with shaking at 100 rpm. The suspension was centrifuged at 15,000 × g for 10 min at 4°C, and the supernatant was analyzed. To determine the intracellular concentrations of galactose and tagatose, the cell pellet from the above step was resuspended in saline solution and then centrifuged at 15,000 × g for 20 min at 4°C. Lysis solution (Turbolysis cell lysis reagent; Genefocus, Daejeon, Korea) was added to the washed cells, which were then incubated at 70°C for 15 min. After cellular debris was removed by centrifugation at 15,000 × g for 10 min at 4°C, the concentrations of galactose and tagatose in the cell- and protein-free supernatant were determined.

Purified L-arabinose isomerase was incubated at 20°C for 4 h with 1 mM MnCl₂ and then dialyzed against 50 mM Tris-HCl buffer (pH 7.0) at 4°C. The equilibrium concentration of tagatose determined as the average of the fraction obtained after incubation of the enzyme solution at 37°C for 1 h in 50 mM Tris-HCl buffer (pH 7.0) containing a 10 mM concentration of the sugars at five different initial ratios of galactose to tagatose (0.100, 25.75, 50.50, 75.25, and 100.00). The enzyme concentration used was 10 mg/ml.

Determination of kinetic parameters. Substrate was mixed with whole cells (19 mg/ml) or purified L-arabinose isomerase (5 mg/ml) in 50 mM Tris-HCl buffer (pH 7.0) and incubated for 1 h at 37°C. The tagatose concentration used ranged from 50 to 1,000 mM. The tagatose concentration used ranged from 50 to 500 mM for whole cells and from 5 to 100 mM for the purified enzyme. The Michaelis-Menten constant (Kₘ) and turnover number (kₜₐₜ) of the purified enzyme and cells were calculated using Lineweaver-Burk transformations of the concentration and velocity data. The equilibrium constants of the whole cells and purified enzyme were calculated using the Haldane equation (7).

Measurement of transport rates for uptake and release. The rates of galactose and tagatose uptake into cells were determined using the ΔΔUL strain without pTaraA. Ten milliliters of cells suspension in 50 mM Tris-HCl (pH 7.0) at 19 mg/ml was incubated with 5, 10, 15, or 25 mM galactose or tagatose for 5 min. The cell suspension was then rapidly washed twice at 15,000 × g for 30 s at 4°C to remove extracellular sugar. The concentrations of intracellular galactose and tagatose were measured after the cells were lysed with Turbulys (Genefocus). To determine the cellular release rates of galactose and tagatose, 5 ml of cell suspension (19 mg/ml) was incubated for 1 h with 5, 10, 15, or 20 mM galactose and 2, 4, 6, or 8 mM tagatose. The cells were rapidly washed twice as described above and divided into two portions. One portion was lysed and used to determine...
mine the intracellular concentrations of galactose and tagatose, and the other portion was resuspended in 50 mM Tris-HCl buffer solution without the sugars. This cell suspension was incubated for 5 min and then centrifuged at 15,000 \( \times \) g for 30 s at 4°C. The sugar concentrations of the supernatant were measured, and the intracellular concentrations were calibrated using the reported intracellular volume of 7.3 \( \mu l \) per 10\(^9\) cells (8). One milligram of cells used in this study corresponded to 1.9 \( \times \) 10\(^9\) cells.

**Analytical methods.** Cell mass was determined using a calibration curve of absorbance at 600 nm versus dry cell weight. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (3). The concentrations of galactose and tagatose were determined by high-performance liquid chromatography (SCL-10A; Shimadzu, Kyoto, Japan) with a re-tractor index detector on a Shodex column (Showa Denko, Kawasaki, Japan). The column was eluted at 30°C with distilled water at a flow rate of 0.4 ml/min. Sugar concentrations of less than 10 mM were determined using an high-performance liquid chromatography instrument (Agilent 1100 series; Agilent, Palo Alto, CA) fitted with a Corona charged aerosol detector (ESA Biosciences, Chelmsford, MA) on a Shodex column (Showa Denko, Kawasaki, Japan). The column was eluted at 30°C with 70% acetonitrile at a flow rate of 1.0 ml/min.

**RESULTS**

**Conversion of galactose to tagatose by the galK and ΔgalK strains.** The ΔgalK strain harboring pTaraA did not grow in the minimal medium without glucose, and it converted little galactose to tagatose due to initially low cell mass. Thus, concentrated suspensions of E. coli MG1655 galK and ΔgalK strains harboring pTaraA were used in 50 mM Tris-HCl buffer (pH 7.0) containing 50 mM galactose and compared with respect to their ability to convert galactose to tagatose. The E. coli MG1655 galK strain gave a very low conversion yield of tagatose at 37°C was 57.0% in the ΔgalK strain harboring pTaraA and 30.2% in a crude extract of this strain. The stability, meaning the decrease of activity during the conversion, was similar for whole cells and the crude extract (Fig. 3B), suggesting that the difference in the conversion yield between the two was not due to a difference in stability. The high tagatose yield in whole cells (57%) compared with crude extract (30%) and purified enzyme (36%) suggests that the presence of the cell membrane affected the shift of equilibrium toward tagatose.

