JOURNAL OF BACTERIOLOGY, Mar. 2007, p. 1597–1603 Vol. 189, No. 5
0021-9193/07/$08.00+0 doi:10.1128/JB.01402-06

The L-Tartrate/Succinate Antiporter TtdT (YgjE) of L-Tartrate Fermentation in *Escherichia coli*▽

Ok Bin Kim and Gottfried Unden*

Institut für Mikrobiologie und Weinforschung, Johannes Gutenberg-Universität Mainz, Becherweg 15, 55099 Mainz, Germany

Received 1 September 2006/Accepted 11 December 2006

Escherichia coli **ferments L-tartrate under anaerobic conditions in the presence of an additional electron donor to succinate. The carrier for L-tartrate uptake and succinate export and its relation to the general C_4-dicarboxylate carriers DcuA, DcuB, and DcuC were studied. The secondary carrier TtdT, encoded by the *ttdT* (previously called *ygjE*) gene, is required for the uptake of L-tartrate. The *ttdT* gene is located downstream of the *ttdA* and *ttdB* genes, encoding the L-tartrate dehydratase TtdAB. Analysis of mRNA by reverse transcription-PCR showed that *ttdA*, *ttdB*, and *ttdT* are cotranscribed. Deletion of *ttdT* abolished growth by L-tartrate and degradation of L-tartrate completely. Bacteria containing TtdT catalyze L-tartrate or succinate uptake and specific heterologous L-tartrate/succinate antiporting. D-Tartrate is not a substrate for TtdT. TtdT operates preferentially in the direction of tartrate uptake and succinate excretion. The Dcu carriers do not support anaerobic growth on L-tartrate or L-tartrate transport. TtdT is related in sequence and function to CitT, which catalyzes heterologous citrate/succinate antiporting in citrate fermentation.**

Enteric bacteria such as *Escherichia coli* are able to use C_4-dicarboxylates, such as fumarate, malate, and aspartate, for anaerobic growth (29). Under anaerobic conditions, the C_4-dicarboxylates are converted to fumarate, which is then used for fumarate respiration. Conversion of malate and aspartate to fumarate is catalyzed by the dehydratases fumarase and aspartase (7, 13, 16). Due to repression of the citric acid cycle under these conditions, succinate cannot be oxidized further and is excreted (for reviews, see references 2, 12, 19, and 27). The transport of the C_4-dicarboxylates is effected by carriers which are specifically produced under anaerobic conditions (5, 12, 26, 32, 33, 34). DcuB functions as a C_4-dicarboxylate/succinate antiporter which catalyzes electroneutral antiporting of the external C_4-dicarboxylates (generally fumarate, malate, and aspartate) against succinate as the end product of fumarate respiration (6). DcuB is synthesized under conditions of fumarate respiration, i.e., under anoxic conditions, in the presence of external C_4-dicarboxylates (8, 9, 12, 14, 29, 33). DcuB can be replaced or supported by the carrier DcuA or DcuC. DcuA is homologous to DcuB but is expressed constitutively (8). DcuC normally functions as a succinate efflux carrier during glucose fermentation, but it can take over fumarate/succinate antiporting when required (32, 34).

Anaerobic tartrate degradation was recognized early as a significant microbiological process, but it has not been studied in much detail (1, 10, 21, 25, 30). Utilization of tartrate requires the presence of an oxidizable cosubstrate, such as glucose or glycerol. L-Tartrate is dehydrated by L-tartrate dehydratase (TtdAB) to oxaloacetate, which is converted via malate to fumarate (Fig. 1) (24). The reducing equivalents are required for the function of malate dehydrogenase and fumarate reductase. The carrier for the uptake of L-tartrate (and the export of succinate) in tartrate fermentation is not known. DcuB would be an obvious candidate for the transport of L-tartrate, since it has a broad substrate specificity and transports C_4-dicarboxylates, such as fumarate, malate, and aspartate, in antiport against succinate (5, 6). In addition, DcuB is expressed under anaerobic conditions in the presence of tartrate (14). From database analysis, it has been suggested, however, that the *ygjE* (the putative *ttdT* gene) gene, which is located downstream of the *ttdA* and *ttdB* genes (encoding L-tartrate dehydratase), encodes a specific secondary carrier for tartrate/succinate antiporting (22). It was tested, therefore, whether TtdT is required for tartrate/succinate antiporting or whether one of the Dcu carriers is used for this purpose. It turned out that L-tartrate, the major form of tartrate in nature, requires TtdT for transport. The properties of L-tartrate transport by the TtdT carrier and the differences from the general Dcu carriers were determined.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are shown in Table 1. Growth of the bacteria for mRNA isolation and transport experiments was performed in M9 medium (17) supplemented with acid-hydrolyzed casein (Gibco BRL) (0.1% [wt/vol]) and L-tryptophan (0.005% [wt/vol]) (15). For anaerobic growth, cultures were incubated at 37°C in degassed medium in rubber-stoppered infusion bottles under N_2. For growth tests, strains with a deleted *ttdT* gene or inactivated genes for other C_4-dicarboxylate carriers were grown in supplemented M9 medium under anoxic conditions. A 20 mM concentration of L-tartaric acid (potassium-sodium salt), D-tartaric acid (sodium salt), *meso*-tartrate (sodium salt), or fumarate (fumaric acid disodium salt) was included as an electron acceptor, and 20 mM of glycerol was included as an electron donor.

Genetic methods. (i) Inactivation of *ttdT* (*ygjE*). The *ttdT* gene was deleted by the method of Datsenko and Wanner (4), using the PCR product of the chloramphenicol resistance (Cm^r) cassette from plasmid pKD3 flanked by FRT sequences. For inactivation of *ttdT*, primers ttdTH1P1/2 (5′-TAA CCC TCC CGG AGA GGC TCA CCC CTC TCC TTT TTC GCA GGC ATA ACA CGG TGT AGG CTG GAG CTG CTT C-3′) and ttdT_H2P2/2 (5′-TGC GTA AAA CTA TTG GGT GCG CCA GAG CAA TTT CCG GCA CCG TCC TCA CTC ATA TGA ATA TCC TCC TTA G-3′), which contain parts of the regions adjacent to

* Corresponding author. Mailing address: Gottfried Unden, Institut für Mikrobiologie und Weinforschung, Johannes Gutenberg-Universität Mainz, Becherweg 15, 55099 Mainz, Germany. Phone: 49-6131-3923550. Fax: 49-6131-3922695. E-mail: unden@uni-mainz.de.

