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Transcriptional Regulation and Organization of the *dcuA* and *dcuB* Genes, Encoding Homologous Anaerobic C_4-Dicarboxylate Transporters in *Escherichia coli*

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The *dcuA* and *dcuB* genes of *Escherichia coli* encode homologous proteins that appear to function as independent and mutually redundant C_4-dicarboxylate transporters during anaerobiosis. The *dcuA* gene is 117 bp downstream of, and has the same polarity as, the aspartase gene (*aspA*), while *dcuB* is 77 bp upstream of, and has the same polarity as, the anaerobic fumarase gene (*fumB*). To learn more about the respective roles of the *dcu* genes, the environmental and regulatory factors influencing their expression were investigated by generating and analyzing single-copy *dcuA*- and *dcuB-lacZ* transcriptional fusions. The results show that *dcuA* is constitutively expressed whereas *dcuB* expression is highly regulated. The *dcuB* gene is strongly activated anaerobically by FNR, repressed in the presence of nitrate by NarL, and subject to cyclic AMP receptor protein (CRP)-mediated catabolite repression. In addition, *dcuB* is strongly induced by C_4-dicarboxylates, suggesting that *dcuB* is under the control of an uncharacterized C_4-dicarboxylate-responsive gene regulator. Northern blotting confirmed that *dcuA* (and *aspA*) is expressed under both aerobic and anaerobic conditions and that *dcuB* (and *fumB*) is induced anaerobically. Major monocistronic transcripts were identified for *aspA* and *dcuA*, as well as a minor species possibly corresponding to an *aspA-dcuA* cotranscript. Five major transcripts were observed for *dcuB* and *fumB*: monocistronic transcripts for both *fumB* and *dcuB*; a *dcuB-fumB* cotranscript; and two transcripts, possibly corresponding to *dcuB-fumB* and *fumB* mRNA degradation products. Primer extension analysis revealed independent promoters for *aspA*, *dcuA*, and *dcuB*, but surprisingly no primer extension product could be detected for *fumB*. The expression of *dcuB* is entirely consistent with a primary role for DcuB in mediating C_4-dicarboxylate transport during anaerobic fumarate respiration. The precise physiological purpose of DcuA remains unclear.

Escherichia coli can utilize C_4-dicarboxylates for energy generation under both aerobic and anaerobic conditions (8). Under aerobic conditions, the uptake of C_4-dicarboxylates (fumarate, malate, and succinate) and L-aspartate is mediated by a secondary transporter and/or a binding protein-dependent system, designated Dct (19, 22). Three mutations (*cbt* at 16.2 min, *dctA* at 79.2 min, and *dctB* at 16.1 min) have been reported to result in the inactivation of components involved in aerobic C_4-dicarboxylate transport (23). The *dctA* gene has been sequenced, and the role of its product (DctA) in the utilization of C_4-dicarboxylates (and the cyclic monocarboxylate orotate) is supported by complementation studies of *Salmonella typhimurium dctA* or *outA* mutants (2, 37). The *dctB* and *cbt* genes are predicted to encode inner membrane and periplasmic binding proteins, respectively (21), but the genes have yet to be identified in the *E. coli* genome sequence.

Uptake, exchange, and efflux of C_4-dicarboxylic acids under anaerobic conditions are mediated by the Dcu systems (K_m for fumarate uptake, 50 to 400 μM), which are genetically distinct from the aerobic Dct system (8, 9, 45). Transport studies suggest that the Dcu systems are expressed exclusively under anaerobic conditions, activated by the anaerobic activator protein FNR, and repressed in the presence of nitrate. Three independent Dcu systems have been identified, DcuA, DcuB, and DcuC (36, 45). DcuA and DcuB are homologous proteins (36% identical), whereas DcuC is only 22 to 24% identical to DcuA and DcuB. The DcuA protein is encoded by a gene (*dcuA*, at 94.0 min) located 117 bp downstream of the anaerobically expressed aspartase gene *aspA* (see Fig. 1). DcuB is encoded by *dcuB* (at 93.5 min), which is 77 bp upstream of the gene (*fumB*) encoding the anaerobic fumarase, FumB (see Fig. 1). DcuC is encoded by the *dcuC* gene at 14.1 min.

