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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

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In *Escherichia coli*, two carriers (DcuA and DcuB) for the transport of C_4 dicarboxylates in anaerobic growth were known. Here a novel gene *dcuC* was identified encoding a secondary carrier (DcuC) for C_4 dicarboxylates which is functional in anaerobic growth. The *dcuC* gene is located at min 14.1 of the *E. coli* map in the counterclockwise orientation. The *dcuC* gene combines two open reading frames found in other strains of *E. coli* K-12. The gene product (DcuC) is responsible for the transport of C_4 dicarboxylates in DcuA-DcuB-deficient cells. The triple mutant (*dcuA dcuB dcuC*) is completely devoid of C_4-dicarboxylate transport (exchange and uptake) during anaerobic growth, and the bacteria are no longer capable of growth by fumarate respiration. DcuC, however, is not required for C_4-dicarboxylate uptake in aerobic growth. The *dcuC* gene encodes a putative protein of 461 amino acid residues with properties typical for secondary procaryotic carriers. DcuC shows sequence similarity to the two major anaerobic C_4-dicarboxylate carriers DcuA and DcuB. Mutants producing only DcuA, DcuB, or DcuC were prepared. In the mutants, DcuA, DcuB, and DcuC were each able to operate in the exchange and uptake mode.

In *Escherichia coli*, various transport activities for C_4 dicarboxylates are known. Under aerobic growth conditions, unidirectional uptake of C_4 dicarboxylates (fumarate, succinate, and malate) and aspartate, but no export, is catalyzed (5, 12). This transport is effected by a binding protein-dependent carrier or by a secondary carrier which is driven by the electrochemical H^+ gradient over the membrane (10, 17). The *dctA* and *dctB* genes have been shown to be related to the aerobic carriers (3, 17, 23). The *dctA* gene has been sequenced (23), but none of the carriers has been clearly defined so far by genetic or biochemical means. Bacteria grown under anaerobic conditions, on the other hand, catalyze exchange, uptake, and efflux of C_4 dicarboxylates (5, 6). Fumarate/succinate exchange is required during fumarate respiration where the acceptor fumarate has to be taken up and the product succinate has to be excreted. Net C_4-dicarboxylate uptake is required for anaerobic growth with C_4 dicarboxylates as the C source. Citrate fermentation, on the other hand, which produces 1 succinate per citrate, depends on a C_4-dicarboxylate efflux system. The exchange reaction of the C_4 dicarboxylates is an electroneutral process, whereas uptake and efflux are electrogenic symport reactions, presumably of the dicarboxylate^{2-} with 3 H^+ (6). The anaerobic transport activities were found only in bacteria grown under anaerobic conditions, and the synthesis requires intact FNR (named FNR for fumarate nitrate reductase regulator), the transcriptional regulator of anaerobic metabolism (5, 6, 25).

Recently two homologous genes (*dcuA* and *dcuB*) were identified in *E. coli* which encode two C_4-dicarboxylate carriers, DcuA and DcuB (22). The carriers (DcuA and DcuB) were complementary to each other, and each was sufficient for fumarate/succinate exchange and growth by fumarate respiration. Only the double mutant *dcuA dcuB* was severely affected in both functions. This mutant still showed some fumarate/succinate exchange (about 28% of the parental strain) and slow growth by fumarate respiration, indicating the presence of a further fumarate/succinate exchange activity. To identify the third system, we have generated a mutant in which the residual growth on fumarate and the exchange activity were completely lost. By using this mutant, a third C_4-dicarboxylate carrier (designated DcuC) which is responsible for growth on fumarate by the *dcuA dcuB* double mutant was identified.

The presence of three isoenzymes for the anaerobic transport of C_4 dicarboxylates raises the question as to whether the three different transport modes (exchange, uptake, and efflux) are effected by each of the carriers or whether the various transport modes can be attributed to specific carrier types. The availability of the three *dcu* genes allowed us to construct strains which produced only one of the carriers. In the mutants, uptake and exchange activities were measured to see whether any of the carriers shows a specificity or preference for uptake or exchange activity. In addition, growth experiments were performed with the mutants under conditions where either C_4-dicarboxylate exchange or uptake is required specifically. The experiments demonstrated the capability of each of the carriers for exchange and uptake activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used are shown in Table 1. For growth experiments and transport assays, the bacteria were grown in M9 mineral medium supplemented with acid-hydrolyzed casein (1 g liter^{-1}) and L-tryptophan (0.05 g liter^{-1}) unless stated otherwise (6, 18). The carbon sources glucose (50 mM), sodium fumarate (50 mM), glycerol (50 mM), and nitrate (50 mM) were added as stated for the individual experiments. Cell densities were measured as the absorbance at 578 nm (A_{578}). An A_{578} of 1 corresponds to 1.5×10^9 cells ml^{-1}. Bacteria were grown under anaerobic conditions in bottles sealed with gas-tight stoppers in degassed buffers under an O_2-free atmosphere of N_2. Bacteria were grown aerobically, in flasks containing medium filled to 5 to 10% of the maximal volume, with vigorous shaking. For other experiments, Luria-