▽ Published ahead of print on 15 December 2006.

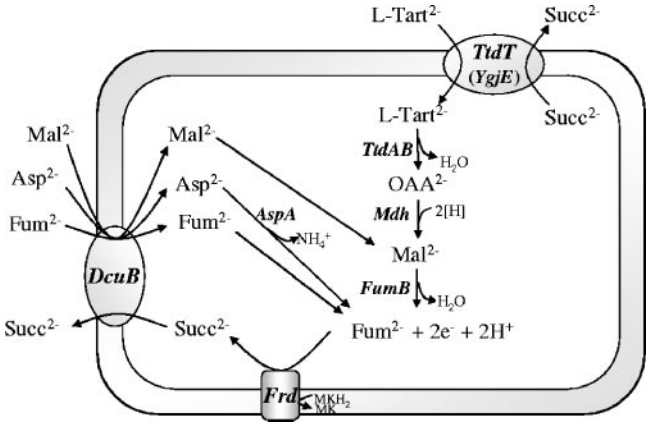

FIG. 1. Comparison of the pathways for anaerobic conversion of L-tartrate and other C_4-dicarboxylates (fumarate, malate, and aspartate) in *Escherichia coli*. The pathways require a supply of external electron donors for fumarate and oxaloacetate reduction. The enzymes and carriers for the pathways are (i) a C_4-dicarboxylate/succinate antiporter (DcuB), (ii) fumarase (FumB), (iii) aspartase (AspA), (iv) fumarate reductase (Frd), (v) (putative) tartrate carrier (TtdT, YgjE), (vi) L-tartrate dehydratase (TtdAB), and (vii) malate dehydrogenase (Mdh), menaquinone (MK), and menaquinol (MKH_2).

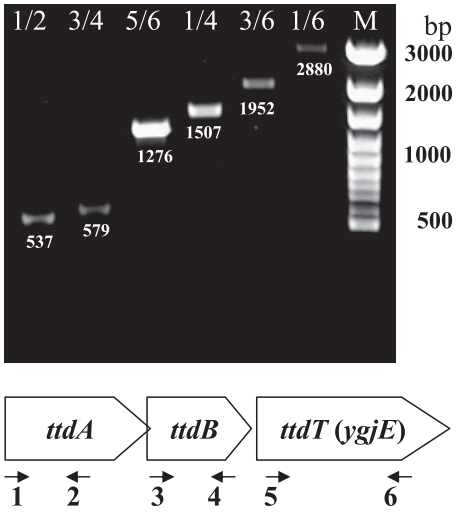

FIG. 2. Detection of *ttdA*, *ttdB*, and *ttdT* transcripts in *E. coli* LJ1 mRNA by RT-PCR. Isolated total mRNA from bacteria grown anaerobically on L-tartrate and glycerol was reversely transcribed by RT-PCR, using primer ttdT_rev_RT, and the product cDNA was used for PCRs using primers located in the *ttdA*, *ttdB*, and *ttdT* genes in various combinations (see lower part of the figure). The primer combinations are given at the top of each track, and the calculated lengths of the products (bp) are given below the bands. M, 1-kb DNA ladder.

FRT and of the target gene *ttdT*, were used. The PCR product was purified, concentrated, and used for transformation. Cm^r colonies were tested for loss of the helper plasmid (pKD46) by ampicillin sensitivity. For deletion of the Cm^r cassette, the *ttdT*::Cm^r mutant was transformed with the FLP helper plasmid pCP20 and selected at 30°C (3). The *ttdT*::Cm^r and Δ*ttdT* mutant genotypes were verified by PCRs with test primers ttdT_test_frd (5′-CGA CAA CCA GTA TGC GTA AG-3′), ttdT_test_rev (5′-AGT GCT AAC CGT ACG TTA CG-3′), cat_frd (5′-GAG ATT ATG TTT TTC GTC TCA GCC AAT CC-3′), cat_rev (5′-CTA TCC CAT ATC ACC AGC TCA CCG TCT TTC-3′), and cat_mitte (5′-CTC TGG AGT GAA TAC CAC GAC-3′). For construction of mutants IMW528 and IMW529, *dcuA*::Spc^r, *dcuB*::Kan^r, *dcuC*::mini-Tn*10* (Cm^r), and *citT*::Kan^r were transduced successively by bacteriophage $P1_{kc}$ in strains LJ1 and IMW522 (Table 1).

(ii) RT-PCR. Total mRNA was isolated from *E. coli* LJ1 grown anaerobically on L-tartrate (50 mM) and glycerol (50 mM) to an optical density at 578 nm (OD_{578}) of 0.7, using Protect Bacteria reagent (QIAGEN) and an RNeasy Mini kit (QIAGEN). The mRNA was transcribed into cDNA with SuperScript III reverse transcriptase (Invitrogen), using primer ttdT_rev_RT (5′-CGT ATG GTG TAA GAA TGC TC-3′). The cDNA was amplified by PCRs using three forward (ttdA_frd_RT [5′-GAT GAG CGA AAG TAA TAA GC-3′], ttdB_frd_RT [5′-CGA TCA AAG CTG AAG ATC TG-3′], and ttdT_frd_RT [5′-GTC ATC GCC ATT ATT GCT CT-3′]) and three reverse (ttdA_rev_RT [5′-TCG AAG ACG AAT TTC ACC AC-3′], ttdB_rev_RT [5′-TGA TGT AAT GGA CGT GCT CG-3′], and ttdT_rev_RT [5′-CGT ATG GTG TAA GAA TGC TC-3′]) primers, which are located in the genes *ttdA*, *ttdB*, and *ttdT*, in various combinations (Fig. 2).