Growth tests and transport studies using *dcuA*, *dcuB*, and *dcuC* single, double, and triple mutants have shown that DcuA, DcuB, and DcuC each mediate exchange as well as uptake (36, 45). The triple mutant was almost completely devoid of Dcu activity and was unable to use fumarate for anaerobic respiratory growth, but growth could be supported by fermentation. The single mutants exhibited no phenotype, but the *dcuA dcuB* double mutant was markedly defective in both C_4-dicarboxylate transport and fumarate respiratory growth, suggesting that DcuA and DcuB have analogous and mutually complementary transport functions in the anaerobic uptake of C_4-dicarboxylates (36, 45). The affinities of DcuA and DcuB for C_4-dicarboxylates are similar, except for the lower affinity of DcuA for malate (45).

Expression of the *aspA* gene is repressed by glucose (~10-fold) under aerobic conditions and enhanced anaerobically (~10-fold) in a manner which is partially (~2-fold) FNR dependent (16, 44). Expression of *fumB* is also induced anaerobically (1.5- or 5-fold) in a manner which is dependent on both FNR and ArcA (41, 44). The anaerobic expression of *fumB* is reduced by ~10- or 2-fold in an *fnr* background, and unlike

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

Strain, plasmid, or phage	Relevant genotype	Source or reference
E. coli strains		
DH5α	Δ(*argF-lac*)*U169* (ϕ80Δ*lac*ZM15) *recA*	30
JRG1999	MC1000 Δ*crp*T8	S. T. Cole, Paris, France
JRG1728	MC1000 Δ(*tyrR-fnr-rac-trg*) *17 zdd-230*::Tn*9*	39
JRG3834	MC4100 [λRS45::*dcuA′-lacZYA*]	This work
JRG3835	MC4100 [λRS45::*dcuB′-lacZYA*]	This work
JRG3836	MC1000 [λRS45::*dcuA′-lacZYA*]	This work
JRG3837	MC1000 [λRS45::*dcuB′-lacZYA*]	This work
JRG3838	JRG3834*fnr*	This work
JRG3839	JRG3835*fnr*	This work
JRG3840	JRG3834*arc*	This work
JRG3841	JRG3835*arc*	This work
JRG3842	JRG3835*narL*	This work
JRG3843	JRG3835*narP*	This work
JRG3845	JRG1999 [λRS45::*dcuA′-lacZYA*]	This work
JRG3846	JRG1999 [λRS45::*dcuB′-lacZYA*]	This work
MC1000	Δ*lacX74* Δ(*araABC-leu*)	33
MC4100	Δ(*argF-lac*)*U169 rpsL*	33
RKP3580	*narL215*::Tn10	R. K. Poole, Sheffield, United Kingdom
RKP3655	*narP258*::Tn*10d*	R. K. Poole, Sheffield, United Kingdom
RM315	MC4100 Δ*arcA1 zjj*::Tn*10*	31
Plasmids		
pGS73	*aspA dcuA*	13
pGS78	*dcuB fumB*	12
pGS744	pBR322 + 4.7-kb *Bam*HI-*Eco*RI *dcuB-fumB* fragment	This work
pGS745	pBR322 + 6.2-kb *Sph*I-*Sal*I *aspA-dcuA* fragment	This work
pGS748	pUC118 + 1.3-kb *dcuA* fragment	This work
pGS749	pUC119 + 1.3-kb *dcuB* fragment	This work
pGS929	pRS415 + *dcuA′-lacZYA*	This work
pGS931	pRS528 + *dcuB′-lacZYA*	This work
pRS415/528	*lacZYA* transcriptional fusion vectors	35
Phages		
P1*vir1*		26
λRS45		35

aspA expression, *fumB* expression is not subject to glucose-mediated repression (41, 44). The anaerobic induction of *aspA* and *fumB* is appropriate since both aspartase and fumarase B are thought to function in the generation of fumarate for utilization as an anaerobic electron acceptor. Predicted FNR and cyclic AMP receptor protein (CRP) sites at suitable distances from putative promoter sequences are located immediately upstream of *aspA* and *dcuB* (36, 44). However, a previous analysis of the *aspA-dcuA* and *dcuB-fumB* intergenic regions showed no potential CRP sites and the putative FNR sites were poorly placed, suggesting that the *dcuB-fumB* and *aspA-dcuA* gene pairs are cotranscribed (36).