The intra- and extracellular equilibrium fractions of galactose and tagatose were measured using whole cells of the ΔgalK strain harboring pTaraA incubated in 10 mM sugars at three different initial ratios of galactose to tagatose (100:0, 50:50, and 0:100) (Fig. 4). The whole cells did not grow due to the absence of galactose kinase. The intra- and extracellular equilibrium fractions of tagatose converged at 25% and 68%, respectively, reflecting an extracellular equilibrium shift toward tagatose but an intracellular equilibrium shift toward tagatose. The ΔgalK strain grew poorly and consumed little galactose because of the absence of galactose kinase (Fig. 2).

**Equilibrium constants for conversion of galactose to tagatose by whole cells and purified enzyme.** The target spot on a 2D gel was identified as \( \alpha \)-arabinose isomerase by mass analysis and comparison with purified \( \alpha \)-arabinose isomerase (data not shown). The amount of intracellular \( \alpha \)-arabinose isomerase in the ΔgalK strain harboring pTaraA, as determined by image analysis of a 2D gel, was 3.46 mg/g of cells. The cellular \( k_{cat} \) value was calculated from the maximum velocity of the conversion of galactose to tagatose due to initially low cell mass.

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version reaction and the amount of intracellular L-arabinose isomerase. The kinetic parameters were measured using galactose concentrations ranging from 50 to 1,000 mM and tagatose concentrations ranging from 50 to 500 mM for whole cells and from 5 to 100 mM for the purified enzyme. High concentrations of galactose and tagatose were used for determining $K_m$ values because the authentic substrate and product for L-arabinose isomerase are not D-galactose and D-tagatose but L-arabinose and L-ribulose, respectively. The equilibrium constant ($K_{eq}$) for whole cells and purified enzyme was determined using the Haldane equation:

$$K_{eq} = \left[ T \right]_{eq} / \left[ G \right]_{eq} = \left( \frac{k_{cat}}{K_m} \right)_{G} / \left( \frac{k_{cat}}{K_m} \right)_{T},$$

where $G$ is galactose and $T$ is tagatose (7) (Table 1). In whole cells, the $K_{eq}$ was 2.30, which corresponds to a predicted tagatose equilibrium fraction of 69.7%; the $K_{eq}$ for the purified enzyme was 0.53, which corresponds to a predicted tagatose equilibrium fraction of 34.6%. These predicted equilibrium fractions are within 2% of the experimental values obtained for whole cells (67.9%) and the purified enzyme (36.0%). The equilibrium shift toward tagatose isomerization resulted primarily from increased catalytic efficiency ($k_{cat}/K_m$) for galactose owing to an increase in the $k_{cat}$ for galactose.

Uptake and release rates of galactose and tagatose. The uptake and release rates for galactose and tagatose can be measured in the ΔgalK strain without pTaraA. In this strain, the intra- and extracellular concentrations of the sugars are affected only by the sugar transport rates; no interconversion occurs between the two sugars in the absence of pTaraA, and the galK knockout prevents the metabolism of galactose and tagatose via galactose. The slopes of the linear regressions for uptake at 0 to 15 mM sugar and for release at 0 to 0.32 mM sugar were compared between galactose and tagatose in cells. The galactose uptake rate into cells was 2-fold the tagatose uptake rate, whereas the tagatose release rate was 4.4-fold the galactose release rate (Fig. 5). Thus, the extracellular equilibrium shift toward tagatose outside the cells was the result of its

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**TABLE 1. Equilibrium constants and kinetic parameters of cells and purified enzyme for galactose and tagatose**

<table>
<thead>
<tr>
<th>Catalyst and substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$ (min$^{-1}$ mM$^{-1}$)</th>
<th>$K_{eq}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Galactose</td>
<td>256 ± 3.8</td>
<td>253 ± 4.3</td>
<td>0.99 ± 0.022</td>
<td>2.30 ± 0.090</td>
</tr>
<tr>
<td>Tagatose</td>
<td>114 ± 3.2</td>
<td>49 ± 0.88</td>
<td>0.43 ± 0.014</td>
<td></td>
</tr>
<tr>
<td>Purified enzyme</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>365 ± 9.3</td>
<td>62 ± 1.05</td>
<td>0.17 ± 0.005</td>
<td>0.53 ± 0.024</td>
</tr>
<tr>
<td>Tagatose</td>
<td>11 ± 0.28</td>
<td>3.5 ± 0.08</td>
<td>0.32 ± 0.011</td>
<td></td>
</tr>
</tbody>
</table>

*Data represent the means of three separate experiments.*
lower cellular uptake rate and higher cellular release rate relative to the rates of galactose.