Transport of D,L-[^{14}C]tartrate and [^{14}C]succinate. For transport experiments, the bacteria were grown anaerobically on L-tartrate (50 mM) and gluconate (50 mM) in supplemented M9 medium to an OD_{578} of 0.7 to 0.8. After being washed with degassed ice-cold phosphate buffer (100 mM sodium-potassium phosphate, 1 mM $MgSO_4$, pH 7), the bacteria were suspended to an OD_{578} of 8 in the same

TABLE 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Genotype	Reference or cross
E. coli strains		
LJ1	MG1655, but *fnr*$^+$	K. Jahreis (Osnabrück)
JRG2814	AN387, but *dcuA*::Spc^r *dcuB*::*Kan*^{r}	26
IMW157	AN387, but *dcuC*::mini-Tn*10* (Cm^r)	32
IMW159	AN387, but *dcuA*::Spc^r *dcuC*::mini-Tn*10* (Cm^r)	32
IMW277	AN387, but *dcuA*::Spc^r *dcuB*::Kan^r *dcuC*::mini-Tn*10* (Cm^r) *citT*::Kan^r	11
IMW516	MG1655, but *dctA*::Spc^r *dcuA*::Spc^r *dcuB*::Kan^r *ttdT*::Cm^r	This work
IMW518	LJ1, but *dcuA*::Spc^r *dcuB*::Kan^r	P1 (JRG2814) × LJ1
IMW519	LJ1, but *ttdT*::Cm^r	P1 (IMW516) × LJ1
IMW520	LJ1, but *dcuA*::Spc^r *dcuB*::Kan^r *dcuC*::Tn*10* (Cm^r)	P1 (IMW157) × IMW518
IMW521	LJ1, but *dcuA*::Spc^r *dcuB*::Kan^r *ttdT*::Cm^r	P1 (JRG2814) × IMW519
IMW522	IMW521, but Δ*ttdT*	This work
IMW524	LJ1, but *dcuA*::Spc^r *dcuB*::Kan^r *dcuC*::Tn*10* (Cm^r) Δ*ttdT*	P1(IMW157) × IMW522
IMW528	LJ1, but *dcuA*::Spc^r *dcuB*::Kan^r *dcuC*::Tn*10* (Cm^r) *citT*::Kan^r	P1(IMW277) × IMW518
IMW529	LJ1, but *dcuA*::Spc^r *dcuB*::Kan^r *dcuC*::Tn*10* (Cm^r) *citT*::Kan^r Δ*ttdT*	P1(IMW277) × IMW522
Plasmids		
pKD3	*oriR*γ *cat bla* Δ(*phoB-phoR*)*580 galU95* Δ*uidA3*::*pir*$^+$ Δ*endA*::FRT	4
pKD46	*oriR101 repA101*(ts) *araBp-gam-bet-exo bla*	4
pCP20	FLP$^+$ λ*cI857* λ p_R*Rep*^{ts} *cat bla*	4

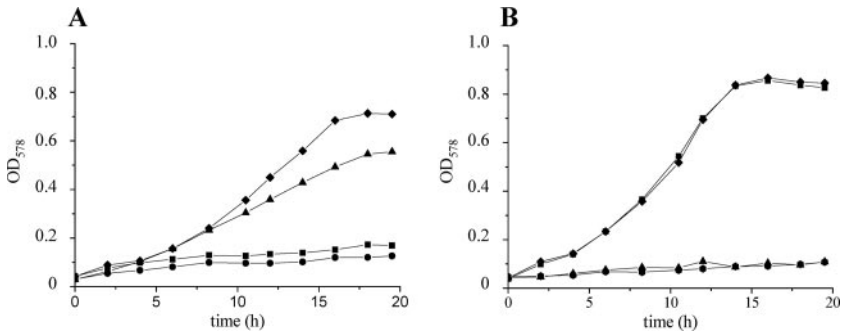

FIG. 3. Anaerobic growth of *E. coli ttdT* mutant strains on (A) glycerol and L-tartrate and (B) glycerol and fumarate. Growth was performed in supplemented M9 medium with the addition of glycerol (20 mM) and L-tartrate (20 mM) (A) or glycerol (20 mM) and fumarate (20 mM) (B). Strains of *E. coli* used were LJ1 (◆, wild type), IMW519 (■, *ttdT* mutant), IMW528 (▲, *dcuA dcuB dcuC citT* mutant), and IMW529 (●, *dcuA dcuB dcuC citT ttdT* mutant).

buffer, which was subsequently degassed. Glucose was added, and the suspension was incubated at 37°C for 5 min (energization). If required, the protonophore CCCP (carbonyl cyanide *m*-chlorophenylhydrazone; Sigma) (20 μM) was added after 3 min of energization and incubated for 2 min before use in transport measurement. For the other experiments, anaerobic solutions and suspensions were used, but the transport experiments were performed under air. The identity and purity of D,L-[^{14}C]tartrate (D,L-[1,4-^{14}C]tartaric acid at 3,700 MBq/mmol; American Radiolabeled Chemical, Inc.) were tested and verified by high-performance liquid chromatography (HPLC) on an Aminex HPX87H column (300 × 7.8 mm; Bio-Rad), with authentic D- and L-tartrate, and by thin-layer chromatography on Merck F254 silica gel 60 with the solvent *n*-propanol–saturated ammonium hydroxide–distilled water (6/3/1 [vol/vol]) (http://www.nat-working.uni-jena.de/NaTWorkingMaterialien.htm).

For measurements of uptake activity, 50 μl of energized cell suspension was added to 50 μl of D,L-[^{14}C]tartrate (3,700 MBq/mmol) or [2,3-^{14}C]succinate (Moravek Biochemicals, Inc.) (1,628 MBq/mmol) at various concentrations at 37°C in 1.5-ml reaction tubes. After various times, the reaction was stopped by the addition of 0.9 ml of ice-cold 0.1 M LiCl followed by rapid vacuum filtration through membrane filters (mixed cellulose ester, type ME 24; diameter, 25 mm; 0.2-μm pore size) (Schleicher & Schuell MicroScience). The filters were washed three times with ice-cold 0.1 M LiCl, transferred to scintillation vials with 4 ml of scintillation liquid, and counted for D,L-[^{14}C]tartrate or [^{14}C]succinate. Generally, all transport assays and experiments were performed at least in triplicate.