This paper describes studies on the transcriptional regulation and organization of the *dcuA* and *dcuB* genes that provide further information concerning the roles of DcuA and DcuB. The results show that the *dcuB* gene is expressed exclusively under anaerobic conditions in a manner that is FNR dependent and that it is repressed by NarL in the presence of nitrate and is subject to CRP-mediated catabolite repression. Furthermore, *dcuB* is strongly induced by C_4-dicarboxylates presumably mediated by an uncharacterized C_4-dicarboxylate-responsive gene regulator. In contrast, the *dcuA* gene shows little variation in expression under the growth conditions investigated. These findings suggest that DcuB functions primarily as a C_4-dicarboxylate transporter during fumarate respiration, whereas DcuA has a general role in anaerobic C_4-dicarboxylate transport.

MATERIALS AND METHODS

Strains and plasmids. All *E. coli* strains, plasmids, and phages used in this study are listed in Table 1. A plasmid containing the *aspA-dcuA* region was obtained by subcloning the 6.2-kb *Sph*I-*Sal*I fragment of pGS73 (13) into the corresponding sites of pBR322 to generate pGS745 (Fig. 1A). A *dcuA′-lacZYA* transcriptional fusion was produced by subjecting pGS745 to digestion with *Nar*I, treatment with DNA polymerase I (Klenow fragment) to generate blunt-ended fragments, and digestion with *Eco*RI and then subcloning the resulting 1-kb *Eco*RI-*Nar*I *dcuA′*-containing fragment into *Eco*RI- and *Sma*I-digested pRS415 (35) to generate pGS929 (Fig. 1A).

A plasmid containing the *dcuB-fumB* region was made by subcloning the 4.7-kb *Bam*HI-*Eco*RI fragment of pGS78 (12) into *Bam*HI- and *Eco*RI-digested pBR322 to generate pGS744 (Fig. 1B). A *dcuB′-lacZYA* transcriptional fusion was generated by subjecting pGS744 to digestion with *Hin*dIII, treatment with DNA polymerase I (Klenow), and then digestion with *Bam*HI and then subcloning the resulting 1-kb *Bam*HI-*Hin*dIII *dcuB′* fragment into *Bam*HI- and *Sma*I-digested pRS528 (35) to generate pGS931 (Fig. 1B). The *dcuA* and *dcuB-lacZ* fusions were transferred to the phage λRS45 (35) by homologous recombination in vivo as described by Simons et al. (35), and the resulting Lac^+-conferring phages, λRS45(*dcuA′-lacZYA*) and λRS45(*dcuB′-lacZYA*), were used to produce monolysogenic derivatives of appropriate strains of *E. coli* (Table 1).

A 1.3-kb *dcuA* fragment, suitable for use as a hybridization probe in Northern blot analysis, was PCR amplified by using the plasmid pGS73 (13) as a template, *Pfu* DNA polymerase (Stratagene), and the following two primers: DCUA1, 5′-CC**GAATTC***AT*^{2129}*ATG*CTAGTTGTAGAACTC^{2146}-3′; and DCUA4, 5′-CC**GGAT CC**^{3436}TGATCATTACAGCATGAAG^{3418}-3′ (where underlining indicates the *dcuA* start codon, small capitals indicate mismatches, boldface type indicates *Eco*RI and *Bam*HI sites, italics indicate *Nde*I site, and coordinates are from the work of Six et al. [36]; primers were designed to introduce flanking *Eco*RI-*Nde*I and *Bam*HI sites). The *Eco*RI- and *Bam*HI-digested PCR product was subcloned into plasmid pUC118 (42) to generate the plasmid pGS748 (Fig. 1A). A similar strategy was adopted for subcloning the *dcuB* gene in pUC119 (42), except that a *dcuB*-containing template, pGS78 (12), and different PCR primers were used;

