The Fumarate/Succinate Antiporter DcuB of Escherichia coli Is a Bifunctional Protein with Sites for Regulation of DcuS-dependent Gene Expression

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DcuB of Escherichia coli catalyzes C₄-dicarboxylate/succinate antiporter during growth by fumarate respiration. The expression of genes of fumarate respiration, including the genes for DcuB (dcuB) and fumarate reductase (frdABCD) is transcriptionally activated by C₄-dicarboxylates via the DcuS-DcuR two-component system, comprising the sensor kinase DcuS, which contains a periplasmic sensing domain for C₄-dicarboxylates. Deletion or inactivation of dcuB caused constitutive expression of DcuS-regulated genes in the absence of C₄-dicarboxylates. The effect was specific for DcuB and not observed after inactivation of the homologous DcuA or the more distantly related DcuC transporter. Random and site-directed mutation identified three point mutations (T394I, D398N, and K353A) in DcuB that caused a similar derepression as dcuB deletion, whereas the transport activity of the DcuB mutants was retained. Constitutive expression in the dcuB mutants depended on the presence of a functional DcuS-DcuR two-component system. Mutation of residues E79A, R83A, and R127A of DcuB, on the other hand, inactivated growth by fumarate respiration and transport of [¹⁴C]succinate, whereas the expression of dcuB''-lacZ was not affected. Therefore, the antiporter DcuB is a bifunctional protein and has a regulatory function that is independent from transport, and sites for transport and regulation can be differentiated.

The fumarate/succinate antiporter DcuB (dicarboxylate/uptake) of Escherichia coli catalyzes the uptake of external C₄-dicarboxylates like fumarate, l-malate, or aspartate (1–3). Fumarate is used as an electron acceptor in fumarate respiration by fumarate reductase, which carries the active site at the cytoplasmic side of the membrane (for reviews, see Refs. 4 – 6). l-Malate and aspartate are converted to fumarate by fumarase and aspartase, respectively, and are then metabolized in the same way as fumarate. Fumarate respiration results in the generation of a proton potential and drives ATP synthesis and growth of the bacteria. The product of fumarate respiration, succinate, is not further catabolized in anaerobic growth and is excreted by DcuB. DcuB, therefore, is responsible for the substrate/product antiport of fumarate, l-malate, or aspartate against succinate. DcuB and fumarase B are encoded by the dcuB fumB gene cluster (1, 7).

E. coli contains two further carriers for C₄-dicarboxylates during anaerobic growth, DcuA and DcuC (1, 8). DcuA shows high sequence identity (36%) and similarity (63%) to DcuB and constitutes with DcuB the DcuAB family of C₄-dicarboxylate transporters. The dcuA gene encoding DcuA is expressed constitutively under aerobic and anaerobic conditions (7). DcuC has been suggested to serve as a backup of DcuB or as a C₄-dicarboxylate transporter for anabolic and other purposes. DcuC, on the other hand, is produced only during anaerobic growth (8). Expression of dcuC is (in contrast to dcuB) not subject to catabolite repression by glucose. DcuC is used under conditions of glucose fermentation when succinate is formed and functions as a succinate efflux transporter.

The Dcu transporters have different roles in the metabolism of E. coli and catalyze in the bacteria antiporter (DcuB), uptake (DcuA), and efflux (DcuC) (1, 3, 8). Each of the carriers can, however, operate in any of the three transport modes (1, 8). The transporters, therefore, can replace each other in mutants that are deficient in one or two of the Dcu carriers. The corresponding single and double mutants retain high activities for antiport and uptake (as well as for efflux) of C₄-dicarboxylates. Only the triple mutant loses the capacity for uptake and antiport of C₄-dicarboxylates and for growth by fumarate respiration (8, 9).

Expression of dcuB and of dcuC depends on gene activation by the FNR (fumarate nitrate reductase regulator) protein (7–9). FNR activates expression of genes of anaerobic respiration in the absence of O₂. Expression of dcuB requires in addition transcriptional activation by the DcuS-DcuR (dicarboxylate uptake sensor and regulator) two-component system (10, 11). The system consists of the membrane-bound sensor kinase DcuS, which is activated in the presence of C₄-dicarboxylates (12, 13). The presence of stimuli like fumarate, l-malate, or aspartate causes phosphorylation of DcuS and transfer of the phosphate group to the response regulator DcuR, which then stimulates the expression of the target genes (14, 15). These include the genes for fumarate reductase (frdABCD), DcuB (dcuB), fumarase B (fumB), and of the aerobic C₄-dicarboxylate carrier DctA (dctA). Expression of dcuA and dcuC, on the other hand, is transcriptionally independent of DcuS-DcuR. The sensor site of DcuS responding to the C₄-dicarboxylates is located in a periplasmic domain and has been characterized by structural and mutational studies (13, 16, 17). Recently, the cytoplas-
Regulation by DcuB

