

Inactivation and Regulation of the Aerobic C₄-Dicarboxylate Transport (*dctA*) Gene of *Escherichia coli*

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The gene (*dctA*) encoding the aerobic C₄-dicarboxylate transporter (DctA) of *Escherichia coli* was previously mapped to the 79-min region of the linkage map. The nucleotide sequence of this region reveals two candidates for the *dctA* gene: *f428* at 79.3 min and the *o157a-o424-o328* (or *orfQMP*) operon at 79.9 min. The *f428* gene encodes a homologue of the *Sinorhizobium meliloti* and *Rhizobium leguminosarum* H⁺/C₄-dicarboxylate symporter, DctA, whereas the *orfQMP* operon encodes homologues of the aerobic periplasmic-binding protein-dependent C₄-dicarboxylate transport system (DctQ, DctM, and DctP) of *Rhodobacter capsulatus*. To determine which, if either, of these loci specify the *E. coli* DctA system, the chromosomal *f428* and *orfM* genes were inactivated by inserting Sp^r or Ap^r cassettes, respectively. The resulting *f428* mutant was unable to grow aerobically with fumarate or malate as the sole carbon source and grew poorly with succinate. Furthermore, fumarate uptake was abolished in the *f428* mutant and succinate transport was ~10-fold lower than that of the wild type. The growth and fumarate transport deficiencies of the *f428* mutant were complemented by transformation with an *f428*-containing plasmid. No growth defect was found for the *orfM* mutant. In combination, the above findings confirm that *f428* corresponds to the *dctA* gene and indicate that the *orfQMP* products play no role in C₄-dicarboxylate transport. Regulation studies with a *dctA-lacZ* (*f428-lacZ*) transcriptional fusion showed that *dctA* is subject to cyclic AMP receptor protein (CRP)-dependent catabolite repression and ArcA-mediated anaerobic repression and is weakly induced by the DcuS-DcuR system in response to C₄-dicarboxylates and citrate. Interestingly, in a *dctA* mutant, expression of *dctA* is constitutive with respect to C₄-dicarboxylate induction, suggesting that DctA regulates its own synthesis. Northern blot analysis revealed a single, monocistronic *dctA* transcript and confirmed that *dctA* is subject to regulation by catabolite repression and CRP. Reverse transcriptase-mediated primer extension indicated a single transcriptional start site centered 81 bp downstream of a strongly predicted CRP-binding site.

Escherichia coli can utilize C₄-dicarboxylates as a carbon and energy source under aerobic and anaerobic conditions (9, 50, 56). Anaerobically, the uptake, exchange, and efflux of C₄-dicarboxylates (fumarate, malate, maleate, and succinate) and L-aspartate are mediated by the three independent dicarboxylate uptake (Dcu) systems, DcuA, DcuB, and DcuC (9, 12, 13, 50, 56). These Dcu systems appear to be active solely under anaerobic conditions (9).

Aerobically, uptake of C₄-dicarboxylates is mediated by a secondary transporter and/or a binding-protein-dependent system, designated Dct (20, 24). The Dct system has an apparent *K_m* of 10 to 20 μM for C₄-dicarboxylates and is driven by the electrochemical proton gradient (15), and its activity is induced by succinate and is subject to catabolite repression (20, 27). The corresponding *dctA* mutants cannot utilize the C₄-dicarboxylates malate and fumarate but grow normally on the monocarboxylate lactate (27). Transport across the outer membrane may be mediated by a C₄-dicarboxylate-binding protein (Cbt; *K_d* for C₄-dicarboxylates of 30 to 50 μM) and a porin (3, 4, 25–30).

Three genetic loci (*cbt* at 16.6 min, *dctA* at 79.3 min, and *dctB* at 16.4 min) are involved in aerobic C₄-dicarboxylate transport (27). The nucleotide sequence of the 76- to 81.5-min region revealed a putative *dctA* gene (*f428*) encoding a protein (DctA) most closely resembling the DctA proteins of *Sinorhizobium meliloti* and *Rhizobium leguminosarum* (62 to 63% identity) that function as H⁺/C₄-dicarboxylate symporters (51). The DctA proteins are members of a family that includes the Na⁺/H⁺ glutamate symporters (GltP/GltT). A role for the putative *dctA* gene of *E. coli* in the utilization of C₄-dicarboxylates (and the cyclic monocarboxylate orotate) has been suggested by complementation studies with *Salmonella typhimurium* *dctA* or *outA* mutants (2, 51). The coding regions corresponding to the *dctB* (predicted to encode an inner membrane protein) and *cbt* (predicted to encode the binding protein) genes have yet to be identified (23).

In addition to the *dctA* (*f428*) gene, *E. coli* contains three apparently cotranscribed genes (*o157-o424-o328* or *orfQMP*) at 79.9 min that encode products 23 to 32% identical to the DctPQM components of the periplasmic binding protein-dependent C₄-dicarboxylate transport system of *Rhodobacter capsulatus* (11, 46, 51). The *orfQMP* genes are apparently part of a large operon involved in pentose sugar metabolism (11, 42). This suggests that the *orfQMP* products form a pentose sugar transporter, although, given their similarity to the *R. capsulatus* DctPQM components, it is also possible that they transport C₄-dicarboxylates.

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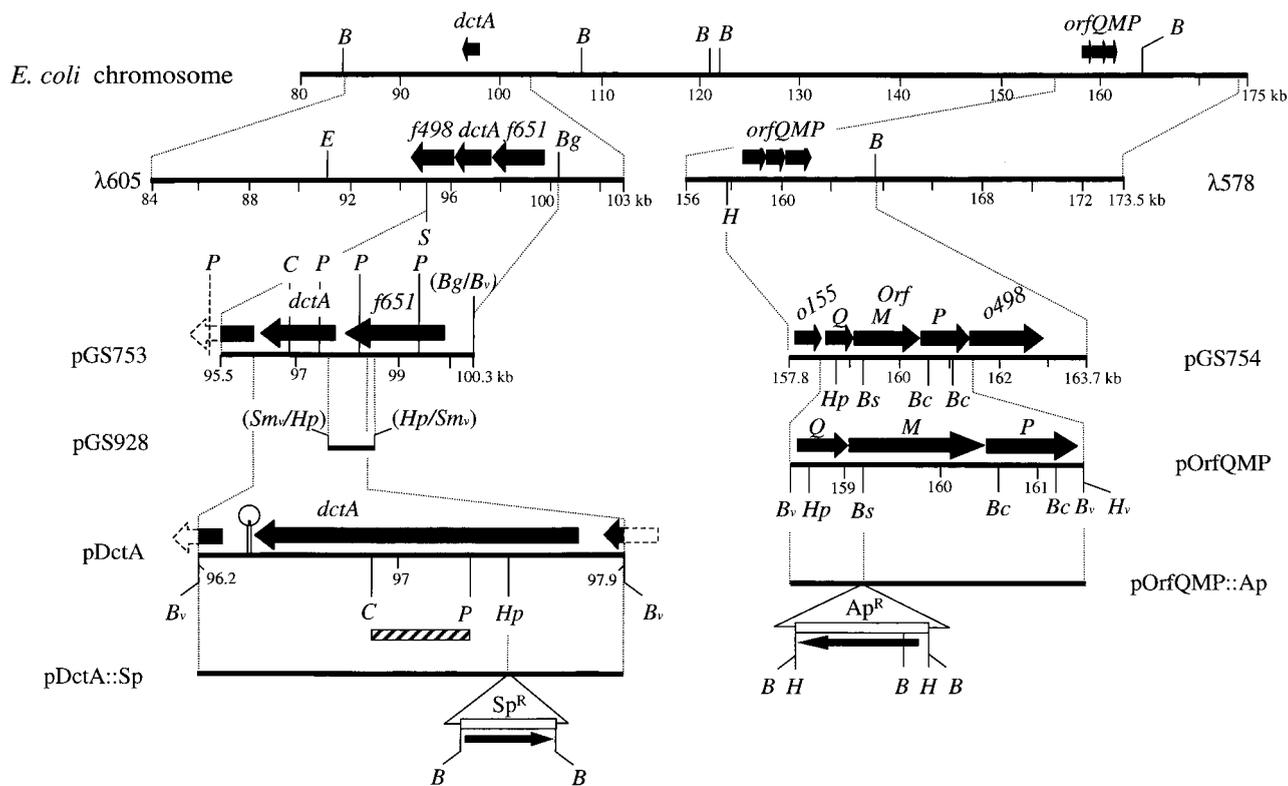


FIG. 1. Restriction maps of the *dctA-orfQMP* region of the *E. coli* chromosome. The inserts cloned in λ 578, λ 605, pGS753, pGS754, pGS928, pDctA, pOrfQMP, pDctA::Sp and pOrfQMP::Ap are shown along with *E. coli* DNA (thick black lines) and the Ap^r and Sp^r resistance cassettes (open bars). Relevant restriction sites are indicated: B, BamHI; Bc, BclI; Bg, BglII; Bs, BspHI; C, ClaI; E, EcoRI; H, HindIII; Hp, HpaI; P, PstI; S, SalI; and Sm, SmaI. Restriction sites within vector DNA are denoted by a v, and hybrid restriction sites no longer recognized by the corresponding enzymes are in parentheses. Solid arrows indicate the positions and polarities of relevant structural genes. The hatched bar represents the DNA fragment used as a hybridization probe, and a strongly predicted stem-loop structure is also indicated. Coordinates are from reference 51.

To investigate the potential roles of the *dctA* and *orfQMP* genes of *E. coli* in C₄-dicarboxylate transport, the corresponding genes were inactivated and the phenotypes of the resulting mutants were studied. The results showed that the *dctA* (*f428*) product is required for aerobic growth on malate and fumarate and mediates the transport of C₄-dicarboxylates. Interestingly, *dctA* mutants were still able to grow aerobically on succinate, indicating the presence of an uncharacterized transporter with specificity for succinate. In contrast, the *orfQMP* products play no apparent role in C₄-dicarboxylate utilization and transport. Transcript mapping and regulatory studies with a *dctA-lacZ* transcriptional fusion showed that the *dctA* gene is monocistronic, has a single transcriptional start site, and is activated by cyclic AMP receptor protein (CRP) in the absence of glucose, repressed by ArcA during anaerobiosis, and weakly activated by the recently identified DcuS-DcuR system (13, 57) in the presence of C₄-dicarboxylates. In addition, inactivation of *dctA* led to constitutive *dctA-lacZ* expression with respect to C₄-dicarboxylates, suggesting that DctA regulates its own synthesis through an interaction with DcuS in a manner similar to that proposed for DctA- and DctB-dependent regulation of *dctA* in *S. meliloti* and *R. leguminosarum*.