In the antiport assay, energized bacteria from the anaerobic cell suspension (460 μl) were preloaded by being mixed with 460 μl [^{14}C]succinate or D,L-[^{14}C]tartrate (200 μM). After 10 min, 405 μl of the loaded cell suspension was removed and added to 8.2 μl of antiport substrate (L-tartrate or D-tartrate potassium sodium salt, succinate disodium salt, fumarate disodium salt, citrate trisodium salt, and butyrate sodium salt at 100 mM [each]) to give a final concentration of 2 mM antiport substrate. The reaction was stopped after 20 s and 1, 2, and 5 min by mixing 100 μl of the cell suspension with LiCl as described above, followed by vacuum filtration to determine the amount of intracellular [^{14}C]succinate or [^{14}C]tartrate. The transport activities were calculated from the changes in the intracellular concentration of the D,L-[^{14}C]tartrate or [^{14}C]succinate (25.7 and 33.4 Bq/nmol, respectively) by measuring the radioactivity of the cells, assuming that an OD_{578} of 1 corresponds to 281 mg dry weight/liter of *E. coli* cells (32).

Identification of fermentation products by HPLC. The fermentation products in the supernatants of media from growth experiments were determined after removal of bacteria by centrifugation. The substrates and products in the supernatant were analyzed by HPLC on an Aminex HPX87H column (300 × 7.8 mm; Bio-Rad) at 65°C with buffer (6.5 mM H_2SO_4) at a flow rate of 550 μl/min (28) and were quantified by UV (215 nm) and refractive index detection.

RESULTS AND DISCUSSION

The *ttdT* (or *ygjE*) gene is part of the *ttd* operon. The *ttdA* and *ttdB* genes encode the O_2-labile L-tartrate dehydratase (L-Ttd or TtdAB) (24). The *ygjE* (presumptive *ttdT*) gene downstream of *ttdAB* shows similarity to genes for secondary carriers of the CitT family and was suggested to encode a tartrate carrier for anaerobic growth (22). Total mRNA was isolated from a wild-type strain of *E. coli* grown anaerobically on L-tartrate plus glycerol. The *ttd* mRNA was reversely transcribed by RT-PCR, using a primer specific for the 3′ end of the *ttdT* gene. The cDNA was then amplified with three forward and three reverse primers in various combinations, one of which was located in each of the three genes (Fig. 2). This should allow reverse transcription of each of the genes (*ttdA*, *ttdB*, and *ttdT*) separately, in pairs, or as the complete set of genes, depending on the mRNA present. Each of the primer pairs provided products, including transcripts corresponding to *ttdA-ttdB*, *ttdB-ttdT*, and the *ttdA-ttdB-ttdT* total transcript. The products were of the predicted sizes if one assumes cotranscription of the corresponding single genes. The amounts of the cDNAs containing *ttdA* and the complete fragment *ttdABT* were comparable, indicating that the *ttdA-ttdB-ttdT* mRNA is not a minor form.

Prediction of promoter regions by PRODORIC (18; http://www.prodoric.de), which is based on the G+C content and the calculated stacking energy of DNA segments to predict T+A-rich promoter regions, suggested a promoter region in front of *ttdA* only, not in front of the other *ttd* genes. In addition, the intergenic region between *ttdB* and *ttdT* is small (47 bp), and the *ttdA* and *ttdB* genes even overlap by 4 bp, supporting the view that no additional promoter site is present in the *ttdABT* region.

Growth defects in *ttdT* mutants. Strains with a deleted *ttdT* gene were tested for anaerobic growth on L-tartrate, with glycerol as an electron donor (Fig. 3). For the experiments, a strain deleted in genes encoding DcuA, DcuB, DcuC, and CitT, representing the carriers for C_4-dicarboxylates in anaerobic growth (12, 26, 29, 32) and for citrate/succinate antiporting (22), was used as the genetic background to avoid interference from other C_4-dicarboxylate carriers. The *dcuA dcuB dcuC citT* strain was only slightly impaired for growth on L-tartrate with glycerol (Fig. 3A). A mutant with a deleted *ttdT* gene in the *dcuA dcuB dcuC citT* background completely lost the capability for growth by L-tartrate fermentation (Fig. 3A). The same result was observed for a *ttdT* deletion in the wild-type background. Anaerobic growth on fumarate plus glycerol, on the other hand, was not affected by inactivation of *ttdT* (Fig. 3B) but required the presence of the *dcuA*, *dcuB*, and *dcuC* genes, as described earlier (26, 32). Therefore, growth by L-tartrate

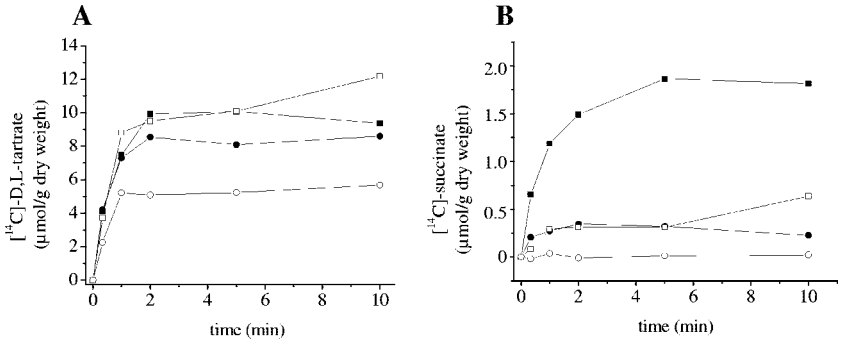

FIG. 4. Kinetics of uptake of D,L-[^{14}C]tartrate (A) and [^{14}C]succinate (B) in cell suspensions of *E. coli* IMW528 (■ and ●, *dcuA dcuB dcuC citT* mutant) and IMW529 (□ and ○, *dcuA dcuB dcuC citT ttdT* mutant). Growth was performed in supplemented M9 medium under anoxic conditions with gluconate (50 mM) and L-tartrate (50 mM) (■ and □) or with gluconate (50 mM), L-tartrate (50 mM), and glucose (20 mM) (● and ○). Intracellular concentrations of D,L-[^{14}C]tartrate and [^{14}C]succinate were determined by filtration after incubation of the cell suspensions (OD_{578} = 4) of the bacteria with a 100 μM concentration of the labeled substrates.

fermentation depends specifically on the presence of TtdT, which cannot be replaced by the Dcu carriers, and vice versa.