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TABLE 1. Bacterial strains and λ phage used

Bacterial strain or phage	Genotype	Reference or construction
E. coli K-12		
AN387	Parental	27
W3110[a]	F$^-$ λ$^-$ IN(*rrnD-rrnE*)*1 rph-1*	2
JRG2813	AN387 but *dcuB*::Kan^r	22
JRG2821	AN387 but *dcuA*::Spc^r	22
JRG2814	AN387 but *dcuA*::Spc^r *dcuB*::Kan^r	22
IMW157	AN387 but *dcuC*::mini-Tn*10*(Cam^r)	IMW153(P1) × AN387
IMW158	AN387 but *dcuB*::Kan^r *dcuC*::mini-Tn*10*(Cam^r)	IMW153(P1) × JRG2813
IMW159	AN387 but *dcuA*::Spc^r *dcuC*::mini-Tn*10*(Cam^r)	IMW153(P1) × JRG2821
IMW152	AN387 but *dcuA*::Spc^r *dcuB*::Kan^r IS*5* upstream of *dcuC*	30
IMW153	AN387 but *dcuA*::Spc^r *dcuB*::Kan^r *dcuC*::mini-Tn*10*(Cam^r)	IMW152 with mini-Tn*10* introduced
IMW171	W3110b but *dcuA*::Spc^r *dcuB*::Kan^r	
λ phage		
λNK1324	λ with mini-Tn*10*(Cam^r)	13

[a] Two varieties were identified (W3110a and W3110b) from strain W3110. W3110a differs from W3110b by an IS*5* insertion in *dcuC* (Fig. 1).

Bertani (LB) broth supplemented with glucose (20 and 50 mM for aerobic and anaerobic growth, respectively) was used (18).

Genetic procedures and DNA manipulation. Transduction with $P1_{kc}$ was performed essentially as described by Miller (18). Transductants were identified by selecting for the transfer of antibiotic resistance. The antibiotics chloramphenicol, kanamycin sulfate, and spectinomycin were used at 20, 50, and 50 μg ml^{-1}, respectively. Recombinant DNA techniques were performed as described by Sambrook et al. (21). Restriction endonucleases and DNA-modifying enzymes were used as suggested by the manufacturers (Boehringer, Pharmacia, and MBI Fermentas). Genomic DNA was isolated by the method of Chen and Kuo (4). Plasmid DNA was prepared from 1.5 ml of an overnight culture by lysis by boiling (21) or from 15 ml with the Jetstar kit (Genomed). DNA sequencing was performed with the ABI 373 sequencer from plasmid DNA or PCR-amplified DNA after purification (PCR purification kit from Qiagen). For DNA and protein sequence analysis, DNASIS for Windows (version 2) was used (Hitachi).

PCR amplification of the *dcuC* region. For PCR amplification, the following primers were used: primer A, 5′-CCGCGCATCGATTAAAGATG-3′; and primer B, 5′-CACTGCAGCCATCGATATGG-3′. The positions of the corresponding sequences (primer B, 460 bases downstream of *dcuC*; primer A, 441 bases upstream of *dcuC*) are shown in Fig. 1. The underlined sequences show where *Cla*I sites were introduced. Amplification of the respective fragments was done on *Bam*HI-digested genomic DNAs of strains AN387, W3110b, and IMW152 with *Tfl* polymerase (Biozym). The fragments obtained with the primers were purified, digested with *Cla*I, and cloned into the *Cla*I sites of pBluescriptKS$^-$ (1).