MATERIALS AND METHODS

Subcloning the *dctA* (*f428*) and *orfQMP* genes. The *dctA* (*f428*) and *orfQMP* genes were subcloned from phages λ 605 and λ 578, respectively (21), by standard procedures (36). DNA was isolated from the liquid lysates as described by Miller (36). A 4.9-kb BglII-SalI restriction fragment containing *dctA* was subcloned from λ 605 into the BamHI and SalI sites of pSU18, generating plasmid pGS753 (Fig. 1 and Table 1). Similarly, a 5.9-kb HindIII-BamHI fragment, containing

orfQMP, was isolated from λ 578 and inserted into pSU18, generating pGS754 (Fig. 1 and Table 1).

Inactivation of *dctA* (*f428*) and *orfQMP*. A 1.7-kb fragment containing the putative *dctA* gene was PCR amplified from pGS753 by using *Pfu* DNA polymerase (Stratagene) and primers annealing ~300 bp upstream and ~100 bp downstream of the *dctA* coding region, and the 1.7-kb product was subcloned into the *Sma*I site of pSU18 to generate pDctA (Fig. 1). The cloned *dctA* gene was disrupted by inserting a 1.9-kb *Sma*I fragment containing the *spc* cassette of pHP45ΩSpc into the *Hpa*I site of pDctA, generating pDctA::Sp (Fig. 1). A similar strategy was used to disrupt the *orfQMP* operon. A 3.1-kb PCR product was subcloned into the *Sma*I site of pSU18, generating pOrfQMP (Fig. 1), and the operon was disrupted by inserting the *amp* cassette contained in the 2.6-kb *Sma*I fragment of pHP45ΩAp into the Klenow-filled *Bsp*HI site of pGS754 to generate pOrfQMP::Ap (Fig. 1). The chromosomal *dctA* and *orfQMP* genes were replaced by the disrupted versions in pDctA::Sp and pOrfQMP::Ap by allelic exchange (37). This was achieved by transforming JC7623 (*recBC sbcB*) with pDctA::Sp or pOrfQMP::Ap and isolating potential *dctA::spc* and *orfM::amp* mutants by screening for Sp^r Cm^s or Ap^r Cm^s colonies. The successful disruption of the *dctA* and *orfM* genes was confirmed by PCR and Southern blot analyses (Fig. 2). PCR amplification and Southern blotting of the *dctA* and *orfM* genes gave the expected band sizes for the parental strain and representative Sp^r Cm^s or Ap^r Cm^s mutants (MDO1 and MDO2, respectively), indicating that the corresponding *dctA* or *orfM* genes had indeed been disrupted with the *spc* or *amp* cassette (Fig. 1 and 2). Finally, the *dctA::spc* and *orfM::amp* mutations of strains MDO1 and MDO2 were transferred to the wild-type strain, AN387, by phage P1-mediated transductions (36) to produce the mutants MDO800 (AN387, *dctA::spc*) and MDO900 (AN387, *orfM::amp*). The identities of the resulting mutants were confirmed by PCR analysis (results not shown).

Southern blot analysis of *dctA* and *orfQMP* mutants. Chromosomal DNA was isolated from JC7623 and the Sp^r Cm^s and Ap^r Cm^s derivatives (34). Southern blotting was performed with 10 μ g of chromosomal DNA digested with *Pst*I (*dctA* analysis) or with *Hind*III and *Bam*HI (*orfQMP* analysis), separated by agarose electrophoresis, and blotted onto nylon membranes (41). Hybridizations

TABLE 1. Strains, phages, and plasmids used in this study

Strain, phage, or plasmid	Relevant genotype	Source or reference
<i>E. coli</i>		
AN387	Wild type	G. Uden, Mainz, Germany
DH5 α	$\Delta(\text{argF-lac})U169$ ($\phi 80\Delta\text{lacZM15}$) <i>recA</i>	41
JC7623	<i>thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galR2 ara-14 xyl-5 ml-1 tsx33 rpsL31 supE44 recB21 recC22 sbcB15</i>	37
JRG1728	MC1000 $\Delta(\text{tyrR-fnr-rac-trg})17$ <i>zdd-230::Tn9</i>	52
JRG1999	MC1000 ΔcrpT8	S. T. Cole, Paris, France
JRG2814	AN387 <i>dcuA::spc dcuB::kan</i>	50
JRG3351	MC4100/ λ RS45(<i>dctA'-lacZYA</i>)	This work
JRG3835	MC4100/ λ RS45(<i>dcuB'-lacZYA</i>)	12
JRG3983	JRG3835 <i>dcuS::mini-Tn10</i>	13
JRG3984	JRG3351 <i>dcuS::mini-Tn10</i>	13
JRG4005	JRG3351 <i>dctA::spc</i>	This work
JRG4011	JRG3351 $\Delta\text{arcA1 zjj::Tn10}$	This work
JRG4013	JRG3351 $\Delta(\text{tyrR-fnr-rac-trg})17$ <i>zdd-230::Tn9</i>	This work
JRG4016	JRG1999/ λ RS45(<i>dctA-lacZYA</i>)	This work
JRG4017	MC1000/ λ RS45(<i>dctA-lacZYA</i>)	This work
MC1000	ΔlacX74 $\Delta(\text{araABC-leu})$	48
MC4100	$\Delta(\text{argF-lac})U169$ <i>rpsL</i>	48
MDO1	JC7623 <i>dctA::spc</i>	This work
MDO2	JC7623 <i>orfM::amp</i>	This work
MDO800	AN387 <i>dctA::spc</i>	This work
MDO900	AN387 <i>orfM::amp</i>	This work
RH90	MC4100 <i>rpoS359::Tn10</i>	R. Hengge-Aronis, Konstanz, Germany
RM315	MC4100 $\Delta\text{arcA1 zjj::Tn10}$	43
SCA344	MDO800 <i>orfM::amp</i>	This work
SCA345	JRG3351 <i>rpoS::Tn10</i>	This work
Phages		
P1 <i>vir1</i>		36
λ RS45		49
λ 578		21
λ 605		21
Plasmids		
pCH21	pBR325 + 1.65-kb <i>Bam</i> HI- <i>Hind</i> III <i>fnr</i> -containing fragment of pGS24; Ap ^r Cm ^r	16
pDctA	pSU18 + 1.7-kb <i>dctA</i> fragment	This work
pDctA::Sp	pDctA + 1.9-kb <i>spc</i> cassette inserted in <i>dctA</i> ; Sp ^r Cm ^r	This work
pGS279	pBR325 + 2.8-kb <i>Bam</i> HI- <i>Sal</i> I <i>crp</i> -containing fragment of pBScrp2; Ap ^r Cm ^r	J. R. Guest, Sheffield, United Kingdom
pGS753	pSU18 + 4.9-kb <i>dctA</i> fragment	This work
pGS754	pSU18 + 5.9-kb <i>orfQMP</i> fragment	This work
pGS928	pRS528 + 0.9-kb <i>dctA'-lacZ</i>	This work
pHP45 Ω Sp	Ap ^r Sp ^r	10
pHP45 Ω Ap	Kn ^r Ap ^r	10
pOrfQMP	pSU18 + 3.1-kb <i>orfQMP</i> fragment	This work
pOrfQMP::Ap	pOrfQMP + 2.6-kb <i>amp</i> cassette inserted in <i>orfM</i>	This work
pRB38	pACYC184 + 4.2-kb <i>arc</i> ⁺ <i>Sal</i> I fragment, Cm ^r	J. R. Guest, Sheffield, United Kingdom
pRS528	Ap ^r	49
pSU18	Cm ^r	35

were carried out at 63°C, and for the *dctA* analysis the hybridization probe was the digoxigenin-labeled 4.9-kb insert of pGS753, whereas for the *orfQMP* analysis the hybridization probe was the digoxigenin-labeled 5.9-kb insert of pGS754.

[¹⁴C]fumarate and [¹⁴C]succinate uptake experiments. Cultures were grown aerobically to late exponential phase in 500 ml of Luria broth (L broth), and the bacteria were harvested by centrifugation (2,000 \times g for 15 min at 4°C), resuspended in 100 ml of M9 salts solution (Sigma) at 4°C, centrifuged as before, and finally resuspended in 5 ml of M9 salts solution and kept on ice for up to 6 h before use. Uptake of [¹⁴C]fumarate or [¹⁴C]succinate (1.8 to 2.2 Gbq mmol⁻¹; NEN) was measured in a stirred Clark-type oxygen electrode assembly at 37°C under aerobic conditions (11, 45, 46). Aliquots (5 μ l) of cell suspension were added to 2 ml of M9 salts solution and equilibrated for 1 min before the addition of the radiolabeled substrate to a final concentration of 20 μ M [¹⁴C]fumarate or [¹⁴C]succinate. Samples (0.1 ml) were taken after 20 s and thereafter at 30-s intervals, immediately added to 5 ml of stop buffer (64 mM phosphate buffer [pH 7.0], 10 mM fumarate, 0.2 mM sodium fluoroacetate), and filtered rapidly through Whatman GF/F filters. The filters were dried and assayed for radioactivity by scintillation counting. The total protein contents of the cell suspensions were determined by the Lowry et al. assay (32).

Construction of the *dctA-lacZ* transcriptional fusion. The 0.9-kb *Hpa*I-*Hpa*I *f651'-dctA'* fragment of pGS753 was subcloned into the *Sma*I site of pRS528 (49) to generate pGS928, carrying a *dctA-lacZ* transcriptional fusion (Fig. 1). The *dctA-lacZ* fusion was transferred to phage λ RS45 (49) by *in vivo* homologous recombination as described by Simons et al. (49), and the resulting Lac⁺ phage, λ RS45(*dctA'-lacZYA*), was used to create a monolysogenic derivative of MC4100, JRG3351 (Δlac $\lambda\text{dctA-lacZYA}$).

To investigate the effects of ArcA, RpoS, and FNR on *dctA* expression, *arcA*, *rpoS*, and *fnr* deletions were transferred from the respective donor strains, ECL585, RH90, and JRG1728, by P1*vir*-mediated transduction, to the *dctA-lacZ* fusion strain, JRG3351, generating strains JRG4011, SCA345, and JRG4013, respectively (Table 1). To study the effects of CRP on *dctA* expression, a pGS279 (*crp*⁺) transformant of strain JRG1999 (Δcrp) was infected with λ RS45(*dctA'-lacZYA*) to generate the monolysogen JRG4016(pGS279), which was subsequently cured of pGS279 by being propagated under nonselective conditions (Table 1).