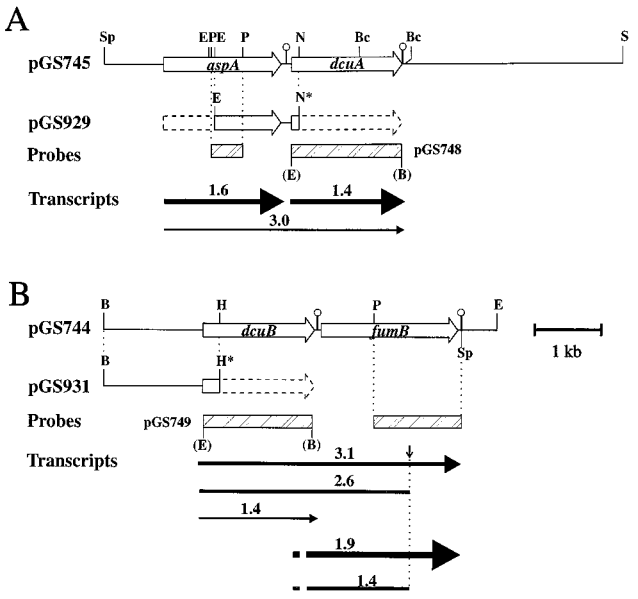

FIG. 1. Restriction maps and transcriptional organization of the *aspA-dcuA* (A) and *dcuB-fumB* (B) regions. The inserts of plasmids pGS745, pGS748, and pGS929 (A) and of plasmids pGS744, pGS749, and pGS931 (B) are shown. Restriction site abbreviations: B, *Bam*HI; Bc, *Bcl*I; E, *Eco*RI; H, *Hin*dIII; N, *Nar*I; P, *Pst*I; S, *Sal*I; Sp, *Sph*I. Restriction sites in parentheses were introduced during PCR amplification. Asterisks indicate restriction sites that have been inactivated by digestion with the corresponding restriction enzyme followed by filling in with the Klenow fragment of DNA polymerase I. Open arrows indicate the positions and polarities of the *aspA*, *dcuA*, *dcuB*, and *fumB* structural genes. Closed arrows indicate primary transcripts and putative processed transcripts (sizes in kilonucleotides) as detected by Northern hybridization experiments. The relative abundance of each transcript is indicated by arrow thickness. The small downward arrow indicates a potential endoribonucleolytic processing site. Hatched bars represent DNA fragments used as hybridization probes, and strongly predicted stem-loop structures are indicated.

the primers were DCUB1, 5′-CCGAATTC$_{AT}^{170}$*ATG*TTATTTACTATCCAAC^{188}-3′, and DCUB2, 5′-CCGGATCC^{1516}GTGCATTTATAAGAACCCG^{1497}-3′. The 1.3-kb *dcuB*-containing fragment was subcloned in pUC119, generating plasmid pGS749 (Fig. 1B).

To investigate the effects of the global regulators Fnr, Arc, NarL, and NarP on *dcuA* and *dcuB* expression, the *fnr*, *arc*, *narL*, and *narP* deletions were transferred from the corresponding donor strains, JRG1728, RM315, RKP3580, and RKP3655, by P1*vir*-mediated transduction to the *dcuA*- and *dcuB-lacZ* fusion strains, JRG3834 and JRG3835 (Table 1). To study the effects of CRP on *dcuA* and *dcuB* expression, a pGS279 (*crp*$^+$) transformant of strain JRG1999 (Δ*crp*) was infected with λRS45(*dcuA′-lacZYA*) and λRS45(*dcuB′-lacZYA*) to generate the monolysogens JRG3845(pGS279) and JRG3846(pGS279), respectively, which were subsequently cured of the plasmid by propagation of the strains under nonselective conditions.

Growth media and conditions. Cultures were grown at 37°C, either aerobically in shaking 250-ml conical flasks or anaerobically in 10-ml bijou bottles (for β-galactosidase measurements) or in 300-ml medical flats (for the preparation of total RNA). Bacteria were usually grown aerobically in L broth for DNA manipulation and in L broth supplemented with glycerol and fumarate for the extraction of total RNA. For measurements of β-galactosidase activity, strains were grown in M9 minimal salts medium (Sigma), unless otherwise stated, with glucose (0.4%) or glycerol (0.4%) as a carbon source, and supplements of 0.5% Casamino Acids, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.5 mg of vitamin B_1 per ml. When used, fumarate, nitrate, and trimethylamine *N*-oxide (TMAO) were present at 50 mM.

β-Galactosidase measurements. Samples with an optical density at 650 nm (OD_{650}) of 0.5 were withdrawn from cultures grown in duplicate at regular intervals during the growth cycle. The samples were cooled on ice, and the bacteria were pelleted by centrifugation, resuspended in ice-cold saline, and resedimented at 4°C. After complete removal of the supernatant by aspiration, the bacteria were frozen at −20°C and used within 3 days.

Cell extracts were prepared from thawed cells and assayed for β-galactosidase activity and protein content by using an iEMS Reader MF microtiter plate spectrophotometer (Labsystems) by the method of Phillips-Jones et al. (27). β-Galactosidase specific activities (micromoles of ONPG [*o*-nitrophenyl-β-D-galactopyranoside] per minute per milligram of protein) were averaged from samples taken from two independent cultures. Each of the two samples was assayed in duplicate.