Kinetics of tartrate and succinate transport by the TtdT carrier. The kinetics of anaerobic tartrate transport was analyzed with cell suspensions of bacteria grown under inducing conditions in the presence of L-tartrate. To exclude interference from other known C_4-dicarboxylate carriers, a strain lacking carriers DcuA, DcuB, DcuC, and CitT was used. After the addition of D,L-[^{14}C]tartrate, representing a mixture of D-[^{14}C]- and L-[^{14}C]tartrate, uptake started rapidly, and cellular contents reached constant levels after about 2 min (Fig. 4A). In the *ttdT* mutant which lacked TtdT in addition to the Dcu and CitT carriers, uptake was not significantly impaired. This is presumably due to alternative uptake carriers (YeaV, YfaV, and others) for tartrate and other C_4-dicarboxylates which are formed in addition to the Dcu and DctA carriers when C_4-dicarboxylates are present (unpublished data). Succinate, the proposed antiporter substrate of L-tartrate, was taken up by the TtdT$^+$ strain as well (Fig. 4B), but at a significantly lower rate. However, in the *ttdT* mutant, the succinate uptake rate was decreased to very low levels, indicating that succinate uptake in the *dcuA*-, *dcuB*-, *dcuC*-, and *citT*-negative strain depends on the presence of TtdT.

Succinate uptake was strongly induced in L-tartrate-grown bacteria and repressed in glucose-grown bacteria (Fig. 4). In contrast, succinate did not induce (not shown). Induction by tartrate and repression by glucose are compatible with a role of TtdT in L-tartrate metabolism. Tartrate uptake was also induced by tartrate and repressed by glucose, though to a lesser degree. The background levels without induction and under glucose repression corresponded to about one-half the maximal activity. This background activity and the high residual activity after deletion of *ttdT* suggest that the bacteria contain a further tartrate uptake carrier which supports tartrate uptake but not antiporting and growth by tartrate fermentation. Expression analysis and transport assays support the presence of a further uptake carrier for tartrate and other C_4-dicarboxylates (11; unpublished data). The alternative carriers which do not support growth by L-tartrate fermentation are presumably uptake carriers and lack tartrate/succinate antiporting capacity (unpublished data).

[^{14}C]succinate and [^{14}C]tartrate accumulated to final concentrations of 640 μM and 4 mM, respectively, within the bacteria, corresponding to 6.4- and 40-fold accumulations of the substrates (100 μM) in the external medium. The transport rates were highest around neutral pH (pH 7 to 8), exceeding the activities at pHs 5 to 6 by factors of 1.8 to 3.7. The initial rates for tartrate and succinate uptake increased with increas-

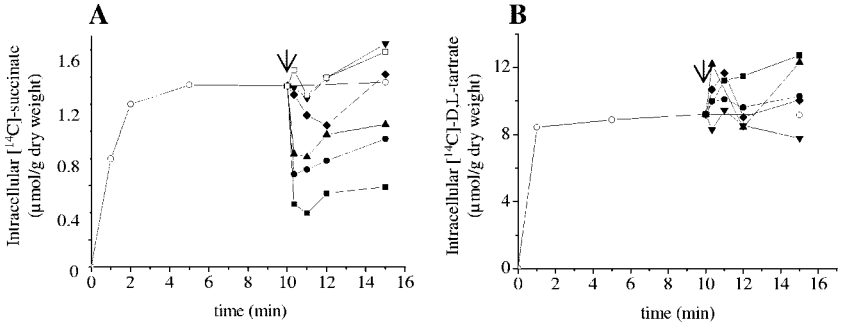

FIG. 5. Efflux of [^{14}C]succinate (A) and D,L-[^{14}C]tartrate (B) from *E. coli* TtdT$^+$ CitT$^-$ strain by the addition of external antiporter substrate (arrow). Degassed cell suspensions of *E. coli* IMW528 (*dcuA dcuB dcuC citT* mutant) were incubated at an OD_{578} of 4 with 100 μM [^{14}C]succinate (A) or 100 μM D,L-[^{14}C]tartrate (B) (open circles). After 10 min of loading, 2 mM (A) or 20 mM (B) L-tartrate (■), D-tartrate (□), succinate (●), fumarate (▲), citrate (◆), or butyrate (▼) was added to the suspension. At the time points indicated, samples were drawn, and the amount of internal [^{14}C]succinate or D,L-[^{14}C]tartrate was determined by a filtration assay and scintillation counting.

TABLE 2. Efflux of [^{14}C]succinate or D,L-[^{14}C]tartrate from loaded cells of *E. coli* TtdT$^+$ CitT$^-$ (IMW528 [*dcuA dcuB dcuC citT* mutant]) after the addition of 2 mM external antiporter substrate

Antiporter substrate (2 mM)	Antiporting activity (μmol/min/g dry wt)[a]	
	[^{14}C]succinate	D,L-[^{14}C]tartrate
L-Tartrate	2.6	<0.2
Succinate	2.0	<0.2
Fumarate	1.5	<0.2
Citrate	0.2	ND[b]
Butyrate	0.1	ND[b]

[a] Activities were determined as described in the experiments shown in Fig. 5 as the release of internal [^{14}C]succinate or D,L-[^{14}C]tartrate 20 s after the addition of the external exchange substrate.
[b] ND, not detectable.

ing concentrations with Michaelis-Menten-type kinetics and showed a linear relation of $1/V$ to $1/S$ in a Lineweaver-Burk plot. The K_m and V_{max} were about 700 μM and 110 μmol/min/g dry weight for tartrate (L- and D-tartrate mixture) and 400 μM and 16 μmol/min/g dry weight for succinate in L-tartrate-grown bacteria.