Growth media and conditions. Cultures were grown at 37°C either aerobically (50 ml in 250-ml conical flasks at 250 rpm) or anaerobically (10 ml in filled and

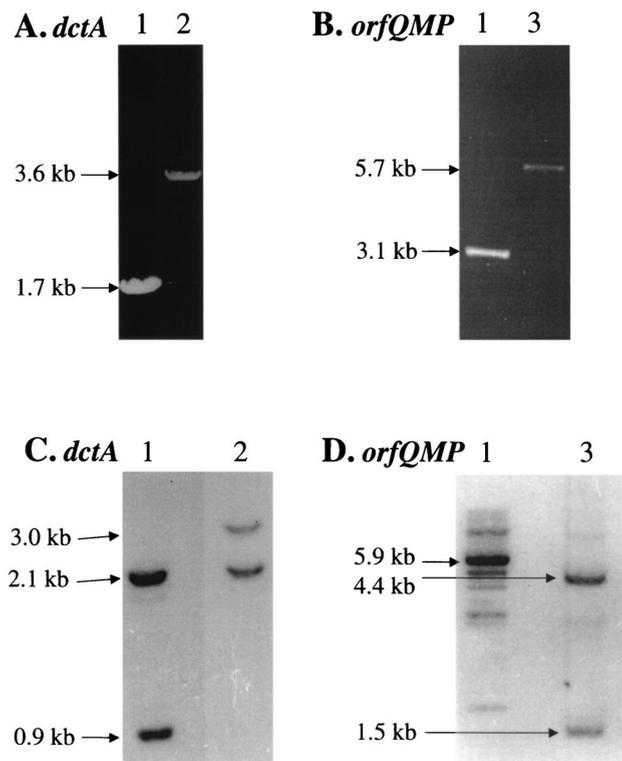


FIG. 2. PCR and Southern blot analysis of *dctA::spc* and *orfM::amp* mutants. PCR (A and B) and Southern blotting (C and D) were performed, as described in Materials and Methods, with chromosomal DNA from JC7623 (lanes 1), JC7623 *dctA::spc* (MDO1) (lanes 2), and JC7623 *orfM::amp* (MDO2) (lanes 3), together with PCR primers or hybridization probes specific for the *dctA* (A and C) and *orfQMP* (B and D) regions. The sizes of the PCR products and major hybridizing bands are shown. Analysis of MDO3 gave results similar to those of MDO2 (data not shown).

sealed Bijou bottles) in L broth or M9 minimal salts (Sigma) with various carbon sources.

β -Galactosidase measurements. β -Galactosidase specific activities (expressed in micromoles of *o*-nitrophenyl- β -D-galactopyranoside [ONPG] per minute per milligram of protein) were determined for samples taken at 0.5- to 1-h intervals from two independent cultures. Each sample was assayed in duplicate, as described previously (12).

Northern hybridization and primer extension analysis. Total RNA was extracted by a method (12) based upon that described by Aiba et al. (1). The 0.46-kb *ClaI-PstI* *dctA* fragment of pDctA (Fig. 1) was used as the hybridization probe. Reverse transcriptase-mediated primer extension analysis was performed (12, 39) with two independent primers for each promoter (see reference 51 for coordinates): P1_{*dctA*} (5'-²²³⁵ATCGCTGTCAGGACCTGAAAGTAAAGGCTT²²⁰⁶-3') and P2_{*dctA*} (5'-²²¹¹TAGAAATGGCCAAGGAGAATACCAATGGCT²¹⁸²-3'). Sequence ladders were generated with the T7 Sequenase DNA sequencing kit (Amersham) together with pGS753 as template and the primers described above. A potential CRP site was identified by using the score-matrix searching option of the xnip program (53) and a score matrix derived from 25 experimentally determined CRP-binding sites.

RESULTS

Growth properties of the *dctA* and *orfM* mutants. The wild type and *dctA* mutant grew identically under aerobic conditions in L broth (data not shown) and minimal medium containing 0.4% glucose (Fig. 3A), 0.4% fructose, 40 mM lactate, 40 mM acetate, or 0.4% glycerol (data not shown) as the sole carbon source. However, the *dctA* mutant (MDO800) failed to grow with malate or fumarate as the sole carbon source and grew more slowly than the wild type with succinate (Fig. 3). The growth difference on succinate was fully complemented by pDctA (Fig. 3B), but the growth defects on fumarate and

malate were only partially restored by pDctA (Fig. 3C and D). However, AN387(pDctA) grew at the same rate as MDO800 (pDctA) in fumarate and malate minimal medium (Fig. 3C and D), showing that pDctA lowers the fumarate- and malate-dependent growth of the parental strain. No growth defects were detected for the *orfM* mutant, MDO900, under the same conditions.

The results indicate that *dctA* (*f428*) does indeed encode the aerobic C₄-dicarboxylate transporter of *E. coli* whereas *orfQMP* plays no apparent role in aerobic C₄-dicarboxylate transport. They also show that the *dctA* mutant retains the ability to utilize succinate as a carbon and energy source, albeit at a reduced rate relative to the wild type. This suggests that *E. coli* possesses a succinate transporter of relatively low activity that enables the *dctA* mutant to grow on succinate. The possibility that aerobic succinate transport in the *dctA* mutant is mediated by the OrfQMP system or the anaerobic C₄-dicarboxylate transporters, DcuA and DcuB, was excluded by showing that the aerobic growth of the *dctA orfM* (SCA344), *dctA dcuA* (MDO1200), and *dctA dcuA dcuB* (MDO1100) mutants on succinate was identical to that of the *dctA* mutant (results not shown). Furthermore, it is unlikely that the other anaerobic C₄-dicarboxylate transporter, DcuC, corresponds to the putative DctA-independent aerobic succinate transporter, since *dcuC* expression is strongly repressed aerobically (58). Therefore, it appears that *E. coli* contains an uncharacterized succinate transporter, designated SucT, distinct from DctA, DcuA, DcuB, DcuC, and the OrfQMP system.

Anaerobic growth of the *dctA* and *orfM* mutants in glycerol (0.4%) plus fumarate (50 mM) was unaffected relative to the wild type, and the *dctA* mutation likewise had no effect on the anaerobic growth of the *dcuA* and *dcuA dcuB* mutants in the same medium (results not shown). This suggests that DctA plays a strictly aerobic role in the transport of C₄-dicarboxylates and that the *orfQMP* products are not involved in either aerobic or anaerobic C₄-dicarboxylate transport.

Fumarate and succinate uptake properties of the *dctA* mutant. To determine whether the *dctA::spc* mutation affects transport or some other metabolic function, [¹⁴C]fumarate uptake was compared in the wild type and *dctA* mutant, and in the corresponding pDctA (*dctA*⁺) transformants, after aerobic growth to late log phase in L broth (Fig. 4). High levels of fumarate transport activity (~56 pmol of fumarate/min/mg of protein with 20 μ M fumarate) were observed for the wild type (AN387), and although no uptake activity was detected with the *dctA* mutant, it was fully restored in the pDctA transformant (Fig. 4). This shows that the growth defects of the *dctA* mutant are due to deficient C₄-dicarboxylate transport (Dct) activity and that the failure to transport fumarate is due to inactivation of the *dctA* gene. The Dct activity of AN387 was inhibited to below detectable levels by a 100-fold excess of unlabeled fumarate but was not affected by 100-fold excess lactate, pyruvate, or acetate (data not shown). This indicates that neither lactate, pyruvate, nor acetate is transported by the Dct system. Dct activity for AN387 in L broth was 19-fold higher during the stationary phase than the early log phase (not shown), probably due to stationary-phase induction of *dctA* transcription (see below).

As anticipated from the growth studies (Fig. 3), the *dctA* mutant was able to transport succinate, but the rate was ~10-fold lower than that of the wild type (data not shown). Thus, the poor growth of the *dctA* mutant on succinate is apparently a consequence of reduced transport capacity.

Environmental factors affecting *dctA* expression. To provide a more complete understanding of the role of DctA in C₄-dicarboxylic acid transport, factors that regulate *dctA* expres-

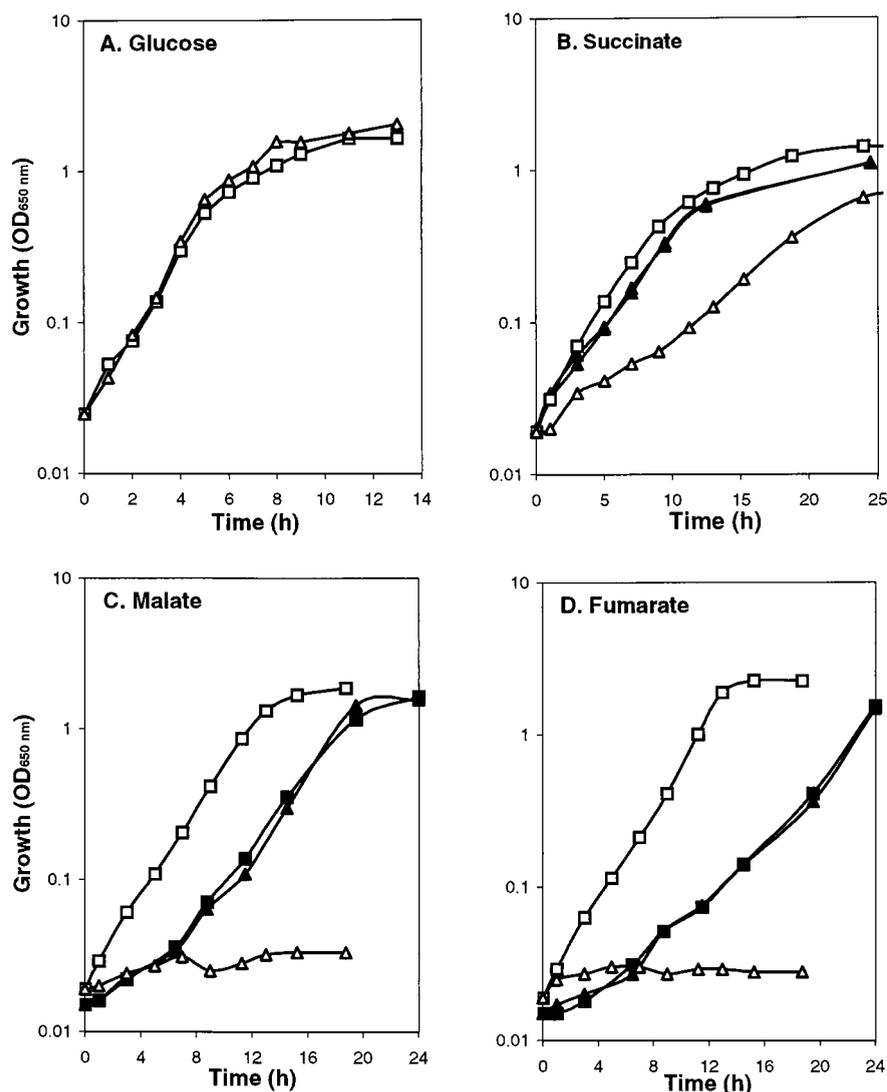


FIG. 3. Effects of the *dctA::spe* mutation on growth. Cultures were grown aerobically in M9 salts medium containing 0.4% glucose (A), 50 mM succinate (B), 50 mM malate (C), or 50 mM fumarate: AN387 (*dctA*⁺) (□), MDO800 (*dctA::spe*) (△), AN387(pDctA) (■), and MDO800(pDctA) (▲). OD_{650 nm}, optical density at 650 nm.

sion were examined by using a strain harboring a single-copy *dctA-lacZ* transcriptional fusion: JRG3351 (MC4100/λRS45 [*dctA'-lacZYA*]). The fusion contains 0.43 kb of the upstream *f651* gene, the 0.18-kb *f651-dctA* intergenic region, and 0.3 kb of the *dctA* coding region (Fig. 1). The activity of the *dctA-lacZ* fusion in single copy (see below) indicated that the *dctA* gene possesses an independent promoter and therefore, contrary to a previous suggestion (51), is not dependent on the upstream *f651* gene for transcription.