### TABLE 1

Strains and plasmids used

| Strain/Plasmid | Genotype | Source or reference |
|----------------|----------|---------------------|
| AN387          | Wild type | Ref. 23             |
| MC4100         | F- araD139 Δ(argF-lac)U169, rpsL50 relA1 flbB530 deoC1 ptsF25 rbsR | Ref. 25         |
| MG1655         | CGSC 6300 for K-, F-, P1-sensitive | Ref. 43         |
| IM105          | thiA endA shkB15 hisd44 Δ(lac-proAB) F' tral36 proAB + lacIq lacZ AM15 | Ref. 44         |
| XL-1 Blue MRF  | Δ(mcrA183 mcrCB-hsdSMR-mrr)173 endA1 supE4 thi-1 rca1 gyrA96 relA1 lacI1 qraB3 bla lacZ KanR | Stratagene       |
| BW25113        | BD792, aber rnmB3 UlasZ747 hisR514U(arabBAD)567 U(rhaBAD)568 rph-1 | Ref. 24         |
| JW4083         | BW25113, but ΔmutB3 | Ref. 45, NIG collection |
| IMW237         | MC4100, but F(dcuB::lacZ)hyb, ampr | Ref. 10         |
| IMW260         | MC4100, but [F(dcuB::lacZ)hyb ampR] dcuC::camR^2 | Ref. 10         |
| IMW329         | MC4100, but dcuB::lacZ dcuC dcuB dcuC | Ref. 8          |
| IMW369         | MC4100, but ducuS::Spec^R [F(dcuB::lacZ)hyb ampR] | Ref. 1          |
| IMW370         | MC4100, but dcuB::Kan^R [F(dcuB::lacZ)hyb ampR] | Ref. 1          |
| IMW371         | MC4100, but dcuC::Cam^R [F(dcuB::lacZ)hyb ampR] | Ref. 8          |
| IMW372         | MC4100, but ducuS::Spec^R dcuB::Kan^R, [F(dcuB::lacZ)hyb ampR] | Ref. 1          |
| IMW373         | MC4100, but ducuS::Spec^R dcuC::Cam^R [F(dcuB::lacZ)hyb ampR] | Ref. 8          |
| IMW374         | MC4100, but dcuB::Kan^R dcuC::Cam^R [F(dcuB::lacZ)hyb ampR] | Ref. 8          |
| IMW375         | MC4100, but ΔmutB [F(dcuB::lacZ)] | This work       |
| IMW376         | MC4100, but ΔmutB [F(dcuB::lacZ)] dcuC::Spec^R | This work       |
| IMW377         | MC4100, but ΔmutB::lacZ dcuC::Spec^R dcuB::Cam^R | P1 (IMW157) × IMW504, this work |
| IMW378         | C7623, but dcuC::Spec^R | This work       |
| IMW379         | C7623, but ΔmutB::lacZ dcuC::Spec^R dcuB::Cam^R | P1 (IMW534) × IMW237, this work |
| IMW380         | C7623, but ΔmutB::lacZ dcuC::Spec^R dcuB::Cam^R | P1 (IMW497) × IMW535, this work |

### Plasmids

- pET-28a: Expression plasmid, His-tag, Kan^R
- pME6010: Low-copy number plasmid, Tet^R
- pMW181: pET28a, dcuS with own promoter, Kan^R
- pMW228: pME6010, dcuB with own promoter, Tet^R
- pMW281: pET28a, dcuB with own promoter, Kan^R
- pMW237: pMW181 with ducuS mutant encoding DcuS(R147A)
- pMW236: pMW181 with ducuS encoding DcuS(H110A)
- pMW314: pMW281, aber dcuB K202A, Kan^R
- pMW449: pBAD18 with dcuA Kan^R
- pMW522: pET28a with fumB, Kan^R

2 The abbreviations used are: PAS, Per-Arnt-Sim; X-gal, 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside; eM9, enriched M9.

mic PAS^2 domain of DcuS has been studied structurally as a part of a membrane-embedded construct containing the periplasmic and cytoplasmic PAS domains together with the transmembrane domain (18). The study showed an important role for the cytoplasmic PAS domain in signal transduction by the sensor kinase DcuS.

Here deletion and point mutants of DcuB were generated for a more detailed understanding of DcuB function. It turned out that deletion and inactivation of DcuB has, in addition to the effects on transport of C4-dicarboxylates, unexpectedly also an effect on the function of the DcuS-DcuR regulatory system. The regulatory function was analyzed by mutagenesis. Regulatory competent sites could be identified and separated from transport essential sites, demonstrating that DcuB is a bifunctional protein with transport and regulatory function.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Media**—The bacterial strains and plasmids used are given in Table 1. For genetic experiments, the bacteria were routinely grown in Luria-Bertani (LB) broth and on LB agar (19). For anaerobic expression studies, M9 medium or enriched M9 (eM9) medium (20, 21), with acid-hydrolyzed casamino acids (0.1%) and tryptophan (0.005%), was used. Glucose (20 mm), glycerol (50 mm), DMSO (50 mm), and sodium fumarate (50 mm) were added as energy substrates, as indicated. For growth experiments, eM9 medium supplemented with 50 mm glycerol and 50 mm fumarate was used, and growth was monitored for at least 30 h. Bacteria for transport assays were grown in the same medium containing 50 mm DMSO in addition. All media were inoculated at 37 °C with 1–5% (v/v) of an overnight culture grown under the same conditions and in the same medium. When required, antibiotics were added as follows: 50 μg ml^-1 kanamycin, 50 μg ml^-1 spectinomycin, 20 μg ml^-1 chloramphenicol, and 15 μg ml^-1 tetracycline.

**Genetic Procedures**—Phage P1 transduction and DNA isolation and manipulation followed standard procedures (19, 22). Plasmid DNA and PCR products were isolated and purified using kits (Qiagen). For cloning of dcauB, a 1.9-kb fragment containing the dcauB gene and its promoter was obtained by PCR from genomic DNA of E. coli AN387 (23) using primers dcauB-EcoRI and dcauB-Xhol (supplemental Table 1). The PCR product was digested with EcoRI and Xhol and cloned into the low copy number plasmid pME6010 with oriV15A as in pACYC (~10 copies/cell), resulting in plasmid pMW228. For mutagenesis, the 1.9-kb fragment was cloned from pMW228 into pET28a by the EcoRI and XhoI restriction sites, generating plasmid pMW281. Mutated forms of dcauB were recloned into pME6010 for in vivo complementation tests. Plasmids encoding wild-type and mutated forms of DcuS (13) are derivatives of plasmid pET28a (Novagen) with an intermediate copy number of 40–50 copies/cell. The fumB gene was amplified from chromosomal DNA of E. coli MG 1655 using oligonucleotide primers fumB-Ndel-for1 and fumB-EcoRI-rev2 (supplemental
Table 1). The 1.6-kbp fragment was cloned into pET28a behind the T7-regulated T7/lac promoter using Ndel and EcoRI restriction sites, resulting in plasmid pMW522.