Transposon mutagenesis and isolation of *dcuC*. Transposon mutagenesis was performed with the mini-Tn*10* (mini-Tn*10*Cam^r) transposon in strain IMW152 as described by Kleckner et al. (13). The transposon was transposed from λNK1324 into IMW152. Two ampicillin enrichment steps were performed under anaerobic conditions in M9 medium supplemented with glycerol plus fumarate (16). The enrichment was followed by selection on M9 agar plates supplemented with chloramphenicol, acid-hydrolyzed casein (1 g liter^{-1}), and glycerol plus fumarate (20 mM each) or glucose (20 mM). Strains deficient for growth on glycerol plus fumarate were isolated and assayed for fumarate reductase activity and transport of [^{14}C]fumarate. One chloramphenicol-resistant strain (IMW153) negative for growth on glycerol plus fumarate and for [^{14}C]fumarate transport but positive for anaerobic growth on glucose and for fumarate reductase activity was used for cloning of the mini-Tn*10*-labelled gene. Genomic DNA of IMW153 was isolated, digested with *Pst*I, and separated by agarose electrophoresis. The *cam* gene (0.8-kb *Bam*HI fragment) from plasmid pOl6 was labelled by random priming with the digoxigenin DNA labelling and detection kit (Boehringer) and used for probing the genomic DNA by Southern hybridization analysis. A 2-kb fragment of the genomic digest was detected by the probe, extracted from the agarose gel, and cloned into the *Pst*I site of pUC18. Cam^r transformants were isolated. The insert was sequenced from the isolated plasmid (pAC1). It contained a 650-bp genomic fragment interrupted by the 1.4-kb mini-Tn*10* (Fig. 1). A *Bam*HI-*Hin*dIII fragment from pAC1 comprising most of the cloned genomic DNA was labelled with [^{33}P]dCTP by random priming and used to probe a gene-mapping membrane carrying the DNAs from a set of phages with the complete genome of *E. coli* in overlapping fragments (Takara, Genevilliers, France).

Transport assays. Transport assays were performed under anaerobic conditions in an anaerobic chamber with an atmosphere of 95% N_2 and 5% H_2 (O_2 < 5 ppm). Silicone oil centrifugation and radioactively labelled [^{14}C]fumarate were used for the determination of the transport activities as described before (22). Fumarate uptake activity was determined for cells that were grown on glycerol and fumarate and washed with buffer A (100 mM Na_2HPO_4-KH_2PO_4, 1 mM $MgSO_4$, 2 mM dithiothreitol, 100 μg of chloramphenicol per ml; pH 7.4) without glucose. Five minutes before the addition of 100 μM [^{14}C]fumarate (44 Bq nmol^{-1}), 20 mM glucose was added to the cell suspensions and incubation at 37°C was started. Uptake activity is given as the initial rate determined from the cytoplasmic level of [^{14}C]fumarate 20 s after the start of the reaction. Exchange of intracellular and extracellular C_4 dicarboxylates was measured by the silicone oil centrifugation method as the uptake of [^{14}C]fumarate (100 μM) into succinate-loaded bacteria (6, 22). Exchange activity is given as the initial rate of [^{14}C]fumarate uptake into succinate-loaded cell suspensions (in micromoles of fumarate per minute per gram [dry weight]) 20 s after the start of the reaction. The cytoplasmic concentration was calculated by the amount of fumarate taken up, using the values of 281 mg (dry weight) corresponding to an A_{578} of 1, and a cytoplasmic volume of 2.15 μl/mg (dry weight).

Nucleotide sequence accession number. The *dcuC* nucleotide sequence has been submitted to the EMBL database under accession number X99112.

RESULTS

Identification of the third anaerobic C_4-dicarboxylate carrier (*dcuC* gene). The previously described *dcuA dcuB* double mutant with inactivated DcuA and DcuB carriers still showed significant growth by fumarate respiration and about 28% of the parental activity for fumarate/succinate exchange (22). To identify the carrier responsible for the residual activity, a mutant which had lost the C_4-dicarboxylate transport activity was isolated. This mutant was generated by mini-Tn*10* transposon mutagenesis of the *dcuA dcuB* double mutant IMW152. After ampicillin enrichment, mutants were selected by the lack of growth on glycerol plus fumarate but wild-type growth on glucose or on glycerol plus nitrate under anaerobic conditions. One of the mutants (strain IMW153) had completely lost the ability to grow on glycerol plus fumarate. The mutant strain was devoid of C_4-dicarboxylate transport after anaerobic growth (see below) but had the same level of activity of fumarate reductase as the wild type. These properties indicated that the growth defect was due to the loss of C_4-dicarboxylate transport.