TdtT-dependent L-tartrate/succinate antiporting. TtdT is suggested to function as an L-tartrate/succinate antiporter, taking up tartrate and excreting succinate. To test the antiporter capacity, anaerobically grown and L-tartrate-induced bacteria were loaded with [^{14}C]succinate or [^{14}C]tartrate by incubation with the substrate. Up to 1.3 or 8.4 μmol succinate or tartrate, respectively, per g dry weight was taken up by bacteria which contained the TtdT carrier but were deficient in the Dcu and CitT carriers (Fig. 5). After the substrates reached maximal levels, unlabeled substrates were added in excess, and the effect on the intracellular levels of the loaded substrates was determined. The [^{14}C]succinate-loaded cells released most of the internal succinate rapidly after the addition of external L-tartrate, which was the most efficient antiporter agent, followed by succinate and fumarate (Fig. 5A and Table 2). Citrate was a poor substrate for antiporting, and D-tartrate and butyrate caused no significant release of the internal [^{14}C]succinate. Thus, internal [^{14}C]succinate is released preferentially by antiporting against external L-tartrate (Table 2). Internal [^{14}C]tartrate, on the other hand, was not released in significant amounts with external C_4-dicarboxylates and related compounds (Fig. 5B and Table 2). The antiporter experiment with loaded [^{14}C]tartrate was performed in a similar way with bacteria which were not energized with glucose (not shown). Under these conditions, loading with [^{14}C]tartrate was slower and slightly lower, but the response to the antiporter substrates was very similar, i.e., the addition of the external C_4-dicarboxylates caused no release of the internal [^{14}C]tartrate. In the absence of glucose, no reducing agent for the conversion of tartrate is present and tartrate is not metabolized, meaning that the external substrates are indeed not able or are able only to a limited extent to release internal [^{14}C]tartrate. Therefore, antiporting in the TtdT$^+$ strain is rather specific for L-tartrate and succinate and functions preferentially in L-tartrate uptake and succinate efflux (L-tartrate$_{ex}$/succinate$_{in}$), whereas the reverse reaction (L-tartrate$_{in}$/succinate$_{ex}$) is not efficient.

In a strain lacking TtdT (in addition to DcuA, DcuB, and DcuC) but containing CitT, the succinate uptake was decreased about 50% (Fig. 6). The addition of citrate caused the most efficient release of succinate (1.32 μmol/min/g dry weight), followed by L-tartrate (0.84 μmol/min/g dry weight) and succinate (0.81 μmol/min/g dry weight), suggesting that in this strain the most efficient antiporter is the citrate/succinate antiporter and that the tartrate/succinate antiporter in strains lacking TtdT indeed relies on the presence of this carrier. When TtdT as well as CitT was missing in addition to the Dcu carriers, the release of intracellular [^{14}C]succinate was very low in similar experiments (not shown).

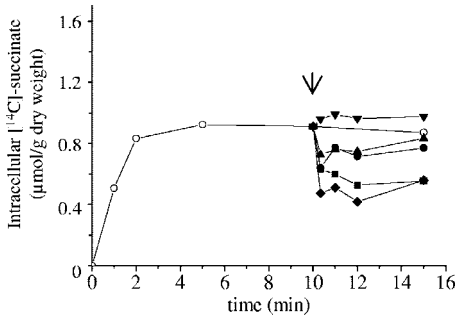

FIG. 6. Efflux of [^{14}C]succinate from *E. coli* TtdT$^-$ CitT$^+$ strain by the addition of external antiporter substrate (arrow). Degassed cell suspensions of *E. coli* IMW524 (*dcuA dcuB dcuC ttdT* mutant) (OD_{578} = 4) were incubated with 100 μM [^{14}C]succinate (open circles) for 10 min. A 2 mM concentration of L-tartrate (■), succinate (●), fumarate (▲), citrate (◆), or butyrate (▼) was added to the suspension. At the time points indicated, samples were drawn, and the amount of internal [^{14}C]succinate was determined by a filtration assay.

Competitive inhibition of [^{14}C]tartrate transport. For more information on the substrate specificity of [^{14}C]succinate and [^{14}C]tartrate uptake by L-tartrate-grown *E. coli*, the uptake was measured in the presence of alternative unlabeled substrates (Fig. 7). The unlabeled substrates were added in a 10-fold excess of the amount of [^{14}C]succinate. In strain IMW528 (*dcuA dcuB dcuC citT*), which contains only TtdT among the known anaerobic C_4-dicarboxylate carriers, the activity for the uptake of [^{14}C]succinate was higher, by a factor of 1.9, than

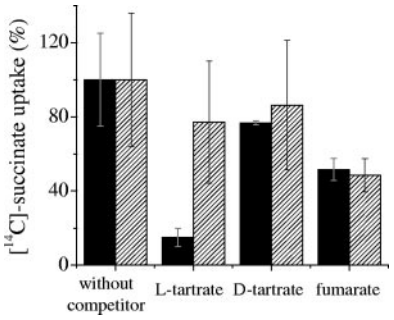

FIG. 7. Effects of competitor substrates on the uptake of [^{14}C]succinate by *E. coli* IMW528 (*dcuA dcuB dcuC citT* mutant) (black bars) and IMW529 (*dcuA dcuB dcuC citT ttdT* mutant) (hatched bars). Uptake of 50 μM [^{14}C]succinate was determined after incubation of anaerobic cell suspensions for 1 min with the substrate, without and in the presence of unlabeled competitors (500 μM). A 100% uptake activity corresponds to 0.53 μmol/min/g dry weight for *E. coli* IMW528 and to 0.28 μmol/min/g dry weight for IMW529.