Deletion of dcuB and Complementation—The dcuB gene was deleted by the method of Datsenko and Wanner (24). A Cam resistance cassette with flanking FRT sequences was amplified from plasmid pKD3 with the 70-nt deletion primers dcuB-H1-P1 and dcuB-H2-P2 (supplemental Table 1). The deletion primers each contained a region of 20 nucleotides before the ATG codon of dcuB homologous to the plasmid, including an FRT site, and another homologous region of 50 nucleotides after the stop codon. The purified PCR product was used to transform strain MC1400 (25) containing the helper plasmid pKD46 (26) for efficient recombination. Recombinants carrying the resistance cassette in place of the dcuB gene were selected on LB agar plates with chloramphenicol (25 µg ml⁻¹). From the resulting insertion mutant IMW497, the helper plasmid was eliminated by a temperature shift from 30 to 37 °C. To eliminate the chloramphenicol resistance cassette, IMW497 was transformed with pCP20 (26), which encodes an FLP recombinase. The dcuB deletion mutant IMW502 was tested for chloramphenicol sensitivity and the loss of the helper plasmid. The dcuB deletion was complemented by the dcuB gene under the control of its native promoter on plasmid pMW228. Electro-competent E. coli IMW503 or IMW505 were transformed with pMW228 or its derivatives (see below), and the transformants were selected for tetracycline resistance. For expression analysis or for growth experiments, cells were grown as described, and the influence of pMW228 or derivatives on expression and growth was determined.

Hydroxylation Mutagenesis and Selection of Mutants—The dcuB gene was mutagenized with hydroxylamine (27). Plasmid pMW228 DNA (20 µg in 100 µl of water) encoding dcuB was mixed with 500 µl of buffer (0.1 M sodium phosphate, pH 6.0, and 1 mM EDTA) and 400 µl of 1 M hydroxylamine hydrochloride (pH 6.0). The mixture was incubated at 70 °C for 10 h, and a 200-µl aliquot was removed every 2 h. Reactions were terminated either by ethanol precipitation or by purification with a PCR purification kit (Qiagen) by elution in 10 µl of water, followed by butanol precipitation. Mutagenized plasmids were electroporated into E. coli IMW505, and transformants were selected by growth on M9 minimal agar plates containing glycerol, DMSO, X-gal (20–40 µg ml⁻¹), spectinomycin (25 µg ml⁻¹), chloramphenicol (10 µg ml⁻¹), and tetracycline (7.5 µg ml⁻¹). Mutant colonies with increased expression of the reporter dcuB'::lacZ were blue in color compared with white colonies of strains with wild-type dcuB. Strains from blue colonies were tested for their ability to grow anaerobically in m9 medium with fumarate plus glycerol. Growth was monitored at least for 30 h. Bacteria capable of growth on fumarate as the sole carbon source reached an A₅₇₈ of 0.7. Reporter gene expression was quantified by measuring β-galactosidase activity after anaerobic growth in m9 minimal medium supplemented with glycerol and DMSO, with or without fumarate (10). Plasmid DNA was extracted from strains with increased β-galactosidase activity that grew on fumarate, and the dcuB gene was sequenced.

Site-directed Mutagenesis of DcuB—Site-directed mutagenesis was carried out with the QuikChange kit (Stratagene). The dcuB coding region, including the complete promoter, was amplified by PCR and cloned into pET28a (Novagen). The resulting plasmid, pMW281, served as the template for site-directed mutagenesis by PCR with synthetic primers containing the desired mutation. The mutations were verified by DNA sequencing. Oligonucleotide primers used for mutagenesis are listed in supplemental Table 1. E. coli strains were transformed with plasmids by electroporation (28). For growth tests, transport assays, and β-galactosidase activity tests, the mutated dcuB gene, including the promoter, was cloned into pME6010, resulting in derivatives of plasmid pMW228. Screening for (blue) regulatory mutants was performed as described above on X-gal agar plates containing glycerol plus DMSO.

DcuB-specific Antiserum and Western Blotting of DcuB—Polyclonal antibodies were produced by GenScript Corp. in rabbits using peptide W¹⁹⁴FRGKDDLKDDEFO²⁰⁰C (sequence in italic type is derived from DcuB). The peptide is part of the large cytoplasmic loop located between transmembrane helices V and VI of DcuB, assuming a topology as for the homologous DcuA (29). Membranes of the bacteria were prepared by breaking the bacteria in a French press (83 bar, two passages) followed by low (10,000 × g) and high speed (45,000 × g) centrifugation. The membrane pellet from the ultracentrifugation was suspended in buffer. The proteins were separated by SDS gel electrophoresis (30), blotted onto nitrocellulose membranes, and detected by immunoblotting with the antiserum.

Transport Assays (Uptake of [¹⁴C]Succinate)—Bacteria were grown anaerobically in m9 medium supplemented with glycerol, DMSO, and fumarate to an A₅₇₈ of 0.6–0.8. After harvesting and washing, cells were resuspended in anoxic buffer (100 mM sodium potassium phosphate, pH 7, 1 mM MgSO₄) to an A₅₇₈ of 6.6–8.0 at 4 °C. After activation with glucose (20 mM) for 5 min at 37 °C, uptake of [¹⁴C]succinate (33.4 Bq/nmol, 100 µM) was tested by a filtration assay with membrane filters (mixed cellulose ester, 0.2 µm, Whatman/Schleicher & Schuell catalog number 10401706) as described (1, 8). The amount of substrate taken up was calculated from the bacterial dry weight (an A₅₇₈ of 1 corresponds to 281 mg dry weight ml⁻¹) and the radioactivity after background subtraction. The transport is given in µmol of [¹⁴C]succinate min⁻¹ (g dry weight⁻¹) taken up or units (g dry weight⁻¹) at 37 °C.