The mini-Tn*10*-labelled gene was cloned from the mutant into a plasmid. The cloned fragment consisted of the mini-Tn*10* transposon and adjacent genomic DNA (not shown). The fragment was labelled and used to identify the position of the gene on the *E. coli* genome. The miniset of Kohara phages (14) covering the complete genome of *E. coli* on a gene-mapping membrane was probed with the fragment. Phages [168]1G6 and [167]3G5 which both carry genomic DNA of min 14.0 to 14.1 reacted with the probe. The sequence of the cloned genomic DNA was identical to sequences determined earlier in

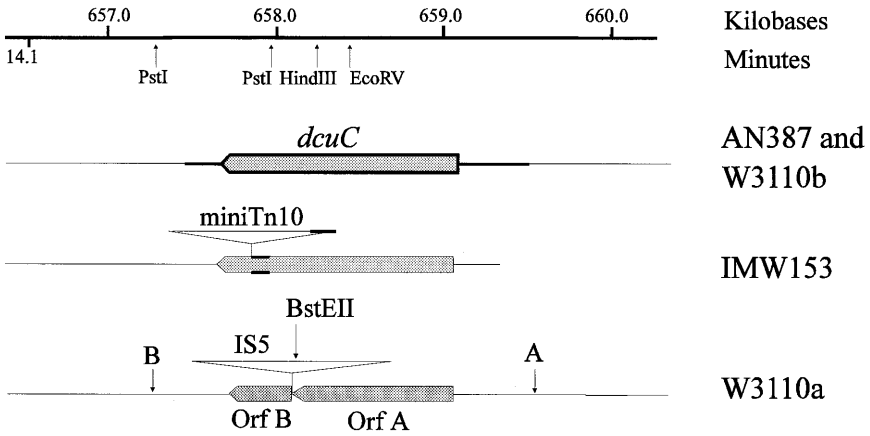

FIG. 1. Gene organization in the *dcuC* region (map position, 14.1 min or 657.0 to 660.0 kb) of *E. coli* K-12 strains (AN387, W3110a and W3110b) and *dcuC* mutant strain (IMW153). The positions of the *dcuC* gene and ORFs A and B are shown. The orientation of *dcuC*, ORF A, and ORF B is counterclockwise on the chromosome. The binding sites of the primers (primers A and B) used for PCR of the genomic DNA and the restriction site for *Bst*EII are shown for W3110a and apply to the others strains correspondingly. The sites for the mini-Tn*10* and the IS*5* insertion are given. Segments sequenced in the various strains in the present study are shown by thick black lines.

the same region from strain W3110a (Fig. 1). Strain W3110a contains two open reading frames (ORFs A and B) and an IS*5* element close to the affected site (19). It was obvious from the results that the mutation was located at min 14.1 of the *E. coli* genome and that this region is required for the transport of C_4 dicarboxylates in the *dcuA dcuB* mutant. The affected gene therefore was termed *dcuC*.

***E. coli* K-12 strains contain the *dcuC* gene at min 14.1 of the genetic map.** For analysis, the DNA region around the supposed *dcuC* gene was amplified by PCR from genomic DNAs of strains W3110b and AN387 with primers A and B located upstream and downstream of the region (Fig. 1). From both strains, PCR products of 2.5 kb were obtained, in contrast to the size of 3.5 kb predicted for the fragment comprising ORF B, IS*5*, and ORF A in strain W3110a. The PCR products from both strains were cloned into a plasmid, and the PCR-amplified DNAs from strain AN387 and a derivative were sequenced throughout. The sequences of both strains were identical over the complete region and differed from that of strain W3110a by the lack of the IS*5* element. The sequence contains an open reading frame of 1,383 bases (*dcuC*). ORF A of strain W3110a, but not ORF B, has the same reading frame as *dcuC*. Cloned fragments from AN387 and W3110b were analyzed for the presence of a *Bst*EII site which is found in the IS*5* element but not in the *dcuC* region (Fig. 1). DNA from AN387 or W3110b was not digested, in contrast to a control strain carrying an IS*5* element (not shown). Therefore, it is obvious from DNA sequence and restriction analysis that *E. coli* K-12 strains AN387 and W3110b carry the *dcuC* gene as depicted in Fig. 1, whereas in strain W3110a, an IS*5* element is inserted in that region.

Genetic characterization of the *dcuC* mutation. The mini-Tn*10* transposon present in strain IMW153 obviously inactivated C_4-dicarboxylate transport. The position of the mini-Tn*10* transposon in the mutant was determined by sequencing respective DNA fragments (Fig. 1). The mini-Tn*10* transposon was inserted into the distal part of the *dcuC* gene (Fig. 1 and 2). The insertion occurred at an AACCTTGGCCGTA insertion sequence (13) present in *dcuC* and interrupts the reading frame of *dcuC*. The mutated DcuC protein is predicted to lack 56 amino acid residues at the C terminus.