Northern hybridization and primer extension analysis. Total RNA present in MC4100 was extracted by using a method based upon that described by Aiba et al. (1). Cultures (500 ml) were harvested by centrifugation and resuspended in 3 to 5 ml of a solution containing 20 mM sodium acetate (pH 5.5), 0.5% SDS, and 1 mM EDTA at 4°C. An equal volume of acid-phenol (phenol equilibrated with 20 mM sodium acetate [pH 5.5]), was added, and the mixture was incubated for 5 min at 65°C. After centrifugation, the aqueous layer was removed, extracted twice more with acid-phenol, and then extracted once with chloroform and once with a mixture of phenol-$CHCl_3$-isoamyl alcohol (25:24:1; equilibrated with 0.1 M Tris-Cl [pH 8.0]). RNA in the aqueous layer was precipitated with 3 volumes of ethanol and dissolved in ~1 ml of water. The RNA content was determined spectroscopically by assuming that an OD_{260} of 1 corresponds to ~40 μg of RNA per ml.

Northern hybridization was performed by fractionating total RNA, along with RNA molecular weight standards (Gibco-BRL), in a 1% agarose gel containing 2.2 M formaldehyde. Denatured RNA was transferred to nitrocellulose membranes and hybridized with DNA probes radiolabeled with [α-^{32}P]dCTP by using the Ready to Go DNA Labeling Kit (−dCTP) (Pharmacia). Hybridization was performed at 65°C as described by Sambrook et al. (30). The hybridization probes used were the 1.3-kb *Eco*RI-*Bam*HI *dcuA* fragment of pGS748, the 1.3-kb *Eco*RI-*Bam*HI *dcuB* fragment of pGS749, the 0.375-kb *Pst*I *aspA* fragment of pGS745 (10), and the 1-kb *Pst*I-*Sph*I *fumB* fragment of pGS744 (Fig. 1). DNA-RNA hybrids were detected by autoradiography using BioMax MS (Kodak) autoradiography film.

Reverse transcriptase-mediated primer extension analysis was performed as described by Quail et al. (28) by using two oligonucleotide primers for each promoter. The primers used and the corresponding base pair coordinates (2, 36) were as follows: for *dcuA*, P1$_{dcuA}$, 5′-^{2235}GCAAGAACCAGCACCCCCAATC CGCCTGCA^{2206}-3′, and P2$_{dcuA}$, 5′-^{2211}CCTGCAAAACCAATACCTATTCCC CCCAAT^{2182}-3′; for *dcuB*, P1$_{dcuB}$, 5′-^{294}AGGTGGAAGACGAAGACCAGA ATGACCAGA^{265}-3′, and P2$_{dcuB}$, ^{270}ACCAGACCGATACCGCCTAATAAA CCCAGC^{241}-3′; for *aspA*, P1$_{aspA}$, ^{693}TTGTTGTTGCTGATATAGAAGTTTA CAATC^{664}-3′, and P2$_{aspA}$, 5′-^{669}ACAATCGCTCTCAGAGTGTGAACACCA TAG^{640}-3′; and for *fumB*, P1$_{fumB}$, 5′-^{1336}GGTTTCGCCGTCGAAGTCGGCA ACGCTAAC^{1307}-3′, and P2$_{fumB}$, 5′-^{1309}AACGTAATCGGAAGTGAGTAGA TAGTA^{1283}-3′. Sequence ladders were generated by using the T7 Sequenase DNA sequencing kit (Amersham), together with plasmids pGS744 and pGS745 as templates, and the primers described above. Primer extension products and sequencing ladders were visualized by autoradiography. Autoradiographic images were digitized by use of a model GS-690 imaging densitometer (Bio-Rad) linked to a computer equipped with Molecular Analyst (Bio-Rad) software. Potential FNR, CRP, and NarL binding sites were identified by using the score matrix searching option of the xnip program (40) and score matrices derived from 22 FNR, 25 CRP, and 8 NarL experimentally determined binding sites.