β-Galactosidase Assay—Expression of the dcuB'::lacZ reporter gene fusions was determined by measuring the β-galactosidase activity (22) of exponentially growing cultures (A₅₇₈ of 0.7) at 37 °C under anaerobic conditions in m9 medium supplemented with glycerol and DMSO, with or without fumarate.

RESULTS

DcuB Is Required in Vivo for the Response of DcuSR to C₅ dicarboxylates—After initial indications that inactivation of Dcu transporters has an effect on the expression of Dcu-dependent genes, the effect was studied systematically by inactivating the dcuA, dcuB, or dcuC genes in a strain that carries a chromosomal dcuB'::lacZ reporter gene fusion (Table 2).
Expression of *dcuB* is a good indicator for the functional state of the DcuS-DcuR system in *E. coli* when the bacteria are grown under anaerobic conditions on glycerol plus fumarate (10, 11). DMSO was included in the medium as an alternative electron acceptor to support growth when no fumarate was included or when mutations inhibited growth by fumarate respiration. The expression of *dcuB* is stimulated about 15-fold by fumarate or other C₄-dicarboxylates in the strain with wild-type *dcuA dcuB dcuC* genes (Table 2). When the *dcuB* gene was inactivated by insertional disruption or deletion, expression of *dcuB*-lacZ unexpectedly was fully induced without the need for C₄-dicarboxylates. The addition of fumarate even caused some repression. The effect was specific for *dcuB* and not observed after inactivation of *dcuA* or *dcuC*. In mutants with two or three of the *dcu* genes inactivated, expression became C₄-dicarboxylate-independent whenever *dcuB* was one of the inactivated genes. The fumarate-independent (or constitutive) expression of *dcuB*-lacZ in the *dcuA dcuB dcuC*-negative strain IMW505 (Table 2) or in the *dcuB*-negative strain IMW370 (not shown) was reversed by introducing *dcuB* on plasmid, whereas plasmid-encoded *dcuA* restored only anaerobic growth on fumarate but not fumarate regulation of *dcuB*-lacZ expression.

For the above mentioned growth experiments, the mineral medium was supplemented with low amounts of casamino acids (eM9 medium) to enhance anaerobic growth, which is low in mineral media. The same experiments were performed without casamino acids to exclude effects by components like asparagine and 1-malate that can be present in low amounts in the casamino acids (and function as effectors of DcuS). Glucose was used under these conditions as the substrate to increase growth. In the experiments, deletion of *dcuB* had the same stimulating effect as the addition of fumarate or 1-malate (Table 3). The activity of *dcuB*-lacZ expression, however, was generally low due to glucose repression (7, 10). The constitutive expression without fumarate was lost here, too, after complementation with *dcuB*.

The *fumB* gene encoding the anaerobic fumarase B is located downstream of *dcuB*. The *fumB* gene can be transcribed from promoters in front of *fumB* and of *dcuB*, since monocistronic *fumB* and bicistronic *dcuB fumB* transcripts were identified (7). It is not clear which is the major transcript of *fumB* expression. It was checked that the effect of *dcuB* deletion was not caused by loss of *FumB* due to polar effects of *dcuB* deletion on *fumB*. A strain that was negative for *fumB* expression (*E. coli* JW4083) still required fumarate (or 1-malate) for full expression of the *dcuB*-lacZ fusion (Table 3). On the other hand, introducing *fumB* on plasmid in the strain with the chromosomally inactivated *dcuB* (*E. coli* IMW503) did not relieve the constitutive (fumarate-independent) expression of *dcuB*-lacZ. In control experiments, the same *fumB* gene on plasmid was able to complement the *fumB*-deficient strain JW4083 when anaerobic consumption of 1-malate was tested (not shown). Overall, the effect of *dcuB* deletion on *dcuB*-lacZ expression was specific for the loss of *dcuB* and independent from *fumB* or fumarase B.

A further target for transcriptional stimulation by DcuS-DcuR is the *fredABCD* operon, which is stimulated to a low but significant extent (1.6–2-fold) by the addition of C₄-dicarboxylates (10, 11). Expression of *fredA* was stimulated 2.3-fold in the *dcuB*-negative strain IMW505 (Table 2) or in the *dcuB*-negative strain IMW370 (not shown) was reversed by introducing *dcuB* on plasmid, whereas plasmid-encoded *dcuA* restored only anaerobic growth on fumarate but not fumarate regulation of *dcuB*-lacZ expression.

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The Regulatory Effect of DcuB Is Independent from Its Transport Function—The transport activity of the dcu mutants was determined, and it was tested whether the constitutive expression in the dcuB negative strain was related to the loss of transport activity or to the loss of DcuB. As an indicator for the transport capacities, the uptake activity of the strains was determined, since each of the transporters is able to catalyze antiporter, uptake, or efflux of C₄-dicarboxylates (1, 3, 8). In the dcuA, dcuB, and dcuC single mutants, uptake of [¹⁴C]succinate was only slightly impaired (maximally 26% decrease in activity compared with the wild type). Even in the double mutants, the activity decreased maximally by 51% in relation to the wild type, which is in agreement with earlier studies (1, 8). None of the single or double mutants was significantly impaired in growth by fumarate respiration (Table 2), which shows that the residual transport activities are sufficient for fumarate/succinate antiport. Only in the triple mutant, the transport activity decreased to background levels (11% of wild type), which prevented also growth by fumarate respiration. Therefore, the fumarate-independent expression of dcuB-lacZ is related to the loss of the DcuB protein but not to the activity of C₄-dicarboxylate transport.