Properties of the *dcuC* gene and of the gene product DcuC. The *dcuC* gene is preceded by a promoter consensus sequence with a conserved −10 region and a −35 region 172 bases upstream of the first (ATG) codon (Fig. 2). The supposed gene product is a hydrophobic protein of 461 amino acids with a molecular mass of 48.4 kDa. N-terminal signal sequences apparently are lacking. The hydropathy plots predict a protein with 12 transmembrane helices separated by small hydrophilic loops (not shown). The N- and C-terminal ends are thought to be located on the cytoplasmic aspect of the membrane. The molecular mass and the supposed topology within the membrane therefore are similar to DcuA and DcuB (22) and typical for procaryotic secondary carriers.

The proteins with the highest sequence similarity to DcuC were DcuA and DcuB (22 and 24% identical residues) and a hypothetical protein YhcL from *E. coli* with unknown function (35% identity). The similarity between DcuC and YhcL extended over the complete sequence (63% similarity) without significant gaps or clustering (Fig. 3). For optimal alignment of DcuB (and DcuA) to DcuC, some gaps had to be introduced.

The *dcuC* gene is required for C_4-dicarboxylate transport in *dcuA dcuB* double mutants. The role of the DcuC protein for C_4-dicarboxylate transport and its relation to the DcuA and DcuB transporters were studied in mutant strains lacking the carriers in different combinations. Strains carrying the inactivated genes in any combination were constructed by P1 transduction, including strains containing only one of the carriers DcuA, DcuB, or DcuC at the same time (Table 1). Of all the single and double *dcu* mutants, only the *dcuA dcuB* double mutant was significantly inhibited in anaerobic growth on glycerol plus fumarate, whereas the other mutants showed similar growth rates (Table 2). The triple mutant, however, was no longer able to grow. The defects in anaerobic growth were not observed with other substrates, like glucose (Table 2) or glycerol plus nitrate.

The set of mutants was assayed for C_4-dicarboxylate exchange activity (Table 3). The single or double mutants showed high transport activities; inactivation of *dcuC* and of *dcuA* even caused stimulated activities. Inactivation of *dcuB*, however, decreased the transport activity, and in the *dcuA dcuB* double mutant, only 25% of the parental activity was left. Thus, as shown earlier (22), DcuB is the most important carrier under these conditions, and DcuA and DcuB are mutually functional in C_4-dicarboxylate transport. A clear role for DcuC could be seen only in the triple mutant which had lost ≥96% of the activity of the parental strain. Therefore, the residual transport