RESULTS

Effects of growth conditions on the expression of single-copy *dcuA*- and *dcuB-lacZ* transcriptional fusions. The expression of the *dcuA* and *dcuB* genes was investigated by using derivatives of MC4100 (Δ*lacZ*) containing single copies of the corresponding *lacZ* transcriptional gene fusion (see Materials and Methods). The fusions were transferred from plasmids to λRS45 via homologous recombination for use in constructing the monolysogens JRG3834 (MC4100 [λRS45::*dcuA′-lacZYA*]) and JRG3835 (MC4100 [λRS45::*dcuB′-lacZYA*]). In order to include the entire *dcuA* and *dcuB* operator-promoter regions, 1.1- and 1.4-kb segments of DNA located immediately upstream of the respective structural genes were fused to the *lacZ* reporter gene. Both fusions were active (see below), indicating that *dcuA* and *dcuB* each possess independent promoters and that *dcuA* transcription is not dependent on the upstream *aspA* gene.

The β-galactosidase specific activities of JRG3834 (*dcuA-lacZ*) and JRG3835 (*dcuB-lacZ*) were determined after aerobic and anaerobic growth in minimal medium containing glycerol plus fumarate and Casamino Acids (Fig. 2). Expression of the *dcuA-lacZ* fusion under aerobic conditions (~0.5 μmol/min/mg) increased by only two- to threefold anaerobically (0.9 to 1.5 μmol/min/mg). In contrast, aerobic expression of the *dcuB-lacZ* fusion was very low (~0.02 μmol/min/mg) but was up to 150-fold higher (3 μmol/min/mg) anaerobically (Fig. 2).

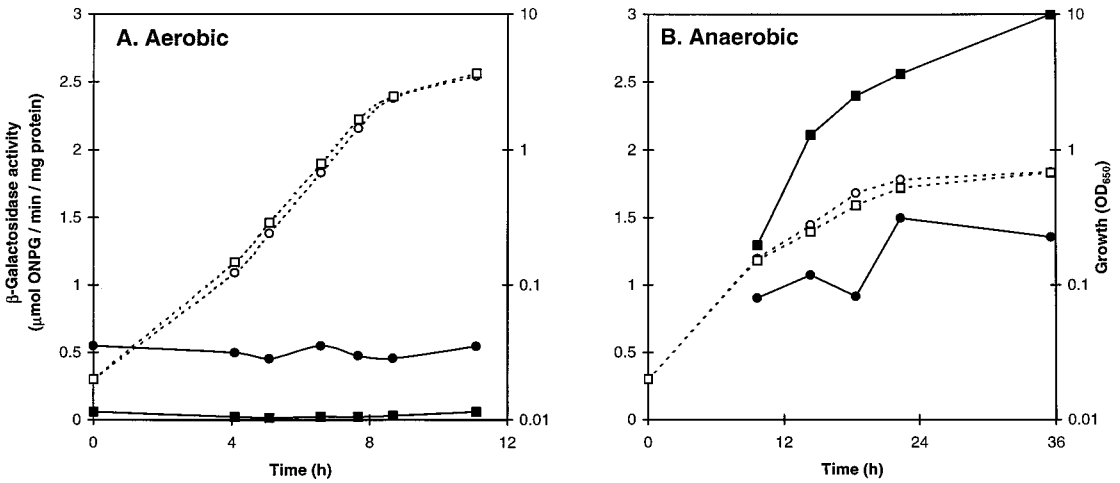

FIG. 2. Expression of *dcuA-lacZ* and *dcuB-lacZ* transcriptional fusions during aerobic (A) and anaerobic (B) growth at 37°C in M9 minimal salts medium containing 0.4% glycerol, 50 mM fumarate, and 0.05% Casamino Acids. The strains used were JRG3834 (*dcuA-lacZ*) (● and ○) and JRG3835 (*dcuB-lacZ*) (■, □). Growth (open symbols) and β-galactosidase activities (closed symbols) are shown.

Thus, *dcuB* expression is strongly induced during anaerobiosis whereas *dcuA* expression is only slightly increased by lack of oxygen. The expression of *dcuA* and *dcuB* increased only slightly (up to ca. twofold) in the transition from exponential to postexponential growth, indicating that expression is weakly affected by the growth phase (Fig. 2). For this reason, all subsequent data (Fig. 3 to 7) relate to samples taken during the mid-logarithmic to late logarithmic phase.