The activity of the *dctA-lacZ* fusion varied in a growth-phase-dependent manner during aerobic growth in L broth (Fig. 5), increasing up to 19-fold in stationary phase (~3.8 μmol/min/mg) relative to the early-log-phase activity (~0.2 μmol/min/mg). This growth-phase-dependent pattern resembles that observed for CRP-regulated genes such as the *cst* genes, *mcc*, and *glgCAP* (22, 31), suggesting that *dctA* is also regulated by CRP (see below). The addition of glucose lowered the expression of the fusion by up to ~30-fold to give a relatively constant activity (~0.13 μmol/min/mg) throughout the growth cycle (Fig. 5). This further supports the view that

dctA is subject to CRP-mediated catabolite repression, which is consistent with studies by Kay and Kornberg (20) showing that CRP, cyclic AMP (cAMP), and glucose regulate Dct activity. Interestingly, *dctA* expression was induced approximately two-fold by the presence of succinate (7.3 and 3.8 μmol/min/mg with and without succinate, respectively) (Fig. 5). Similarly, inductions were observed when citrate, aspartate, fumarate, malate, or maleate was added to the L broth, but no *dctA-lacZ* induction was observed with lactate and pyruvate (Fig. 6A). These data show that *dctA* is weakly induced by its C₄-dicarboxylate transport substrates and by citrate, but not by the monocarboxylates tested. These findings are consistent with recent reports that *dctA* is induced approximately threefold by succinate and twofold by fumarate (13, 57), and they are compatible with a weak C₄-dicarboxylate-dependent activation of *dctA* by the DcuS-DcuR system (13).

Expression of the *dctA-lacZ* fusion during aerobic growth in minimal medium varied with the carbon source (Fig. 7A). Expression of *dctA* was ca. sixfold higher with glycerol than with glucose (~0.8 and ~0.13 μmol/min/mg, respectively, in

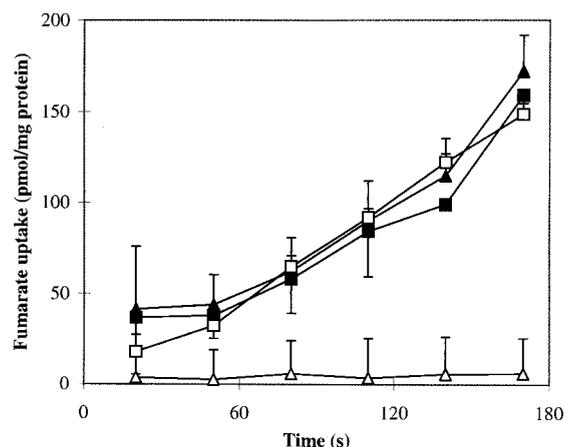


FIG. 4. Effects of the *dctA::spc* mutation on fumarate transport. Cultures were grown aerobically in L broth, harvested, washed in M9 salts solution, and then used to measure the rate of [2,3-¹⁴C]fumarate uptake, in duplicate, as described in Materials and Methods. The strains were AN387 (*dctA*⁺) (□), MDO800 (*dctA::spc*) (△), AN387(pDctA) (■), and MDO800(pDctA) (▲). Standard deviations are shown.

the postexponential phase) as the sole carbon source, presumably due to catabolite repression. However, much higher levels of expression were achieved with C₄-dicarboxylate substrates (2.7 to 4.8 μmol/min/mg in the postexponential phase), further indicating that *dctA* expression is activated by C₄-dicarboxylates. The weak *dctA* expression with glucose was not relieved by succinate, presumably because the catabolite repression effect exerted by glucose is dominant over the activation effect mediated by C₄-dicarboxylates (Fig. 7A). This is fully consistent with strong activation of *dctA* transcription by CRP and weak induction in response to C₄-dicarboxylates.

During anaerobic growth, the *dctA-lacZ* fusion was weakly expressed (0.003 to 0.27 μmol/min/mg) under all conditions used (Fig. 7B). In particular, the activities observed anaerobically in L broth or in minimal medium containing glycerol and fumarate were 7- and 150-fold lower than the corresponding levels observed aerobically (Fig. 7). These findings clearly indicate that *dctA* transcription is repressed in the absence of oxygen.

Effects of global regulators on *dctA-lacZ* expression. The above studies show that *dctA* expression is strongly affected by catabolite repression, repressed in the absence of oxygen, induced in the stationary phase, and induced ca. twofold in the presence of citrate and C₄-dicarboxylates. The roles of the global transcriptional regulators, ArcA, FNR, CRP, and RpoS, in the regulation of *dctA* transcription were investigated by studying the appropriate regulatory mutants. The postexponential anaerobic expression of the *dctA-lacZ* fusion under fumarate respiratory conditions was 18-fold higher in the Δ *arcA* strain, JRG4011, than in the *arcA*⁺ parental strain, JRG3351 (Fig. 8A), but transformation of the Δ *arcA* strain with the *arcA*-containing plasmid, pRB38, restored the anaerobic repression of *dctA* expression, resulting in expression levels similar to those of the wild type (Fig. 8A). In contrast, the Δ *fnr* mutation of JRG4013 increased *dctA* expression by only twofold. This effect was reversed by supplying the multicopy *fnr*⁺ plasmid, pCH21 (Fig. 8A). Similar results were observed with cultures grown under fermentative conditions (L broth plus 0.4% fructose), where postexponential *dctA* expression was 12- and 2-fold higher in the *arcA* and *fnr* mutants (2.4 and 0.4 μmol/min/mg), respectively, than in the wild type (0.2

μmol/min/mg). These results indicate that the anaerobic repression of *dctA* is mediated primarily by ArcA, with a minor contribution from FNR that is likely to be related to the activation of *arcA* expression by FNR (6).

Somewhat surprisingly, the Δ *fnr* mutation caused a 2.4-fold reduction in aerobic *dctA* expression (Fig. 8B), which suggests a minor aerobic role for FNR in the aerobic induction of *dctA* expression, as has been observed previously for eight unidentified *E. coli* gene products (44). There was only a slight (1.4-fold) reduction in *dctA* expression in the Δ *arcA* strain (Fig. 8B), indicating that Arc has little effect on *dctA* expression aerobically.

The ~30-fold postexponential repression of *dctA* expression by glucose in L broth observed under aerobic conditions was largely reversed by the addition of cAMP (from 0.17 to 2.9 μmol/min/mg), which provides a strong indication that *dctA* is activated by the cAMP-CRP complex (Fig. 8C). Furthermore, aerobic *dctA* expression in L broth was virtually abolished by the Δ *crp* mutation of JRG4016 (Fig. 8D), resulting in a 275-fold reduction of *dctA* expression from 5.5 to 0.02 μmol/min/mg. Complementation of the Δ *crp* mutation by the *crp*⁺ plasmid, pGS279, increased *dctA* expression ~130-fold to a level similar to that of the corresponding parental transformant, JRG3351 (Fig. 8D). Expression of *dctA* was unaffected by the *rpoS* mutation of SCA345, showing that *dctA* transcription is RpoS independent (data not shown). These observations strongly suggest that the cAMP-CRP complex is a major transcriptional activator for *dctA* expression and is responsible for the observed stationary-phase induction.

The strong CRP-mediated catabolite repression of *dctA* expression is in agreement with the prevention of induction of Dct activity by glucose: the presence of glucose in succinate minimal medium caused a ~50-fold reduction in Dct activity (20), which compares well with the ~67-fold reduction in aerobic postexponential *dctA* expression caused by the presence of glucose in succinate minimal medium (Fig. 7A). The observed CRP regulation is also consistent with the inability of mutants lacking phosphotransferase system components to grow on succinate (54) and the reestablishment of Dct activity in *cya* (adenyl cyclase) mutants by the addition of cAMP to the

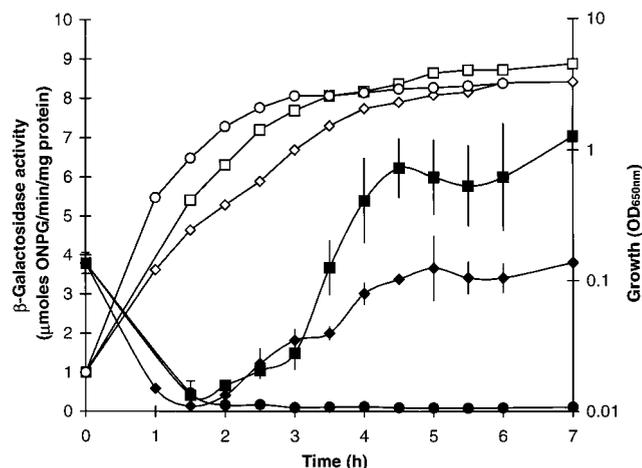


FIG. 5. Expression of a *dctA-lacZ* transcriptional fusion during aerobic growth in L broth. The β-galactosidase activities (solid symbols) and culture densities (open symbols) of JRG3351 (*dctA-lacZ*) are shown after growth at 37°C in L broth (◇ and ◆), L broth with 50 mM succinate (□ and ■), and L broth with 1% glucose (○ and ●). Error bars represent standard deviations for two cultures, each assayed in duplicate. OD_{650 nm}, optical density at 650 nm.