Mutations in DcuB Conferring Fumarate-independent Expression of dcuB-lacZ—In order to identify sites or amino acid residues that are responsible for the regulatory effect of DcuB, the dcuB gene was mutagenized in vitro randomly with hydroxylamine, which introduces C/G transitionalities. An E. coli reporter strain with the dcuB-lacZ fusion and inactivated dcuA, dcuB, and dcuC genes was transformed with a plasmid encoding the mutated dcuB gene. The transformants were screened for blue colonies on X-gal agar after anaerobic growth in the absence of fumarate. The strain with wild-type dcuB on plasmid produced blue colonies only when fumarate was included in the agar. A small percentage of the colonies containing hydroxylamine-treated dcuB (<1%) showed blue staining in the absence of fumarate, indicating dcuB-lacZ expression without C₄-dicarboxylates. For differentiation of the desired deregulated from dcuB-deficient mutants (for which the same phenotype of increased β-galactosidase activity would be expected), the strains from the blue colonies were tested for anaerobic growth on fumarate. Five independent mutants were obtained from two mutagenesis experiments that were able to grow on glycerol plus fumarate but required no fumarate for dcuB-lacZ induction. The isolates contained two types of mutations in DcuB, mutation T394I (ACT → ATT transition, four independent isolates) and mutation D398N (GAT → ATT transition, one mutant). The same mutations generated by directed mutagenesis in cloned dcuB showed the same phenotype as the random mutants, confirming that mutations T394I and D398N of DcuB are responsible for the phenotype.

Mutations DcuB(T394I) and DcuB(D398N) Affect Regulation by DcuB but Not Transport—A strain of E. coli producing DcuB(T394I) or DcuB(D398N) from plasmid as the only Dcu carrier was able to grow on glycerol plus fumarate similar to wild-type DcuB supplied from plasmid, confirming that the mutant forms of DcuB are transport-active (Fig. 1). Both mutants retained full activity for the uptake of [¹⁴C]succinate into the bacterial cells (Fig. 2), whereas in the parental dcuA dcuB dcuC-deficient strain, the transport activity was close to background levels. When the expression of dcuB-lacZ was measured, both mutants showed full induction of the reporter gene fusion without fumarate. The expression of dcuB-lacZ was even higher than in the wild type after fumarate induction, and the presence of fumarate caused some decrease in expression. The DcuB(T394I) and DcuB(D398N) mutants therefore have the same regulatory phenotype as the ΔdcuB mutant, but C₄-dicarboxylate transport is retained. This indicates that residues Thr394 and Asp398 play a specific role in the regulation of DcuS-DcuR function but not in transport by DcuB.

Residues Thr394 and Asp398 were replaced by various residues (Table 5). In the DcuB(T394S) and DcuB(T394N) mutants, dcuB-lacZ expression was very similar to that in the wild type and required fumarate for induction, and the constitutive phenotype of DcuB(T394I) was no longer observed. The DcuB(T394A) mutant showed partial expression of dcuB-lacZ without C₄-dicarboxylates, and fumarate caused further increase of the expression to wild-type levels. Since
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DcuB(T394N) has wild-type activity, the hydroxyl group of Thr is not essential for the function of DcuB in sensing. Replacement of Thr by nonpolar amino acid residues (Ile and in part also Ala) appears to be required for the constitutive phenotype, whereas polar and hydrophilic side groups (Thr, Asn, or Ser) confer C₄-dicarboxylate responsiveness to DcuB.

The DcuB(D398E) and DcuB(D398A) mutants did not retain the constitutive phenotype of DcuB(D398N) and were fumarate-responsive, similar to wild-type DcuB (Table 5), which indicates that the constitutive phenotype of DcuB(D398N) is specific for the Asp/Asn exchange. Most other residues are apparently tolerated without significant functional change. Residue Asp₃⁹⁸ might be required for its negative charge or another chemical or structural property of the side group. Altogether, the data suggest that the regulatory mutations T₃⁹⁴I and D₃⁹⁸N specifically depend on the exchange of the Thr and Asp residues by Ile and Asn.

The mutant forms of DcuB were tested after complementation by plasmid-borne dcuB for their activity in the uptake of [¹⁴C]succinate in a dcaA dcuB dcuC-negative transport test strain (Table 5). Strains producing DcuB with mutations T₃⁹⁴I, T₃⁹⁴S, T₃⁹⁴N, D₃⁹⁸N, and D₃⁹⁸A showed transport activities similar to the corresponding wild-type DcuB strain. Only strains with mutations T₃⁹⁴A and D₃⁹⁸E were impaired for transport to some extent (decrease of wild-type activity by 17–40%), but none of the strains lost the transport activity completely. Thus, mutants DcuB(T₃⁹⁴I) and DcuB(D₃⁹⁸N) were regulation-incompetent but had wild-type phenotype with respect to anaerobic growth on fumarate and C₄-dicarboxylate transport. The loss in regulatory competence therefore was specific not only for the site of mutation but also for the type of exchange (Thr → Ile and Asp → Asn replacements, respectively).