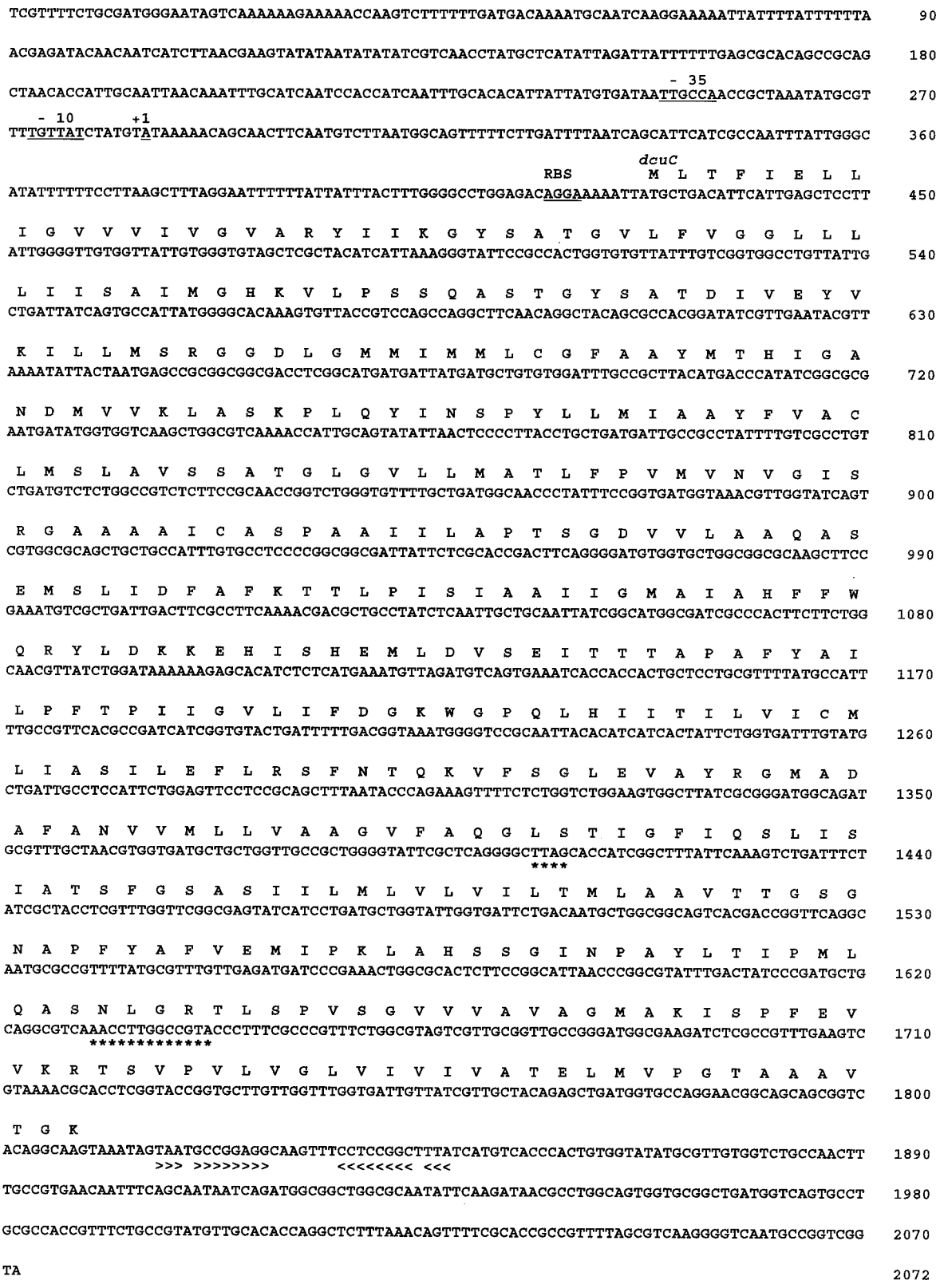

FIG. 2. Nucleotide sequence of the *dcuC* region and derived amino acid sequence of DcuC. Identical nucleotide sequences of the region were obtained for two strains of AN387. Potential promoter (−10 and −35), transcriptional start (+1), and ribosome binding (RBS) sites, and a potential terminator structure (<<<>>>) are indicated. The sites for the insertion of mini-Tn*10* (strain IMW153, bp 1630 to 1642) and of the IS*5* element (strain W3110a, bp 1406 to 1409) are shown (****).