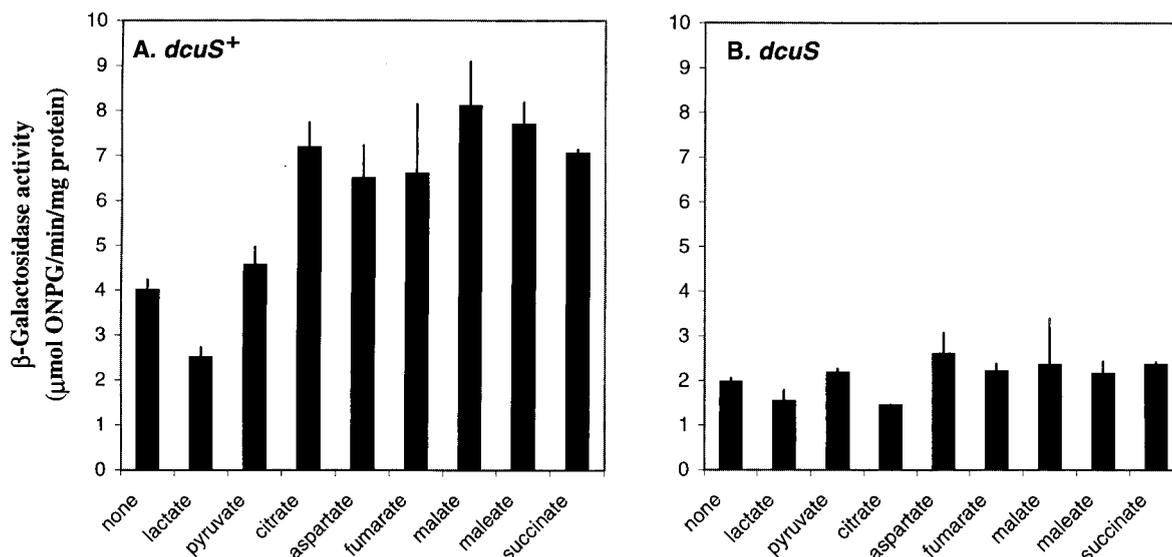


FIG. 6. Effects of various carboxylates and the *dcuS* mutation on *dctA-lacZ* expression. Cultures of JRG3351 (*dcuS*⁺) (A) and JRG3984 (*dcuS*) (B) were grown aerobically to the postexponential phase in L broth supplemented with carboxylates (50 mM) as indicated. The β -galactosidase activities are shown with standard deviations.

growth medium (27). In addition, the ca. twofold induction of *dctA* expression by C₄-dicarboxylates matches the two- to threefold maleate-dependent induction of Dct activity in minimal medium containing various carbon sources (20). Therefore, Dct activity and *dctA* transcription are regulated in parallel in response to catabolite repression and C₄-dicarboxylates, indi-

cating that Dct activity is regulated mainly at the level of transcription.

Roles of DcuS-DcuR and DctA in the C₄-dicarboxylate-dependent regulation of *dctA-lacZ* expression. The possibility that the C₄-dicarboxylate-dependent induction of *dctA* expression is mediated by the recently discovered two-component C₄-

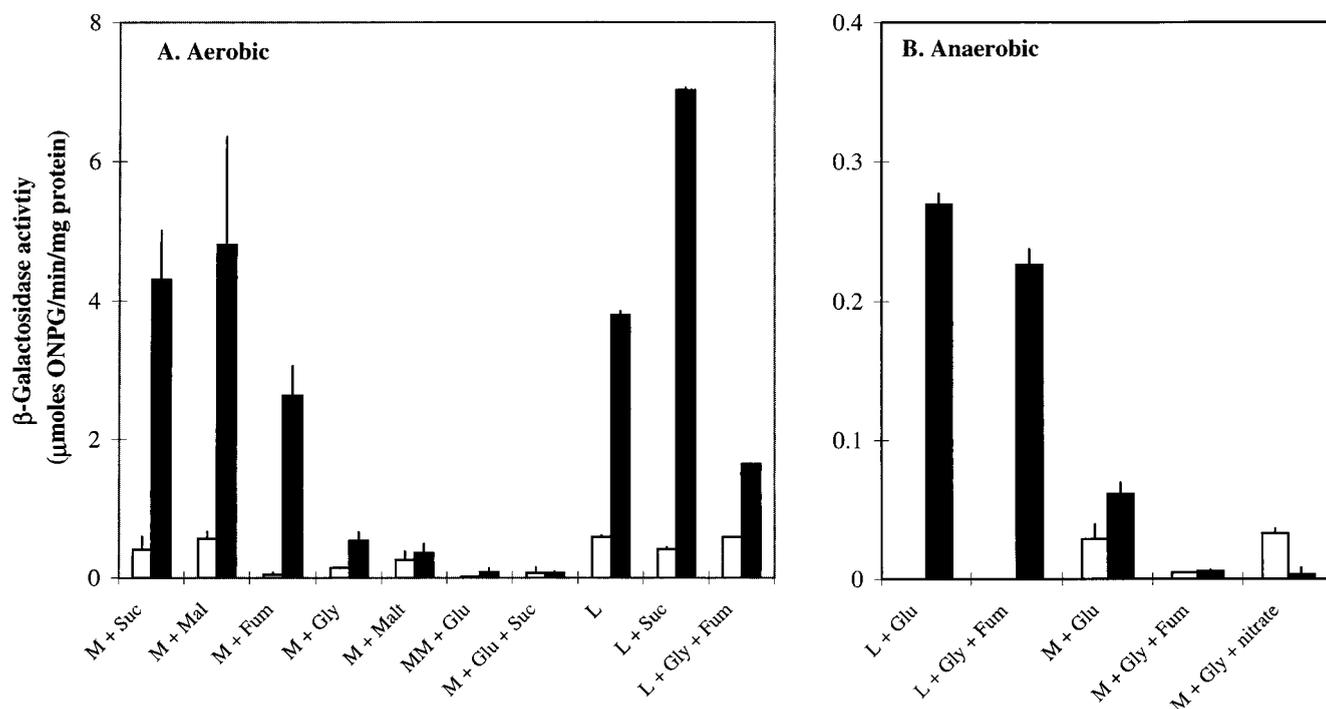


FIG. 7. Expression of *dctA-lacZ* during aerobic (A) and anaerobic (B) growth. Growth of JRG3351 took place in L broth or M9 minimal medium with or without 50 mM succinate (Suc), malate (Mal), fumarate (Fum), or nitrate or 0.4% glycerol (Gly) or maltose (Malt), or 0.4% glucose (Glu) in minimal medium (M) and 1% glucose in L broth (L). β -Galactosidase activities were assayed in duplicate with samples taken from duplicate cultures at 0.5- to 1-h intervals over the entire growth phase, but only those corresponding to early-logarithmic (open bars) and postexponential (solid bars) growth are shown. Standard deviations are indicated.

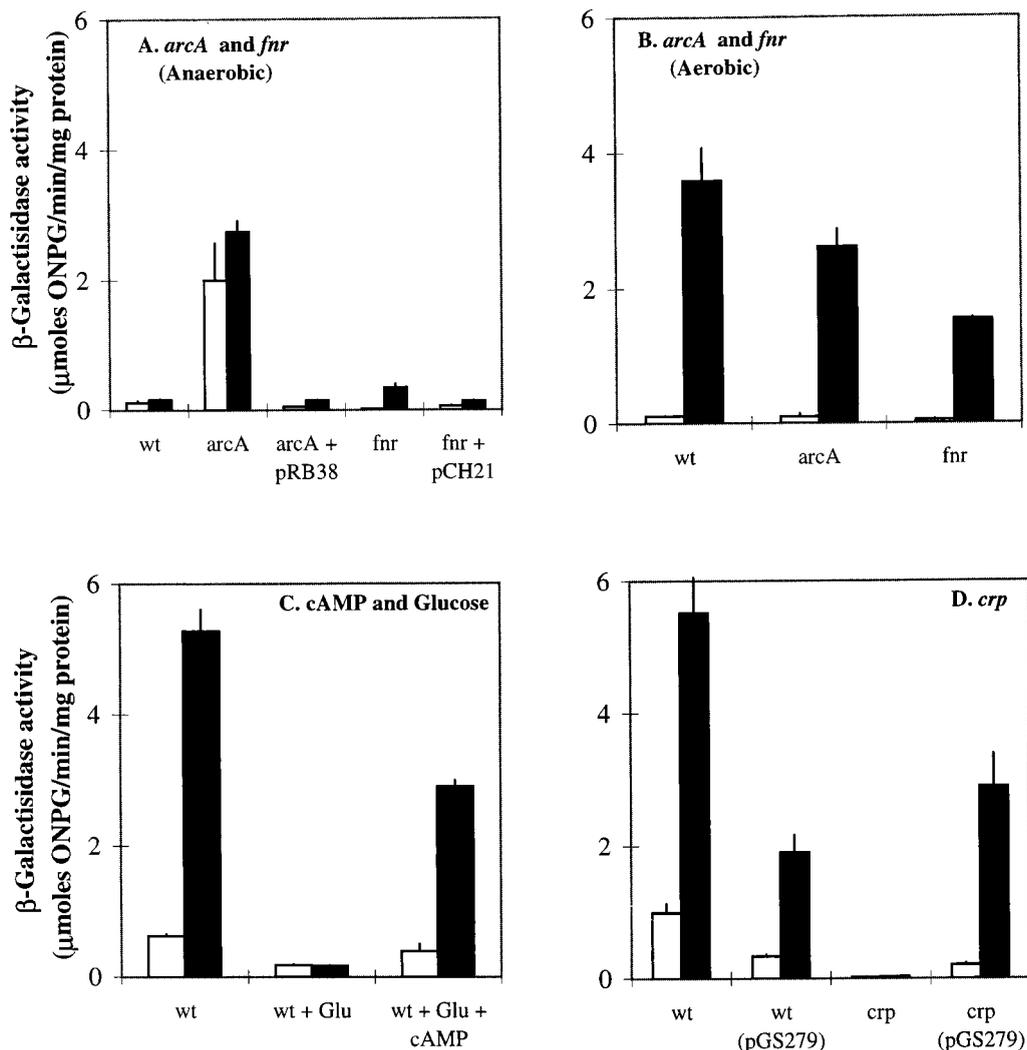


FIG. 8. Effects of ArcA, FNR, cAMP, and CRP on *dctA-lacZ* expression. Cultures were grown anaerobically (A) or aerobically (B to D) at 37°C in L broth plus 0.4% glycerol and 50 mM fumarate (A), in L broth only (B and D), or in L broth with and without 1% glucose (Glu) and 5 mM cAMP (C). The strains were JRG3351 (wild type [wt]), JRG4011 (*arcA*), JRG4013 (*fnr*), and JRG4016 (*crp*) and the corresponding transformants containing plasmids pRB38 (*arcA*⁺), pCH21 (*fnr*⁺), and pGS279 (*crp*⁺). Note that JRG4017 (the corresponding *crp*⁺ control for JRG4016) gave very similar results to those obtained with JRG3351 (data not shown). Other details are as for Fig. 7.

dicarboxylate-responsive DcuS-DcuR system was tested by using a *dcuS* mutant, JRG3984. Under aerobic conditions in L broth, the *dcuS* mutation caused a twofold reduction in post-exponential *dctA* expression and abolished *dctA* induction by C₄-dicarboxylates or citrate (Fig. 6B). These findings are consistent with the recent report that *dctA* is induced fourfold by C₄-dicarboxylates in a DcuS-DcuR-dependent manner (13). The *dctA* gene could thus be a member of the DcuSR regulon, along with the *dcuB-fumB* (specifying an anaerobic C₄-dicarboxylate transporter and the anaerobic fumarase) and *frd-ABCD* (encoding fumarate reductase) operons (13, 57). However, it should be noted that these results contradict those of Zientz et al. (57), who concluded that the C₄-dicarboxylate induction of *dctA* is DcuS-DcuR independent.