that in the TtdT-deficient strain IMW529 (*dcuA dcuB dcuC citT ttdT*). In the TtdT$^+$ strain, the presence of 500 μM L-tartrate inhibited 85% of the uptake activity for [^{14}C]succinate, whereas D-tartrate and fumarate had much smaller inhibitory effects. Unlabeled succinate (not shown) decreased the uptake of [^{14}C]succinate in the same strain to 45% of the original activity. The presence of additional (uptake) carriers for succinate which function in the presence of high concentrations of succinate (11) can explain the relatively low degree of competitive inhibition. Overall, the competition experiments are complicated by (so far unknown) carriers for C_4-dicarboxylates which are present in *E. coli* in addition to the DctA, DcuA, DcuB, DcuC, CitT, and TtdT carriers, but the experiments support the specificity of TtdT for L-tartrate. In the *ttdT*-negative strain, the activity was not significantly inhibited by L-tartrate, and the effects of the other C_4-dicarboxylates were comparable to those in the TdtT$^+$ strain (Fig. 7). Therefore, in this background, another C_4-dicarboxylate carrier which is not L-tartrate specific probably operates. However, interference from other carriers was only observed for tartrate or C_4-dicarboxylate uptake by TtdT, i.e., for partial reactions. No interference or nonspecificity was found for the complete reaction, as demonstrated by the lack of antiporting of L-tartrate$_{ex}$/succinate$_{in}$ and of growth on L-tartrate in the *ttdT* deletion strain (Fig. 3A).

TtdT is the L-tartrate/succinate antiporter of L-tartrate fermentation in *E. coli*. The TtdT (previously called YgiE) carrier has been shown here to be the L-tartrate/succinate antiporter which operates in anaerobic L-tartrate fermentation. The carrier is essential for growth by L-tartrate fermentation and for anaerobic L-tartrate degradation, and it preferentially catalyzes heterologous L-tartrate/succinate antiporting, which proves its function as the L-tartrate/succinate antiporter of L-tartrate fermentation. The antiporter activity in the physiological direction (L-tartrate$_{ex}$/succinate$_{in}$) is higher, by a factor of >13, than the reverse activity (succinate$_{ex}$/tartrate$_{in}$). The transport experiments demonstrated that *E. coli* contains an additional (unknown) tartrate carrier which catalyzes uptake only, with no antiporting. This additional transport does not play a role in anaerobic growth on tartrate.

Transport of L-tartrate appears to be the only transport of a C_4-dicarboxylate in the anaerobic metabolism of *E. coli* which does not use the general Dcu carriers. DcuB has broad substrate specificity and accepts all physiologically relevant C_4-dicarboxylates (succinate, fumarate, malate, and aspartate) apart from L-tartrate. Surprisingly, the two vicinal -OH groups at L-tartrate obviously are not accepted by the Dcu carriers. TtdT is much more specific and accepts only L-tartrate, not D-tartrate, as the external substrate. In addition, TtdT is selective with respect to the transport mode and preferentially catalyzes heterologous antiporting (L-tartrate/succinate), similar to CitT catalyzing heterologous citrate/succinate antiporting. The Dcu carriers, in contrast, are able to shift between the uptake, antiporter, and efflux transport modes and catalyze homo- or heterologous antiporting (6, 12, 32). By the use of a tartrate-specific carrier (TtdT) and tartrate-specific transcriptional regulation (20), L-tartrate metabolism is physiologically separated from general C_4-dicarboxylate metabolism of fumarate, malate, and aspartate, although L-tartrate ends up in fumarate respiration as well.

TtdT is a member of the carboxylate/C_4-dicarboxylate carrier family, which is a subgroup of the DASS family (divalent anion: Na^+ symporter) (12, 23; http://www.tcdb.org/index.php). The best-characterized bacterial member of this family is the citrate/succinate antiporter CitT of *E. coli* (22), which shares 45% sequence identity with TtdT of *E. coli*. CitT can alternatively use citrate, fumarate, or tartrate as an antiporter substrate with low affinity and activity. Homologs of TtdT/YgjE have been identified in enteric and other bacteria (22, 31), and all preferentially catalyze heterologous antiporting of carboxylic acids. For CitT and TtdT/YgjE, 12 or 13 transmembrane helices are predicted, with similar arrangements. Both proteins show similar distributions of polar amino acid residues, which are concentrated in the loops between the transmembrane helices. The polar residues include a considerable number of positively or negatively charged amino acid residues, many of which are conserved in CitT and TtdT/YgjE. This conservation suggests a role in binding of the carboxylates and/or a structural role.

ACKNOWLEDGMENTS

This work was supported by grants from Deutsche Forschungsgemeinschaft and the Innovationsstiftung Rheinland-Pfalz.

We are grateful to I. G. Janausch for construction of strain IMW277, K. Jabreis (Osnabruck) for supply of strains, and S. Lux for support in transport measurements.