Transport systems for galactose and tagatose in cells. Deletions of SETs (ΔsetA, ΔsetB, and ΔsetC) and galactose transporters (ΔmglB and ΔgalP) were combined with the E. coli BW25113 ΔgalK strain harboring pTaraA. For the conversion reaction of galactose to tagatose, the resulting transporter knockout strains were examined. The conversion yields of galactose to tagatose in the ΔsetA, ΔsetB, ΔsetC, ΔgalP, ΔmglB, and ΔgalP ΔmglB mutants were almost the same as those in the original strain (BW25113 ΔgalK strain harboring pTaraA), which was used as the control (Fig. 6). The ΔmglB and ΔgalP ΔmglB mutants showed reduced conversion of galactose to tagatose (40%) compared with the control strain. The conversion yield in a ΔgalP ΔmglB double mutant was almost the same as that in the ΔmglB mutant, suggesting that GalP is not a critical transport system for galactose and tagatose. In the ΔmglB mutant without pTaraA, the uptake rates for galactose and tagatose were 26% and 25%, respectively, of those in the original strain without the transporter knockout (Fig. 5). Since the ΔmglB mutant exhibited significant decreases in conversion yield and uptake of galactose to tagatose, we conclude that the primary transport system for galactose and tagatose uptake is MglABC.

**DISCUSSION**

An increase in reaction temperature combined with use of a hyperthermophilic enzyme resulted in an equilibrium shift toward a tagatose product, resulting in a higher conversion yield (12). To shift the equilibrium position of an isomerization reaction toward tagatose using an alternative method, we exploited the membrane selectivity of cells resulting in intra- and extracellular concentration changes for a galactose substrate and tagatose product at equilibrium. The equilibrium shift toward tagatose was caused by the compartmentalization of intra- and extracellular regions of the cell membrane, with a lower cellular uptake rate relative to that of galactose and the higher cellular release rate for tagatose relative to that of galactose.

In whole cells of the ΔgalK strain, the isomerase-catalyzed reaction exhibited an equilibrium shift toward tagatose, producing a tagatose fraction of 68% at 37°C, whereas the purified L-arabinose isomerase gave a tagatose equilibrium fraction of 36%. These equilibrium fractions are close to those predicted from the measured equilibrium constants of the isomerization reaction catalyzed in whole cells and by the purified enzyme. The values of the equilibrium constant $K_{eq}$ were determined from the kinetic parameters for galactose and tagatose using the Haldane equation. Although the kinetic parameters were dependent on the sugar concentrations, the $K_{eq}$ values appear to be reasonable because the relative values of the kinetic parameters are used. The predicted equilibrium fractions obtained from the $K_{eq}$ values accorded well with the experimental values of equilibrium fractions.

MglABC is a β-methylgalactoside transport system that is a member of the ABC superfamily of transporters. The mglA, mglB, and mglC genes encode the ATP-binding, galactose-binding, and integral membrane components of the ABC transporter, respectively (9). Insertion mutations in each gene indicate that all three components are necessary for galactose transport function. Therefore, the ΔmglB mutant is function-
FIG. 7. A schematic diagram of the conversion from galactose to tagatose by the purified enzyme and the ΔgalK strain harboring pTaraA. For cells, the galactose uptake rate was 2.0-fold the tagatose uptake rate, whereas the tagatose release rate was 4.4-fold the galactose release rate.

The same ally with respect to galactose transport as a mutant lacking the MglABC transporter. A different transporter, GalP, is a member of the major facilitator superfamily of transporters, which act as a major route for galactose transport (22), and SetA, SetB, and SetC are probable efflux transporters for sugars (21). The finding that the knockout of GalP and SET transporters did not affect the cellular conversion of galactose to tagatose suggests that the MglABC transporter plays a major role in galactose uptake and that SET transporters have nothing to do with the release of galactose and tagatose. However, these results may be due to the continued operation of other unidentified transporters, similar to the MglABC transporter, in the mutants, thereby maintaining transport rates in the transporter knockout cells. The effect of the mglB gene knockout on both the galactose and tagatose uptake rates suggests that the MglABC transporter is a primary transport system for not only galactose but also tagatose, although its uptake rate for galactose is higher. The higher selectivity of the ATP-binding MglABC transporter for galactose over tagatose may be attributable to a higher preference for a common sugar over a rare sugar in cells using ATP. In the present study, the equilibrium shift was identified based on direct experimental evidence; it was also explained theoretically using equilibrium constants for a galactose substrate and tagatose product derived from the kinetic parameters of whole cells and the purified enzyme. Moreover, this study also demonstrated that MglABC is a primary transporter for tagatose as well as galactose in E. coli. The conversion of galactose to tagatose by whole cells compared with the purified enzyme was summarized as a schematic diagram (Fig. 7). Whole-cell conversion is proposed as a new alternative method for shifting the equilibrium of the isomerization reaction instead of using temperature to control enzymatic processes. If other sugar isomerization reactions converting a common sugar to a rare sugar were also to be shifted by changing membrane selectivity, the production of rare sugars could be improved.

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