The possibility that the DctA protein is involved in regulating its own synthesis either directly or indirectly (via its transport activity) was tested by comparing *dctA* expression in JRG3351 (*dctA-lacZ* *dctA*⁺) and JRG4005 (*dctA-lacZ* *dctA::spc*) during aerobic growth in glycerol (0.4%) minimal

medium with and without 50 mM malate, succinate, or maleate (Fig. 9). The *dctA* mutation resulted in a 2.6-fold increase in *dctA* expression in the absence of C₄-dicarboxylates, suggesting that DctA represses its own synthesis. Interestingly, the C₄-dicarboxylates no longer induced *dctA-lacZ* expression in the *dctA* mutant. Therefore, the *dctA* mutation results in the constitutive expression of *dctA-lacZ* with respect to the presence or absence of C₄-dicarboxylates. A similar phenomenon has been reported for the *dctA* genes of *R. leguminosarum* and *S. meliloti* (17, 40, 55). It has been suggested that in these species, in the absence of C₄-dicarboxylates DctA interacts with the C₄-dicarboxylate-sensing histidine-kinase (DctB) in a way that inhibits signal transduction from DctB to the cognate response regulator (DctD). In the presence of substrate, the DctA-mediated inhibition of DctB activity is thought to be relieved, leading to the induction of *dctA* by DctD (18, 55). It is possible that an analogous mechanism operates aerobically in *E. coli*, whereby the C₄-dicarboxylate-sensing histidine kinase activity

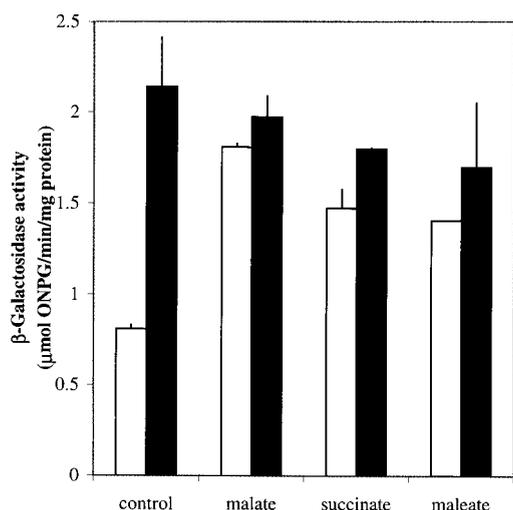


FIG. 9. Effects of the *dctA* mutation on *dctA-lacZ* expression. Cultures of JRG3351 (*dctA*⁺) (open bars) or JRG4005 (*dctA::spc*) (solid bars) were grown aerobically at 37°C in M9 salts medium containing 0.4% glycerol with or without 50 mM malate, succinate, or maleate. Other details are as for Fig. 7.

of DcuS is inhibited by DctA in the absence, but not the presence, of C₄-dicarboxylates (and presumably citrate).

Citrate induces *dcuB* expression in a DcuS-DcuR-dependent manner. Although the DcuS-DcuR system is known to respond to external C₄-dicarboxylates (aspartate, fumarate, malate, maleate, succinate, and tartrate) (12, 13, 57), it had not been shown to respond to citrate until tested here (Fig. 6). To determine whether other members of the DcuSR regulon are also induced by citrate in a DcuS-DcuR-dependent fashion, expression of a *dcuB-lacZ* transcriptional fusion was measured following anaerobic growth to the late log phase (25 h of growth to an optical density at 650 nm of ~0.4) in minimal medium containing 0.4% glycerol and 50 mM trimethylamine oxide, with and without 50 mM citrate. The expression of *dcuB* was induced ~20-fold by citrate in the *dcuS*⁺ strain (JRG3835) (0.59 ± 0.05 and 0.032 ± 0.01 µmol of ONPG/min/mg of protein in the presence and absence, respectively, of citrate) but not in the *dcuS* mutant, JRG3983 (0.009 ± 0.0004 and 0.012 ± 0.002 µmol/min/mg, respectively), and in the presence of citrate, *dcuB* expression was 66-fold greater in the *dcuS*⁺ strain than the *dcuS* mutant. As expected, these results show that *dcuB* (like *dctA*) is induced by citrate in a DcuS-DcuR-dependent manner. This confirms that the DcuS-DcuR system responds to citrate as well as to C₄-dicarboxylates.

Northern blot analysis of *dctA* transcription. Northern hybridization was performed to determine the size of the *dctA* transcript and to correlate its abundance with expression of the *dctA-lacZ* fusion (Fig. 10A). Total RNA was extracted from MC1000 (wild type), JRG1999 (*Δcrp*), and a pGS279 (*crp*⁺) transformant of JRG1999, grown to stationary phase in L broth with and without 0.4% glucose, and then hybridized with a labeled *dctA* fragment (Fig. 10A). A single *dctA*-hybridizing (1,300-nucleotide [nt]) transcript was detected for the wild type grown in the absence of glucose (Fig. 10A). The size corresponds to that expected for a monocistronic *dctA* transcript initiating at the promoter identified in the *f651-dctA* intergenic region (see below) and terminating at an inverted repeat (bp 96402 to 96372) located 15 bp downstream of the *dctA* stop codon (bp 96417) (GenBank accession no. U00039). No *dctA*-hybridizing transcript was detected for the wild type grown with glucose or from the *crp* mutant grown in the absence of

glucose. However, a single major hybridizing band corresponding to the 1,300-nt *dctA* transcript was observed in the pGS279-complemented *crp* strain grown in the absence of glucose. Thus, the *dctA* Northern blot analysis supports the conclusions derived from the studies with the *dctA-lacZ* fusion that *dctA* transcription is strongly activated by the cAMP-CRP complex. Also, contrary to an earlier prediction (51) that *dctA* forms an operon with the upstream *f651* gene (encoding a serpin-like protein), *dctA* appears to be monocistronic (Fig. 1).

Transcriptional start site of the *dctA* gene. The 5' end of the *dctA* transcript was defined by primer extension analysis (Fig. 10B). A single primer extension product corresponding to a start site at G-97752, 51 bp upstream of the anticipated *dctA* translational start codon, was detected (Fig. 10C). It matches the start site determined for the *dctA* gene of *Salmonella typhimurium* (2) and is preceded by appropriately positioned -10 and -35 sites, which are separated by the optimal distance, 17 bp. The -35 site is relatively poor, and this would explain why *dctA* expression is weak in the absence of cAMP-CRP activation. There is a strongly predicted CRP-binding site at bp -81.5, which is consistent with the strong cAMP-CRP activation of *dctA* expression (Fig. 10C), and this indicates that *dctA* is expressed from a class I CRP-dependent promoter (5). Although ArcA binding-site consensus sequences have been proposed (8, 33), they do not allow the accurate prediction of ArcA-binding sites (47). Therefore, it is not possible to locate potential ArcA-binding sites by sequence analysis, although it is presumed that *dctA* is directly repressed by ArcA.

DISCUSSION

The coidentity of *dctA* and *f428* was previously assumed because of the high degree (~63%) of amino acid sequence identity between the translation products of the *E. coli f428* gene and the rhizobial *dctA* genes (51), together with the close locations of the *E. coli f428* and *dctA* genes in the respective physical and linkage maps (19, 25, 51). The data presented here confirm that *f428* does indeed correspond to the *dctA* gene encoding the aerobic C₄-dicarboxylate transporter of *E. coli*. The *dctA::spc* mutant was unable to grow aerobically on fumarate or malate and grew weakly on succinate, which is consistent with the phenotype reported by Kay and Kornberg (19). Anaerobic growth was not affected, nor was aerobic growth on other carbon sources, indicating that *dctA* is strictly concerned with aerobic C₄-dicarboxylate metabolism. The growth defects of the *dctA* mutant were complemented by a multicopy *dctA*⁺ plasmid, confirming that the mutant phenotype is indeed caused by the inactivation of the chromosomal *dctA* gene. No comparable effects were detected for the *orfM::amp* mutant, suggesting that the *orfQMP* genes are not required for C₄-dicarboxylate transport. The overexpressed *orfP*-encoded periplasmic-binding protein has also been shown not to bind C₄-dicarboxylates (11a). The *orfQMP* genes would appear to form part of a nine-gene cluster that is required for L-lyxose utilization (42, 51), and so these genes are likely to be involved in the transport of L-lyxose or some other pentose sugar.

The role of DctA in the aerobic transport of C₄-dicarboxylates was confirmed by the aerobic fumarate transport deficiency of the *dctA* mutant and its complementation by a *dctA*⁺ plasmid. Overall, the results show that DctA is probably the sole mediator of fumarate and malate transport under aerobic conditions and that there is at least one undefined transporter (SucT) mediating the aerobic uptake of succinate, albeit at relatively low rates. Possible candidates are the products of the *yjU* and *o463* genes at 70 and 39 min (respectively), which