REFERENCES

1. **Barker, H. A.** 1936. On the fermentation of some dibasic C_4-acids by *Aerobacter aerogenes*. Proc. Acad. Sci. (Amsterdam) **39:**674.
2. **Böck, A., and G. Sawers.** 1996. Fermentation, p. 262–282. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC.
3. **Cherepanov, P. P., and W. Wackernagel.** 1995. Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp catalyzed excision of the antibiotic-resistance determinant. Gene **158:**9–14.
4. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *E. coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
5. **Engel, P., R. Krämer, and G. Unden.** 1992. Anaerobic fumarate transport in *Escherichia coli* by an *fnr*-dependent dicarboxylate uptake system which is different from the aerobic dicarboxylate uptake system. J. Bacteriol. **174:** 5533–5539.
6. **Engel, P., R. Krämer, and G. Unden.** 1994. Transport of C_4-dicarboxylates by anaerobically grown *Escherichia coli*: energetics and mechanism of exchange, uptake and efflux. Eur. J. Biochem. **222:**605–614.
7. **Falzone, C. J., W. E. Karsten, J. D. Conley, and R. E. Viola.** 1988. L-Aspartase from *Escherichia coli*: substrate specificity and role of divalent metal ions. Biochemistry **27:**9089–9093.
8. **Golby, P., D. J. Kelly, J. R. Guest, and S. C. Andrews.** 1998. Transcriptional regulation and organization of the *dcuA* and *dcuB* genes, encoding homologous anaerobic C_4-dicarboxylate transporters in *Escherichia coli*. J. Bacteriol. **180:**6586–6596.
9. **Golby, P., S. Davies, D. J. Kelly, J. R. Guest, and S. C. Andrews.** 1999. Identification and characterization of a two-component sensor-kinase and response regulator system (DcuS-DcuR) controlling gene expression in response to C_4-dicarboxylates in *Escherichia coli*. J. Bacteriol. **181:**1238–1248.
10. **Grimbert, L., and L. Fiquet.** 1897. Sur un nouveau ferment des tartrates "le *Bacillus tartaricus*". C. R. Soc. Biol. **49:**962–965.
11. **Janausch, I. G., O. B. Kim, and G. Unden.** 2001. DctA and Dcu-independent transport of succinate in *Escherichia coli*: contribution of diffusion and of alternative carriers. Arch. Microbiol. **176:**224–230.
12. **Janausch, I. G., E. Zientz, Q. H. Tran, A. Kröger, and G. Unden.** 2002. C_4-dicarboxylate carriers and sensors in bacteria. Biochim. Biophys. Acta **1553:**39–56.
13. **Karsten, W. E., R. B. Gates, and R. E. Viola.** 1986. Kinetic studies of L-aspartase from *Escherichia coli*: substrate activation. Biochemistry **25:**1299–1303.
14. **Kneuper, H., I. G. Janausch, V. Vijayan, M. Zweckstetter, V. Bock, C. Griesinger, and G. Unden.** 2005. The nature of the stimulus and of the fumarate binding site of the fumarate sensor DcuS of *Escherichia coli*. J. Biol. Chem. **280:**20596–20603.
15. **Lehnen, D., C. Blumer, T. Polen, B. Wackwitz, V. F. Wendisch, and G. Unden.** 2002. LrhA as a new transcriptional key regulator of flagella, motility and chemotaxis genes in *Escherichia coli*. Mol. Microbiol. **45:**521–532.

16. **Miles, J. S., and J. R. Guest.** 1984. Complete nucleotide sequence of the fumarase gene *fumA* of *Escherichia coli*. Nucleic Acids Res. **12:**3631–3642.
17. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
18. **Münch, R., K. Hiller, H. Barg, D. Erldt, S. Linz, E. Wingender, and D. Jahn.** 2003. PRODORIC: prokaryotic database of gene regulation. Nucleic Acids Res. **31:**66–269.
19. **Nimmo, H. G.** 1987. The tricarboxylic cycle and anaplerotic reactions, p. 156–159. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC.
20. **Ohshima, T., and F. Biville.** 2006. Functional identification of *ygiP* as a positive regulator of the *ttdA-ttdB-ygjE* operon. Microbiology **152:**2129–2135.
21. **Pasteur, L.** 1858. Mémorie sur la fermentation de l'acide tartrique. C. R. Biol. **46:**615–618.
22. **Pos, K. M., P. Dimroth, and M. Bott.** 1998. The *Escherichia coli* citrate carrier CitT: a member of a novel eubacterial transporter family related to the 2-oxoglutarate/malate translocator from spinach chloroplasts. J. Bacteriol. **180:**4160–4165.
23. **Prakash, S., G. Cooper, S. Singhi, and M. H. Saier, Jr.** 1998. The ion transporter superfamily. Biochim. Biophys. Acta **1618:**79–92.
24. **Reaney, S. K., C. Begg, S. J. Bungard, and J. R. Guest.** 1993. Identification of the L-tartratase genes (*ttdA* and *ttdB*) of *Escherichia coli* and evolutionary relationship with the class I fumarase genes. J. Gen. Microbiol. **139:**1523–1530.
25. **Sakaguchi, K., and S. Tada.** 1940. On the formation of succinic acid by *Bacterium succinicum*, nov. sp. Zentbl. Bakteriol. Parasitenk. II **101:**341–354.
26. **Six, S., S. C. Andrews, G. Unden, and J. R. Guest.** 1994. *Escherichia coli* possesses two homologous anaerobic C_4-dicarboxylate transporters (DcuA and DcuB) distinct from the aerobic dicarboxylate transport system (Dct). J. Bacteriol. **176:**6470–6478.
27. **Spencer, M. E., and J. R. Guest.** 1987. Regulation of citric acid cycle genes in facultative bacteria. Microbiol. Sci. **4:**164–167.
28. **Tran, H. Q., J. Bongaerts, D. Vlad, and G. Unden.** 1997. Requirement for the proton-pumping NADH I dehydrogenase of *Escherichia coli* in NADH-fumarate respiration and bioenergetic implications. Eur. J. Biochem. **244:**155–160.
29. **Unden, G., and A. Kleefeld.** 30 July 2004, posting date. Chapter 3.4.5, C_4-dicarboxylate degradation in aerobic and anaerobic growth. *In* R. Curtiss III et al. (ed.), EcoSal—*Escherichia coli and Salmonella*: cellular and molecular biology. ASM Press, Washington, DC. http://www.ecosal.org.
30. **Vaughn, R. H., G. L. Marsch, T. C. Stadtmann, and B. C. Cantino.** 1946. Decomposition of tartrates by the coliform bacteria. J. Bacteriol. **52:**311–325.
31. **Weber, A., E. Menzlaff, B. Arbinger, M. Gutensohn, C. Eckerskorn, and U. I. Flügge.** 1995. The 2-oxoglutarate/malate translocator of chloroplast envelope membranes: molecular cloning of a transporter containing a 12-helix motif and expression of the functional protein in yeast cells. Biochemistry **34:**2621–2627.
32. **Zientz, E., S. Six, and G. Unden.** 1996. Identification of a third secondary carrier (DcuC) for anaerobic C_4-dicarboxylate transport in *Escherichia coli*: roles of the three Dcu carriers in uptake and exchange. J. Bacteriol. **176:**7241–7247.
33. **Zientz, E., J. Bongaerts, and G. Unden.** 1998. Fumarate regulation of gene expression in *Escherichia coli* by the DcuSR (*dcuSR*) two-component regulatory system. J. Bacteriol. **180:**5421–5425.
34. **Zientz, E., I. G. Janausch, S. Six, and G. Unden.** 1999. Functioning of DcuC as the C_4-dicarboxylate carrier during glucose fermentation by *Escherichia coli*. J. Bacteriol. **181:**3716–3720.