Identification of Regulatory Mutants of DcuB by Directed Mutation—DcuB contains a considerable number (60) of basic (Arg, Lys, and His) and acidic (Asp and Glu) residues (Fig. 3). Most of the residues are located in cytoplasmic or periplasmic loops that connect the transmembrane helices, when an arrangement of transmembrane helices similar to those in the related DcuA transporter (29) is assumed. From the residues, 37 were selected for directed mutation in addition to residues Asp₃⁹⁸ and Thr₃⁹⁴ that had been identified by random mutagenesis. The selected residues were mostly conserved in DcuB and DcuA or were specific for DcuB (Fig. 3). The basic or acidic residues are candidates for interaction with C₄-dicarboxylate anions. The residues were mutated to Ala residues in cloned dcuB. The mutant forms were tested in vivo in a dcaA dcuB dcuC deletion strain IMW505 for fumarate-independent induction of dcuB-‘lacZ. To this end, the mutant strains were screened for blue colonies on X-gal indicator plates after anaerobic growth on glycerol plus DMSO without fumarate. Strains with mutations DcuB(D119A), DcuB(K353A), and DcuB(D405A) formed blue colonies similar to the DcuB(T₃⁹⁴I) and DcuB(D₃⁹⁸N) mutants. In the β-galactosidase reporter assay, the three

![FIGURE 2. Expression of dcuB-‘lacZ and uptake of [¹⁴C]succinate in IMW505 (dcaA dcuB dcuC) after complementation with plasmids pMW228 coding for wild-type DcuB, without plasmid (ΔDcuB), plasmid pMW397 coding for DcuB(T₃⁹⁴I), or plasmid pMW405 coding for DcuB(D₃⁹⁸N), respectively. β-Galactosidase was measured in exponentially growing cultures without (white bar) or with (black bar) fumarate. [¹⁴C]Succinate uptake (gray bar) was determined in cell suspensions of bacteria grown under the same conditions. MU, Miller units.]

**TABLE 5**

Effect of mutations in Thr₃⁹⁴ and Asp₃⁹⁸ of DcuB on the C₄-dicarboxylate-dependent regulation of dcuB-‘lacZ expression and on transport activity

The regulatory activity was measured as β-galactosidase activity using th dcuB-‘lacZ reporter fusion. Uptake of [¹⁴C]succinate was determined in the transport test strain IMW505 (dcaA ΔdcuB dcuC) transformed with the same plasmid as for the expression studies. The bacteria were grown in eM9 medium with glycerol + DMSO plus fumarate (anoxic conditions).

| Strain (relevant genotype) | β-Galactosidase (dcuB-‘lacZ) | [¹⁴C]Succinate uptake* |
|----------------------------|-------------------------------|------------------------|
|                            | Without fumarate | With fumarate | [μmol g (dry weight)]⁻¹ min⁻¹ |
| IMW505 (ΔdcuB)             | 463 ± 58          | 237 ± 32        | 3.1 ± 0.8                 |
| IMW505 (ΔdcuB dcaA dcuC)   | 131 ± 31          | 125 ± 15        | 0.91 ± 0.1                |
| IMW505 pMW228 (ΔdcuB⁻)     | 20 ± 9            | 348 ± 27        | 3.3 ± 0.4                 |
| IMW505 pMW228 (pdcuB⁻)     | 12 ± 4            | 119 ± 10        | 3.5 ± 0.4                 |
| Mutation at dcuB(Thr₃⁹⁴)    |                  |                |                         |
| IMW505 pMW397 (pdcuB T₃⁹⁴I) | 262 ± 51          | 221 ± 38        | 3.9 ± 1.4                 |
| IMW505 pMW403 (pdcuB T₃⁹⁴S) | 21 ± 6            | 282 ± 57        | 3.9 ± 1.2                 |
| IMW505 pMW402 (pdcuB T₃⁹⁴N) | 33 ± 9            | 278 ± 16        | 3.7 ± 0.9                 |
| IMW505 pMW404 (pdcuB T₃⁹⁴A) | 87 ± 26           | 263 ± 49        | 2.9 ± 0.8                 |
| Mutation at dcuB(Asp₃⁹⁸)   |                  |                |                         |
| IMW505 pMW405 (pdcuB D₃⁹⁸N) | 372 ± 77          | 227 ± 45        | 4.0 ± 0.5                 |
| IMW505 pMW456 (pdcuB D₃⁹⁸E) | 17 ± 1            | 104 ± 11        | 2.1 ± 0.8                 |
| IMW505 pMW455 (pdcuB D₃⁹⁸A) | 17 ± 3            | 125 ± 6         | 4.1 ± 0.6                 |

* Transport activities in the transport test strain IMW505 (dcaA ΔdcuB dcuC).
strains showed high C4-dicarboxylate independent induction of \textit{dcuB}/\textit{lacZ} during anaerobic growth (Table 6), whereas the other mutants had no effect on the expression of \textit{dcuB}/\textit{lacZ}. The strain with DcuB(K353A) was fully induced in the absence of fumarate, like mutants DcuB(T394I) and DcuB(D398N).

\textbf{FIGURE 3.} Sequence alignment of DcuB and DcuA proteins of \textit{E. coli} (Ec), \textit{Salmonella typhimurium} (St), \textit{Erwinia carotovora} (Er), and \textit{Campylobacter jejuni} (Cj). Basic (Arg, Lys, and His) and acidic (Asp and Glu) residues are labeled by red and green background. Basic and acidic residues that were mutated are indicated (filled circles, green). Regulatory residues Thr\textsuperscript{394} and Asp\textsuperscript{398} that were identified by random mutagenesis are highlighted (\textbullet). The numbers on the right give the numbering of the corresponding amino acid residues. In addition some of the mutated residues are numbered according to their position. The supposed positions of transmembrane helices I–X are indicated by gray bars below the sequence, assuming a topology according to DcuA (29).
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TABLE 6
Site-directed mutants of DcuB affecting expression of DcuS-DcuR-dependent genes and DcuB-dependent transport (uptake) of $[^{14}C]$succinate

The experiments were performed with strain E. coli IMW505 ($\text{dcuA} \text{dcuB} \text{dcuC}$), which carried $\text{dcuB}$-encoding plasmids as indicated. Anaerobic growth was determined on glycerol plus fumarate as in Fig. 5 ($+++$, growth rate similar to strain with wild-type $\text{dcuB}$ on plasmid; $+$, slow growth (class II); $-$, no growth (comparable with class III, or $\text{dcuB}$-negative strain). Transport (uptake of $[^{14}C]$succinate) was determined with resting cells as described in the legend to Fig. 2. NA, not available; ND, not determined.