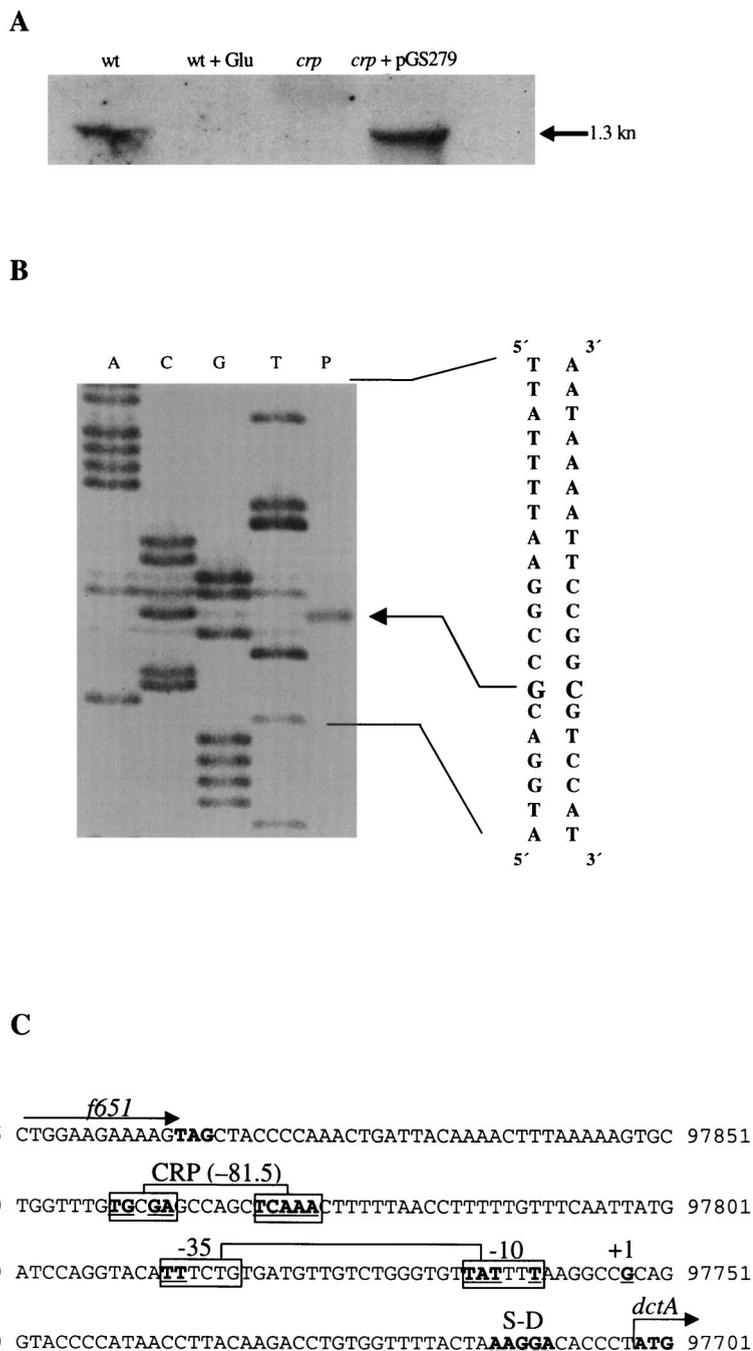


FIG. 10. Analysis of the *dctA* transcript. (A) Northern blotting. Total RNA was extracted from MC1000 (wild type [wt]), JRG1999 (*crp*), and JRG1999 transformed with pGS279 (*crp*⁺). Strains were grown aerobically in L broth with or without 1% glucose (Glu). Following electrophoresis and capillary transfer, RNA was hybridized with a labeled *dctA* fragment. The arrow on the right indicates the specifically hybridizing *dctA* transcripts. The size of the *dctA* mRNA transcript (in kilonucleotides) is indicated. (B) Determination of the *dctA* transcriptional start site by reverse transcriptase-mediated primer extension. RNA was isolated from MC1000 grown aerobically in L broth. Lane P indicates the primer extension product with primer P2_{dctA}. Similar results were obtained with primer P1_{dctA} (results not shown). The sequencing ladder (lanes A, C, G, and T) was generated by using primer P2_{dctA} and pGS753 as template. The corresponding nucleotide sequence, its complement, and the transcriptional start site (indicated by an arrow) are shown. (C) Nucleotide sequence of the *dctA* promoter region. Coordinates are from reference 51. The experimentally determined +1 site is boxed and labeled, as are the deduced -35 and -10 sites and the predicted CRP site. Residues matching the corresponding consensus sequence for the CRP, -35, -10, +1, and Shine-Dalgarno (S-D) sites are in bold and underlined. The positions of the predicted start codon of *dctA* and termination codon of *f651* are in bold.

encode DctA homologues with 27 and 20% sequence identity relative to DctA, or the *ygjE* (a potential tartrate/succinate antiporter), *citT/ybdS* (a citrate/succinate antiporter), and *ybhI* (a potential tricarboxylate transporter) genes (37a, 38).

Previous work has shown that a *dctA* mutant of *Salmonella*

typhimurium cannot use orotate, a cyclic monocarboxylate, for pyrimidine synthesis, but that the lesion could be complemented by a *dctA*⁺ plasmid encoding the *E. coli* DctA protein, which is 95% identical to the *S. typhimurium* protein (2). The *S. typhimurium* *dctA* mutant was reported to be unable to grow

on solid medium containing fumarate and malate, and it grew weakly with succinate, but no transport studies or attempts to complement the nutritional phenotype were made (2), nor was the capacity of the mutant to undergo aerobic growth on other carboxylates and anaerobic growth on C₄-dicarboxylates tested. A role for *E. coli* *dctA* in C₄-dicarboxylate transport has also been implied in a recent study (4a) showing that the inability of an *E. coli* *atp* deletion strain to grow on C₄-dicarboxylates can be complemented by a *dctA*⁺ plasmid.

Expression of a *dctA-lacZ* transcriptional fusion was found to be subject to strong CRP-mediated catabolite repression and ArcA-mediated anaerobic repression. Despite an earlier report to the contrary (56), the present results clearly support the view that the DcuS-DcuR system is involved in the C₄-dicarboxylate-dependent regulation of *dctA* (13). In a *dctA* mutant, *dctA-lacZ* expression was constitutive with respect to the presence or absence of C₄-dicarboxylates, indicating a role for DctA in regulating its own synthesis. By analogy to the mechanism proposed for rhizobial *dctA* autoregulation (17, 18, 40, 55), it is suggested that in aerobically grown *E. coli* in the absence of exogenous C₄-dicarboxylates, DcuS is inhibited by inactive (nontransporting) DctA. Conversely, in the presence of C₄-dicarboxylates, DctA would be active (transporting) and the inhibition of DcuS would be relieved, thus allowing signal transduction from DcuR to DcuS and the consequent induction of *dctA*. Such a mechanism implies that DcuS activity is dictated by the status of DctA. However, anaerobically, DcuS appears to directly sense external C₄-dicarboxylates in way that is independent of DctA or the DcuA, DcuB, and DcuC proteins (13, 57), suggesting that DcuS has two modes of operation, one that acts anaerobically and one that acts aerobically. Switching between the anaerobic and aerobic modes could be mediated by the central PAS domain of DcuS. The proposed redox-sensing role of the PAS domain would be consistent with this suggestion (13).

Previous work had shown that the DcuS-DcuR system is responsive to external C₄-dicarboxylates, namely, aspartate, fumarate, malate, maleate, succinate, and tartrate (13, 57). In this study, citrate was also found to be a coeffector for the DcuS-DcuR system, indicating that DcuS has broader ligand specificity than was previously realized. It is likely that the induction of the DcuSR regulon by citrate is physiologically significant, since, although most *E. coli* strains cannot utilize citrate aerobically, under anaerobic conditions citrate can be converted to fumarate for respiratory purposes (38). The anaerobic utilization of citrate would require the expression of the fumarate reductase operon, *frdABCD*, and the anaerobic fumarase gene of the *dcuB-fumB* operon. Appropriately, these operons are members of the DcuSR regulon (13, 57) and would therefore be induced by external citrate.

The pattern of *dctA* regulation is largely consistent with the role of its product in the aerobic uptake of C₄-dicarboxylates. The strong CRP activation of *dctA* ensures that external C₄-dicarboxylates are taken up for catabolic purposes only in the absence of "more preferable" carbon and energy sources, such as glucose. The anaerobic repression of *dctA* by ArcA limits the function of DctA to aerobic conditions. This is desirable since DctA is thought to be a proton symporter and would therefore consume energy, whereas the anaerobic C₄-dicarboxylate transporters can act in substrate-product exchange (antiport) mode and would therefore be non-energy consuming, a property that is likely to be important under the relatively low-energy-yielding conditions of anaerobic respiration. The relatively weak induction of *dctA* expression by C₄-dicarboxylates (and citrate) is unlikely to have any major impact on the C₄-dicarboxylate metabolism. Interestingly, a *dcuS* mutant

exhibits the same growth defects as the *dctA* mutant, namely, no growth on fumarate and malate, and weak growth on succinate (13). This suggests that the *dcuS* mutant is devoid of Dct activity, which in turn suggests that DcuS (or DcuR, since the *dcuS* mutation is likely to have a polar effect on *dcuR*) could have a direct effect on DctA transport activity. This possibility is supported by the observation that the *dcuS* mutant totally lacks Dct activity (7a), although *dctA* transcription is only halved. The physiological purpose of such a regulatory mechanism is unclear but could be related to the need to ensure that DctA does not mediate net export of C₄-dicarboxylates, which could otherwise occur when external C₄-dicarboxylate concentrations are low.

The regulatory features of the *dctA* gene are reminiscent of those of the genes encoding the aerobic CAC (citric acid cycle) enzymes. Like *dctA*, CAC enzymes are regulated at the level of transcription by CRP-mediated catabolite repression and by the oxygen/redox-responsive, two-component ArcBA system (7). This mode of regulation ensures that CAC activity is relatively low both anaerobically, when the ability of the cycle to supply energy is restricted, and aerobically during growth on glycolytic substrates (such as glucose), when the full cycle is unnecessary because sufficient energy can be derived through glycolysis. Since the role of DctA is to deliver external C₄-dicarboxylates for consumption via the aerobically operating CAC, it is physiologically appropriate that *dctA* regulation should largely match that of the aerobic CAC enzymes.

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REFERENCES

- Aiba, H., S. Adkya, and B. de Crombrughe. 1981. Evidence for a functional *gal* promoter in intact *Escherichia coli* cells. *J. Biol. Chem.* **256**:11905–11910.
- Baker, K. E., K. P. Ditullio, J. Neuhaard, and R. A. Kelln. 1996. Utilization of orotate as a pyrimidine source by *Salmonella typhimurium* and *Escherichia coli* requires the dicarboxylate transport protein encoded by *dctA*. *J. Bacteriol.* **178**:7099–7105.
- Bewick, M. A., and T. C. Y. Lo. 1979. Dicarboxylic acid transport in *Escherichia coli* K12: involvement of a binding protein in the translocation of dicarboxylic acids across the outer membrane of the cell envelope. *Can. J. Biochem.* **57**:653–661.
- Bewick, M. A., and T. C. Y. Lo. 1980. Localization of the dicarboxylate binding protein in the cell envelope of *Escherichia coli* K12. *Can. J. Biochem.* **58**:885–897.
- Boogerd, F. C., L. Boe, O. Michelsen, and P. R. Jensen. 1998. *atp* mutants of *Escherichia coli* fail to grow on succinate due to a transport deficiency. *J. Bacteriol.* **180**:5855–5859.
- Busby, S., and R. H. Ebright. 1997. Transcription activation at class II CAP-dependent promoters. *Mol. Microbiol.* **23**:853–859.
- Compan, I., and D. Touati. 1994. Anaerobic activation of *arcA* transcription in *Escherichia coli*: roles of Fnr and ArcA. *Mol. Microbiol.* **11**:955–964.
- Cronan, J. E., and D. LaPorte. 1996. Tricarboxylic acid cycle and glyoxylate bypass, p. 206–216. 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, 2nd ed., vol. 1. ASM Press, Washington, D.C.
- 7a. Davies, S., S. C. Andrews, and D. J. Kelly. Unpublished data.
- Drupal, N., and G. Sawers. 1995. Purification of ArcA and analysis of its specific interaction with the *pfl* promoter-regulatory region. *Mol. Microbiol.* **16**:597–607.
- Engel, P., R. Kramer, and G. Uden. 1992. Anaerobic fumarate transport in *Escherichia coli* by an *fnr*-dependent dicarboxylate uptake system which is different from aerobic dicarboxylate uptake. *J. Bacteriol.* **174**:5533–5539.
- Fellay, R., J. Frey, and H. Krisch. 1987. Interposon mutagenesis of soil and water bacteria—a family of DNA fragments designed for in vitro insertional mutagenesis of Gram-negative bacteria. *Gene* **52**:147–154.
- Forward, J. A., M. C. Behrendt, N. R. Wyborn, R. Cross, and D. J. Kelly. 1997. TRAP transporters: a new family of periplasmic solute transport sys-