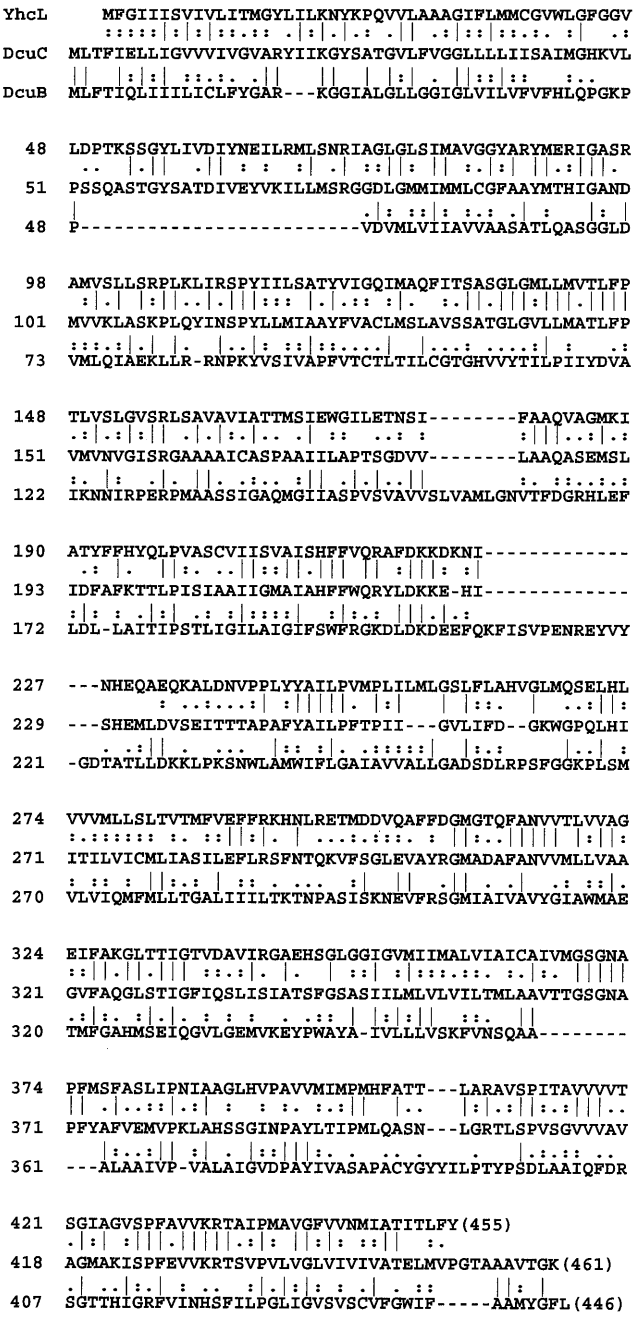

FIG. 3. Alignment of DcuC with YhcL and DcuB amino acid sequences. The sequences are aligned and compared by the method of Needleman and Wunsch (20) with the DNASIS program. Conserved (vertical bars) and conservatively substituted (colons or periods) residues are indicated. Gaps introduced (hyphens) to maximize alignment are shown.

activity in the *dcuA dcuB* double mutant depends on the *dcuC* product. Transport activities were also determined in mutants derived from *E. coli* W3110b (not shown). The activities were similar to those from mutants derived from strain AN387. In particular, the *dcuA dcuB* double mutant (strain IMW171) retained nearly one-third of the parental transport activities of W3110b. This confirms that strain W3110b (in contrast to strain W3110a) contains a functional *dcuC* gene in agreement with the genetic analysis.

TABLE 2. Growth rates of *dcu* mutants by fumarate respiration (glycerol plus fumarate) and by fermentation (glucose plus fumarate)

Strain	Genotype	Growth rates[a] (h^{-1}) on:	
		Glycerol + Fumarate	Glucose + Fumarate
AN387	Parental	0.15	0.87
IMW157	*dcuC*	0.14	0.80
IMW158	*dcuB dcuC*	0.18	1.02
IMW159	*dcuA dcuC*	0.16	0.80
JRG2814	*dcuA dcuB*	0.08	0.73
IMW153	*dcuA dcuB dcuC*	NG[b]	0.73

[a] Growth rates determined from growth curves for bacteria grown in M9 medium.
[b] NG, no growth.

Aerobic growth on succinate on the other hand was similar for the double or triple *dcu* mutants and the parental strain. After growth under these conditions, the uptake rates for [^{14}C]succinate were not significantly affected in the mutants (not shown). Therefore, none of the Dcu carriers is essential for aerobic C_4-dicarboxylate transport.

Uptake versus exchange by DcuA, DcuB, and DcuC. The various transport modes (uptake, exchange, and efflux of C_4 dicarboxylates) of the Dcu carriers were investigated (5, 6). Double mutants, each containing only one of the carriers, were tested for uptake and exchange activities (Table 3). Each of the strains was capable of fumarate uptake in addition to fumarate/succinate exchange. In the strain containing only DcuB, both uptake and exchange activities were increased over those of the wild type. However, in the DcuA$^+$ or DcuC$^+$ background, the activity of both transport modes decreased. In the triple mutant, both transport activities were lost nearly completely. The ratio of the transport activities (exchange/uptake) is a measure of the preference of the carrier for either transport mode. The ratio was between 2.2 and 3.0 for the double mutants. This demonstrates that each of the Dcu carriers was capable of both transport modes (exchange and uptake), with a preference for exchange. Efflux activity, which is also found in the bacteria, was not quantified. The bacteria contained high leak activities after loading with internal substrate, which complicates quantitative measurements of efflux.

TABLE 3. Fumarate/succinate exchange and fumarate uptake in cell suspensions of *dcu* mutants after anaerobic growth on glycerol plus fumarate

Strain	Relevant genotype	[^{14}C]fumarate/succinate exchange[a]	[^{14}C]fumarate uptake[a]	Exchange/uptake ratio[b]
AN387	Parental	21.8	13.9	1.6
JRG2821	*dcuA*	29.5	11.6	
JRG2813	*dcuB*	10.6	5.4	
IMW157	*dcuC*	39.7	16.7	
JRG2814	*dcuA dcuB*	5.4	1.8	3.0
IMW159	*dcuA dcuC*	39.2	16.1	2.4
IMW158	*dcuB dcuC*	17.1	7.9	2.2
IMW153[c]	*dcuA dcuB dcuC*	≤0.9	≤0.2	

[a] Values are given in micromoles per minute per gram (dry weight).
[b] Ratio of transport rates for the wild type and strains containing only one type of carrier.
[c] Growth substrates glucose plus fumarate (anaerobic conditions).