| Type of DcuB | Plasmid | $\beta$-Galactosidase ($\text{dcuB}^{+} \text{lacZ}$) | Growth (glycerol + fumarate) | Transport |
|--------------|---------|---------------------------------|-----------------------------|------------|
|              |         | Without fumarate | With fumarate | units/g dry weight |
| DcuB (wild type) | pMW228 | 552 ± 82 | 550 ± 126 | $+++$ | 0.9 ± 0.6 |
| DcuB(D119A) | pMW532 | 168 ± 25 | 393 ± 35 | $+++$ | 5.8 ± 2.3 |
| DcuB(K353A) | pMW508 | 383 ± 133 | 281 ± 51 | $+++$ | 0.8 ± 0.5 |
| DcuB(D405A) | pMW529 | 224 ± 77 | 295 ± 52 | $-$ | ND |
| DcuB(E79A) | pMW535 | 2 ± 1 | 415 ± 26 | $+$ | 1.5 ± 0.6 |
| DcuB(R83A) | pMW530 | 2 ± 1 | 409 ± 45 | $+$ | 0.9 ± 0.5 |
| DcuB(H419A) | pMW341 | 56 ± 2 | 329 ± 47 | $-$ | 1.6 ± 0.5 |

FIGURE 4. Western blot of the membrane fraction of E. coli IMW505 ($\text{dcuB}$) (lane 1) and the same strain with plasmid encoding wild-type DcuB (pMW228) (lane 2), DcuB(D119A) (pMW532) (lane 3), or DcuB(D405A) (pMW529) (lane 4). Membrane fractions of the strains (500 μg of protein each) were subjected to SDS-PAGE and tested by Western immunoblotting for DcuB using anti-DcuB antibodies. The molecular mass of the positive band is indicated (40,000 Da).

Mutants DcuB(D119A) and DcuB(D405A) were in addition deficient for growth on fumarate, raising the possibility that both properties are caused by the lack of DcuB or complete inactivation of DcuB. The strains were tested by immunoblotting for the presence of the DcuB protein (Fig. 4). The membrane fraction of the strain producing DcuB(D119A) contained about half the levels of DcuB compared with the strain with wild-type DcuB. This suggests that the loss of transport and of the regulatory effect is caused by functional inactivation of DcuB(D119A) and by decreased contents. When residue Asp$^{119}$ was replaced by an Asn residue, DcuB(D119N) was fully active in growth and regulatory competence (not shown). This indicates that residue Asp$^{119}$ is not essential for normal regulatory competence, but some amino acid replacements might affect regulatory and transport properties by structural changes. The amount of DcuB(D405A) in the membranes of the bacteria was very low (Fig. 4), suggesting that the regulatory and transport deficiency of this mutant is due to the low content of the carrier. The same low expression was observed in DcuB(D405N/V/E) mutants. Overall, the directed mutation of DcuB revealed DcuB(K353A) as a further regulatory mutation of DcuB that had lost regulatory competence similar to DcuB(T394I) and DcuB(D398N).

FIGURE 5. Effect of point mutations in DcuB on anaerobic growth with glycerol plus fumarate. E. coli IMW505 (relevant genotype $\text{dcuA} \text{dcuB} \text{dcuC}$) containing mutant forms of $\text{dcuB}$ on a plasmid derived from pMW228 ($\text{dcuB}$) by site-directed mutagenesis was grown in EM9 medium with 50 mM glycerol plus 20 mM fumarate. The growth curves are shown for strain IMW505 encoding wild-type DcuB (□), DcuB(K353A) (△), DcuB(R83A) (○), DcuB(R127A) (●), and no DcuB (■). Class I mutants comprise strains with (DcuB(H41A), DcuB(D50A), DcuB(D72A), DcuB(K80A), DcuB(R84A), DcuB(K87A), DcuB(H108A), DcuB(K123A), DcuB(E129A), DcuB(R130A), DcuB(D173A), DcuB(R196A), DcuB(K198A), DcuB(D199A), DcuB(D201A), DcuB(K202A), DcuB(D203A), DcuB(E204A), DcuB(E214A), DcuB(D222A), DcuB(K230A), DcuB(D257A), DcuB(D259A), DcuB(K289A), DcuB(R302A), DcuB(E319A), DcuB(H325A), DcuB(E328A), DcuB(K353A), DcuB(D375A), DcuB(R406A), or DcuB(R414A); Class II mutants comprise strains with DcuB(E79A) or DcuB(R83A); Class III mutants comprise strains with DcuB(D119A), DcuB(R127A), or DcuB(D405A).
Despite a complete lack of growth on glycerol plus fumarate, the strain catalyzed uptake of $^{[14]C}$succinate with wild-type rates. Since other genes required for fumarate respiration (frdABCD) are not mutated, it is assumed that the mutant is able to catalyze uptake but has lost the capacity for antiport.

**DISCUSSION**

*DcuB Is a Bifunctional Carrier Protein with Regulatory Function—* The C₄-dicarboxylate/succinate antiporter DcuB is a bifunctional protein with a transport and a regulatory function. The regulatory function affects expression of DcuS-DcuR-regulated genes. The latter function was independent from its transport activity and from sites in DcuB that are essential for transport (residues Glu⁷⁹, Arg⁸³, and Arg¹²⁷).

The regulatory function, on the other hand, required sites (Lys³⁵³, Thr³⁹⁴, and Asp³⁹⁸) that were not essential for transport. The transport-specific residues are located in the N-terminal half of DcuB, whereas the regulatory residues concentrate in the other half close to the C-terminal end (Fig. 6). The topology model is hypothetical and based on the homology to DcuA.