- tems encoded by the *dctQMP* genes of *Rhodobacter capsulatus* and by homologs in diverse gram-negative bacteria. *J. Bacteriol.* **179**:5482–5493.
- 11a. **Gibson, M., S. C. Andrews, and D. J. Kelly.** Unpublished results.
 12. **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 C₄-dicarboxylate transporters in *Escherichia coli*. *J. Bacteriol.* **180**:6586–6596.
 13. **Golby, P., S. Davies, D. J. Kelly, J. R. Guest, and S. Andrews.** 1999. Identification and characterization of a two-component sensor-kinase and response-regulator system (DcuS-DcuR) controlling gene expression in response to C₄-dicarboxylates in *Escherichia coli*. *J. Bacteriol.* **181**:1238–1248.
 14. **Guest, J. R., J. Green, A. S. Irvine, and S. Spiro.** 1996. The FNR modulator and FNR-regulated gene expression, p. 317–342. In E. C. C. Lin and A. S. Lynch (ed.), Regulation of gene expression in *Escherichia coli*. Landes Co. and Chapman & Hall, Washington, D.C.
 15. **Gutowski, S. J., and H. Rosenberg.** 1975. Succinate uptake and related proton movements in *Escherichia coli* K12. *Biochem. J.* **152**:647–654.
 16. **Jamison, D. J., and C. F. Higgins.** 1984. Anaerobic and leucine-dependent expression of a peptide transport gene in *Salmonella typhimurium*. *J. Bacteriol.* **160**:131–136.
 17. **Jording, D., P. K. Sharma, R. Schmidt, T. Engelke, C. Uhde, and A. Pühler.** 1992. Regulatory aspects of the C₄-dicarboxylate transport in *Rhizobium meliloti*—transcriptional activation and dependence on effective symbiosis. *J. Plant Physiol.* **141**:18–27.
 18. **Jording, D., C. Uhde, R. Schmidt, and A. Pühler.** 1994. The C₄-dicarboxylate transport-system of *Rhizobium meliloti* and its role in nitrogen-fixation during symbiosis with alfalfa (*Medicago sativa*). *Experientia* **50**:874–883.
 19. **Kay, W. W., and H. L. Kornberg.** 1969. Genetic control of the uptake of C₄ dicarboxylic acids by *Escherichia coli*. *FEBS Lett.* **3**:93–96.
 20. **Kay, W. W., and H. L. Kornberg.** 1971. The uptake of C₄ dicarboxylic acids by *Escherichia coli*. *Eur. J. Biochem.* **18**:274–281.
 21. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *E. coli* genome. *Cell* **50**:495–508.
 22. **Kolter, R., D. A. Siegele, and A. Tormo.** 1993. The stationary phase of the bacterial life cycle. *Annu. Rev. Microbiol.* **47**:855–874.
 23. **Lin, E. C. C.** 1996. Dissimilatory pathways for sugars, polyols, and carboxylates, p. 307–342. 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, 2nd ed. ASM Press, Washington, D.C.
 24. **Lo, T. C. Y.** 1977. The molecular mechanism of dicarboxylic acid transport in *Escherichia coli* K12. *J. Supramol. Struct.* **7**:463–480.
 25. **Lo, T. C. Y., and M. A. Bewick.** 1978. The molecular mechanism of dicarboxylic acid transport in *Escherichia coli* K12. *J. Biol. Chem.* **253**:7826–7831.
 26. **Lo, T. C. Y., and M. A. Bewick.** 1981. Use of a nonpenetrating substrate analogue to study the molecular mechanism of the outer membrane dicarboxylic acid transport in *Escherichia coli* K12. *J. Biol. Chem.* **256**:5511–5517.
 27. **Lo, T. C. Y., K. Rayman, and B. D. Sanwal.** 1972. Transport of succinate in *Escherichia coli* K12. *J. Biol. Chem.* **247**:6323–6331.
 28. **Lo, T. C. Y., and B. D. Sanwal.** 1975. Isolation of the soluble substrate recognition component of the dicarboxylate transport system of *Escherichia coli* K12. *J. Biol. Chem.* **250**:1600–1602.
 29. **Lo, T. C. Y., and B. D. Sanwal.** 1975. Genetic analysis of mutants of *Escherichia coli* K12 defective in dicarboxylate transport. *Mol. Gen. Genet.* **140**:303–307.
 30. **Lo, T. C. Y., and B. D. Sanwal.** 1975. Membrane bound substrate recognition components of the dicarboxylate transport system of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **63**:278–285.
 31. **Loewen, P. C., and R. Hengge-Aronis.** 1994. The role of the sigma factor σ^S (KatF) in bacterial global regulation. *Annu. Rev. Microbiol.* **48**:53–80.
 32. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
 33. **Lynch, A. S., and E. C. C. Lin.** 1996. Transcriptional control mediated by the ArcA two-component response regulator protein of *Escherichia coli*: characterization of DNA binding at target promoters. *J. Bacteriol.* **178**:6238–6249.
 34. **Marmur, J.** 1964. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *Methods Enzymol.* **6**:726–739.
 35. **Martinez, E., B. Bartolomé, and F. De La Cruz.** 1988. pACYC184-derived cloning vectors containing the multiple cloning site and *lacZ α* reporter gene of pUC8/9 and pUC18/19 plasmids. *Gene* **68**:159–162.
 36. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *E. coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 37. **Oden, K. L., L. C. DeVeaux, C. R. T. Vibat, J. E. Cronan, Jr., and R. B. Gennis.** 1990. Genomic replacement in *Escherichia coli* K-12 using covalently closed circular plasmid DNA. *Gene* **96**:29–36.
 - 37a. **Paulsen, Ian T.** January 1999, posting date. Sequences. [Online.] <http://www.biology.ucsd.edu/~ipaulsen/transport/ecoli.html>. [8 February 1999, last date accessed.]
 38. **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.
 39. **Quail, M. A., D. J. Haydon, and J. R. Guest.** 1994. The *pdhR-aceEF-lpd* operon of *Escherichia coli* expresses the pyruvate dehydrogenase complex. *Mol. Microbiol.* **12**:95–104.
 40. **Ronson, C. W., and P. M. Ashwood.** 1985. Genes involved in the carbon metabolism of bacteroids, p. 201–207. In H. J. Evans, P. J. Bottomley, and W. E. Newton (ed.), Nitrogen fixation research progress. Martinus Nijhoff, Dordrecht, The Netherlands.
 41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 42. **Sanchez, J. C., R. Gimenez, A. Schneider, W. Fessner, L. Baldoma, J. Aguilari, and J. Badia.** 1994. Activation of a cryptic gene encoding a kinase for L-xylose opens a new pathway for the utilisation of L-xylose by *Escherichia coli*. *J. Biol. Chem.* **269**:29665–29669.
 43. **Sawers, J., and B. Suppmann.** 1992. Anaerobic induction of pyruvate formate-lyase gene expression is mediated by the ArcA and FNR proteins. *J. Bacteriol.* **174**:3474–3478.
 44. **Sawers, R. G., E. Zeheleih, and A. Böck.** 1988. Two-dimensional gel electrophoretic analysis of *Escherichia coli* proteins: influence of various anaerobic growth conditions and the *fnr* gene product on cellular protein composition. *Arch. Microbiol.* **149**:240–244.
 45. **Shaw, J. G., and D. J. Kelly.** 1991. Binding-protein dependent transport of C₄-dicarboxylates in *Rhodobacter capsulatus*. *Arch. Microbiol.* **155**:466–472.
 46. **Shaw, J. G., M. J. Hamblin, and D. J. Kelly.** 1991. Purification, characterization and nucleotide sequence of the periplasmic C₄-dicarboxylate binding protein (DctP) from *Rhodobacter capsulatus*. *Mol. Microbiol.* **5**:3055–306291.
 47. **Shen, J., and R. P. Gunsalus.** 1997. Role of multiple ArcA recognition sites in anaerobic regulation of succinate dehydrogenase (*sdhCDAB*) gene expression in *Escherichia coli*. *Mol. Microbiol.* **26**:223–236.
 48. **Silhavy, T. J., M. L. Barman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 49. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy lac-based cloning vectors for protein and operon fusions. *Gene* **53**:85–96.
 50. **Six, S., S. C. Andrews, G. Udden, and J. R. Guest.** 1994. *Escherichia coli* possesses two homologous anaerobic C₄-dicarboxylate membrane transporters (DcuA and DcuB) distinct from the aerobic dicarboxylate transport system (Dct). *J. Bacteriol.* **176**:6470–6478.
 51. **Sofia, H. J., V. Burland, D. L. Daniels, G. Plunkett III, and F. R. Blattner.** 1994. Analysis of the *Escherichia coli* genome. V. DNA sequence of the region from 76.0 to 81.5 minutes. *Nucleic Acids Res.* **22**:2576–2586.
 52. **Spiro, S., and J. R. Guest.** 1988. Inactivation of the FNR protein of *Escherichia coli* by targeted mutagenesis in the N-terminal region. *Mol. Microbiol.* **2**:701–707.
 53. **Staden, R.** 1996. The Staden sequence-analysis package. *Mol. Biotechnol.* **5**:233–241.
 54. **Wang, R. J., and M. L. Morse.** 1968. Carbohydrate accumulation and metabolism in *Escherichia coli*. I. Description of pleiotropic mutants. *J. Mol. Biol.* **32**:59–66.
 55. **Yarosh, O. K., T. C. Charles, and T. M. Finan.** 1989. Analysis of C₄-dicarboxylate transport genes in *Rhizobium meliloti*. *Mol. Microbiol.* **3**:813–823.
 56. **Zientz, E., S. Six, and G. Udden.** 1996. Identification of a third secondary carrier (DcuC) for anaerobic C₄-dicarboxylate transport in *Escherichia coli*: roles of the three Dcu carriers in uptake and exchange. *J. Bacteriol.* **178**:7241–7247.
 57. **Zientz, E., J. Bongaerts, and G. Udden.** 1998. Fumarate regulation of gene expression in *Escherichia coli* by the DcuSR (*dcuSR* genes) two-component regulatory system. *J. Bacteriol.* **180**:5421–5425.
 58. **Zientz, E., I. G. Jansusch, S. Six, and G. Udden.** 1999. Functioning of DcuC as the C₄-dicarboxylate carrier during glucose fermentation of *Escherichia coli*. *J. Bacteriol.* **181**:3716–3720.