DISCUSSION

DcuC as the third carrier for C_4 dicarboxylates in anaerobic growth of *E. coli*. The *dcuC* gene has been identified here as the structural gene encoding a third carrier for C_4 dicarboxylates that allows anaerobic growth of *E. coli*. The DcuC carrier is essential for growth by fumarate respiration in the absence of the major carriers DcuA and DcuB. The physiological role of DcuC is not clear, and no phenotype could be identified for the *dcuC* single mutant. DcuC presumably has additional functions to those in fumarate respiration. The earlier known sequence of the 14.1-min region of *E. coli* K-12 showed an IS*5* element dividing *dcuC* in two open reading frames (ORFs A and B in Fig. 1) (19). However, by sequencing, restriction, and functional analysis, it appears that the respective IS*5* element is specific for strain W3110a, which has been used for the *E. coli* sequencing project of the 0- to 33-min region (9, 19, 29). Other strains of *E. coli* K-12, like W3110b and AN387, contain the gene arrangement given in Fig. 1. The *dctB* gene encoding a supposedly aerobic C_4-dicarboxylate carrier has been mapped earlier approximately at min 16 on the *E. coli* map (3). The *dcuC* gene appears to be different from *dctB*, since the *dcuC* mutants were still able to grow aerobically on succinate.

DcuA, DcuB, and DcuC each function as uptake and exchange carriers. The availability of the various double mutants was an important prerequisite for studying the transport mode of each of the carriers in a clear-cut manner without interference from alternative carriers. Each of the carriers turned out to be able to catalyze exchange as well as uptake. The contributions of the DcuA, -B, and -C carriers to the efflux reaction, however, could not be determined so far. However, it appears that C_4-dicarboxylate efflux is decreased in the *dcu* triple mutant also (30). Therefore, the carriers can apparently switch between different transport modes, like the citrate carrier from *Klebsiella pneumoniae* or the lactose permease from *E. coli* (26, 28). Although not studied here, the energetics of the single Dcu carriers is assumed to be the same as for the overall reaction in the wild type, i.e., an electroneutral exchange ($dicarboxylate^{2-}{}_{out} + 3H^+{}_{out} \leftrightarrow dicarboxylate^{2-}{}_{in} + 3H^+{}_{in}$) or an electrogenic uptake of $dicarboxylate^{2-}$ with $3H^+$. This is concluded from the fact that both transport reactions in the wild type were homogenous and monophasic with respect to energetics and kinetics (5, 6).

Types of secondary C_4-dicarboxylate carriers. Secondary C_4-dicarboxylate carriers of different functions and transport mechanisms are known in bacteria. One functional group comprises the Dcu carriers involved in anaerobic fumarate respiration. Their main function is fumarate/succinate exchange, but as shown here these carriers are also able to catalyze uptake and presumably efflux of their substrates. This transport mode is typical for bacteria like *E. coli*, *Wolinella succinogenes*, and *Haemophilus influenzae* growing by fumarate respiration (5, 6, 8, 15, 24). A functionally different group is represented by the DctA carriers from *Rhizobium meliloti* and *E. coli* (3, 7, 23) which operate in aerobic metabolism and presumably only in the uptake mode (5, 10, 12). Sequence comparison indicates that the Dcu proteins form a group of C_4-dicarboxylate carriers (Fig. 4). The hypothetical YhcL protein of *E. coli* shows close similarity to DcuC. The DctA carriers, on the other hand, are very similar to each other (Fig. 4) but only distantly related to the Dcu carriers. DctA from *E. coli* contains only 20% identical and 47% similar residues compared to DcuC.

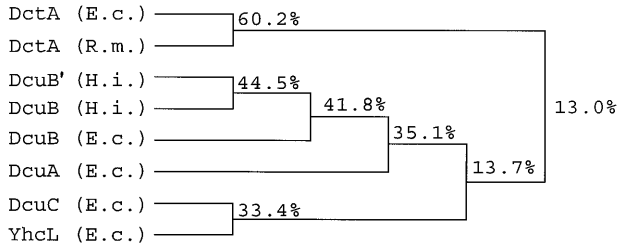

FIG. 4. Dendrogram showing the relation of secondary bacterial C_4-dicarboxylate carriers (Dcu and Dct proteins) and of the hypothetical YhcL protein by the method of Higgins and Sharp (11). The numbers refer to the percentage of identical amino acid residues for the respective proteins or clusters of proteins. E.c., *E. coli*; H.i., *Haemophilus influenzae*; R.m., *Rhizobium meliloti.*

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