**FIGURE 6. Schematic diagram showing DcuB topology and the location of mutations (obtained by random mutagenesis and directed replacement against Ala) that render DcuB regulation- and transport-incompetent.** The topology model is based on the homologous DcuA protein of *E. coli* (29). The approximate positions of the residues that have no effect on the function of DcuB in regulation and anaerobic growth on fumarate are shown in gray. The positions of the amino acid residues that are important for regulation of DcuS-DcuR-dependent gene expression (Lys³⁵³, Thr³⁹⁴, and Asp³⁹⁸) are indicated in red, and those affected by anaerobic growth on glycerol plus fumarate (E79A, R83A, and R127A) are shown in green. DcuB(D119A) and DcuB(D405) (blue circles) were produced in low amounts and had effects on transport and regulation. The position of peptide W¹⁹⁴FRGKOLDKEEFQ²⁰⁷C (sequence in italic type is derived from DcuB), which was used for production of polyclonal antiserum, is indicated (broken line, red).
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conserved in the DcuA and DcuB proteins. Residues Asp\textsuperscript{398} and Lys\textsuperscript{353} with regulatory function were conserved only in the DcuB proteins in agreement with the restriction of regulatory function to the latter. The regulatory residue Thr\textsuperscript{394} is found in DcuB and DcuA, suggesting that it has an additional function that is required also in DcuA.

The acidic and basic amino acid residues that are essential for transport and regulation might be involved in the binding of the C\textsubscript{4}-dicarboxylates. Translocation of substrates by secondary transporters requires specific binding of the substrate at the binding site and in the translocation pathway of the transporter (33, 34). The transport-essential residues might be part of the corresponding sites of DcuB for binding and translocation of the C\textsubscript{4}-dicarboxylates and of protons that are cosubstrates for transport (3). The residues that are essential for the regulatory function of DcuB would be suitable for binding of C\textsubscript{4}-dicarboxylates as well. Alternatively, it is feasible that the regulatory residues are involved in a specific interaction with a protein from the DcuS-DcuR two-component system that is required for exerting the regulatory effect of DcuB. The mode of interaction is not known, but it could involve protein-protein interaction between DcuB and the DcuS-DcuR system. Interestingly, the C\textsubscript{4}-dicarboxylate- and DcuS-DcuR-dependent stimulation of dctA expression became constitutive also in a mutant deficient in dctA (35), indicating that DctA might affect DcuS-DcuR-dependent expression during aerobic growth in a similar way as DcuB during anaerobic growth. It will be interesting to see whether an interaction between DcuB and DcuS-DcuR can be demonstrated. However, DcuB could not be isolated so far in significant amounts for in vitro studies,\textsuperscript{3} whereas the DcuS-DcuR proteins can be isolated and reconstituted functionally (12, 14, 15).

Regulation by DcuB means that the DcuS-DcuR system has a second signal input site in addition to the DcuS sensor. What could be the physiological significance for dual regulation of the DcuS-DcuR system by the same stimulus? DcuS responds to C\textsubscript{4}-dicarboxylate concentration in the periplasm by the periplasmic sensing domain (13, 16, 36), which appears to be the primary sensory site of DcuS. The cytoplasmic PAS domain of DcuS plays an important role in signal transduction of the periplasmically derived stimulus to the kinase domain (18). The DcuB transporter, on the other hand, senses C\textsubscript{4}-dicarboxylate transport and metabolism. Metabolic flux might represent a more direct measure for the status of C\textsubscript{4}-dicarboxylate metabolism than concentration measurement in the periplasm. A transporter catalyzing the first step of metabolism, therefore, is in an optimal position to detect relevant metabolic conditions. Therefore, both sensory devices respond to alternative perspectives of the same regulatory signal.

Transceptors as Sensory Devices for Histidine Kinases and Other Regulatory Systems—Transceptors are able to participate in various modes in sensing and signal perception in transcriptional regulation, but there are only few systems where regulation by transporters has been studied at the molecular level. The sugar transporter of the glucose phosphotransferase system represents a signal input site for catabolite control in \textit{E. coli}. Enzyme II (EI\textsubscript{IIgluc}) of the transport system controls the activity of adenylate cyclase, which then synthesizes the intramolecular signaling molecule cyclic AMP (for a review, see Ref. 37). It is feasible that DcuB interacts with DcuS-DcuR in order to control the sensitivity of the system. Regulation by direct interaction has been shown for the membrane-integrated sensor/regulator CadC, which controls the synthesis of lysine decarboxylase. The function of CadC is regulated by direct interaction with the lysine permease LysP (38).

There are also two-component systems responding to transmembrane carriers. The UhpB-UhpA two-component system of \textit{E. coli} controls the expression of the glucose 6-phosphate uptake transporter UhpT, using the accessory membrane protein UhpC for sensing. UhpC is supposed to represent a former secondary carrier. UhpC stimulates the sensor kinase after binding of glucose 6-phosphate (39, 40). A system similar to the DcuB/DcuS-DcuR system is represented by DctB-DctD of \textit{Rhizobium meliloti}. The DctB-DctD two-component system controls the expression of dctA encoding the C\textsubscript{4}-dicarboxylate carrier DctA (41, 42). DctA is assumed to affect the DctB sensor kinase by direct interaction when it is in the transport-inactive (or C\textsubscript{4}-dicarboxylate-deficient) form.

Transporters, and in particular secondary carriers, represent an interesting device for sensing of stimuli by histidine kinases, but their function in sensing and controlling the kinase activity is generally not clear. Therefore, studies on DcuB of \textit{E. coli} might support our understanding of how secondary carriers can interact with and control the function of sensor kinases in two-component systems and how transporter proteins can function as additional sensory sites of such systems.

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