Although *dcuB* was strongly expressed during anaerobic respiratory growth with fumarate as the terminal electron acceptor (~1.3 μmol/min/mg), expression was 8- and 144-fold lower when TMAO and nitrate (0.16 and 0.009 μmol/min/mg), respectively, were used as sole electron acceptors (Fig. 2B, 3Aii, and 3Bii). However, these values are still 30- and 2-fold higher than the corresponding aerobic activities (0.05 and 0.005 μmol/min/mg) (Fig. 3Aii and Bii). Addition of fumarate to minimal medium containing glycerol plus TMAO and Casamino Acids resulted in a ninefold increase in *dcuB* expression under anaerobic conditions (Fig. 3Aii), but addition of fumarate to minimal medium containing glycerol plus nitrate and Casamino Acids had no effect on *dcuB* expression (Fig. 3Bii). These findings indicate that the anaerobic expression of *dcuB* is strongly repressed by nitrate, and in the absence of nitrate and oxygen, it is strongly induced by fumarate. In contrast, expression of *dcuA* is only slightly (less than twofold) affected by nitrate and fumarate (Fig. 3Ai and Bi).

Expression of the *dcuB-lacZ* fusion was moderate (0.09 μmol/min/mg) during anaerobic fermentative growth in glucose minimal medium plus Casamino Acids and was increased only twofold (to 0.18 μmol/min/mg) by the inclusion of fumarate (Fig. 3Cii). The latter value is sevenfold lower than that (1.3 μmol/min/mg) observed when glucose was replaced by glycerol (Fig. 2B), indicating that *dcuB* transcription is subject to catabolite repression. During aerobic growth, *dcuB* was very weakly expressed (0.005 to 0.014 μmol/min/mg) under all conditions employed (Fig. 3). Expression of the *dcuA-lacZ* fusion (0.35 to 0.75 μmol/min/mg) was only slightly affected by the growth conditions, indicating that *dcuA* is expressed constitutively.

The studies described above show that *dcuB* is strongly repressed by oxygen and nitrate and is moderately repressed by glucose. In the absence of these repressing substrates, *dcuB* is strongly induced by fumarate. To determine whether *dcuB* expression is induced by C_4-dicarboxylic acids other than fumarate, expression was measured in minimal medium containing glycerol plus TMAO and either aspartate, fumarate, malate, maleate, or succinate (Fig. 4A). Casamino Acids was omitted from the media used in these experiments to eliminate any stimulation caused by its aspartate residue content. The expression of *dcuB* was lowered ninefold (from 0.16 to 0.018 μmol/min/mg) by omitting Casamino Acids (Fig. 4A), indicating that the addition of Casamino Acids does indeed induce *dcuB* expression. In the absence of Casamino Acids, *dcuB* expression was induced ~40- to 70-fold by the five C_4-dicarboxylic acids tested (Fig. 4A). However, the carboxylic acids (pyruvate, acetate, and lactate) had no effect (Fig. 4A), indicating that *dcuB* induction is specific for C_4-dicarboxylates as is Dcu transport (8). The degree of *dcuB* induction by C_4-dicarboxylates decreased in the order fumarate (1.2 μmol/min/mg) ≈ malate (1.1) ≈ aspartate (1.0) > succinate (0.8) > maleate (0.6). This order differs from the order of substrate preference for DcuB measured as a function of competitive inhibition of succinate antiport activity in a *dcuA* mutant (36), suggesting that there is no direct link between the substrate binding specificity of DcuB and the regulation of *dcuB* by C_4-dicarboxylates.

The activation of *dcuB* expression by fumarate was directly related to the initial concentration of fumarate in the medium over the range of 1 to 50 mM (Fig. 4B). Fumarate concentrations of ≥250 mM inhibited growth, whereas those of ≤0.1 mM were too low to affect *dcuB* expression (Fig. 4B). The apparent K_m for fumarate of the C_4-dicarboxylate-responsive *dcuB*-regulatory system can be estimated to be 4 to 9 mM. The latter value is 10- to 100-fold greater than the K_m of the Dcu transporters (50 to 400 μM) but is appropriate to ensure induction of DcuB when substrate concentrations are at levels that could be saturating for the DcuA and DcuC transporters. Thus, anaerobic induction of *dcuB* by C_4-dicarboxylates would be expected to result in increased Dcu transport activity, which is indeed what has been observed (8).

Effect of global regulators on *dcuA*- and *dcuB-lacZ* expression. The glucose, oxygen, and nitrate repression of *dcuB* expression suggests that *dcuB* could be subject to regulation by the global transcriptional regulatory proteins FNR, CRP,

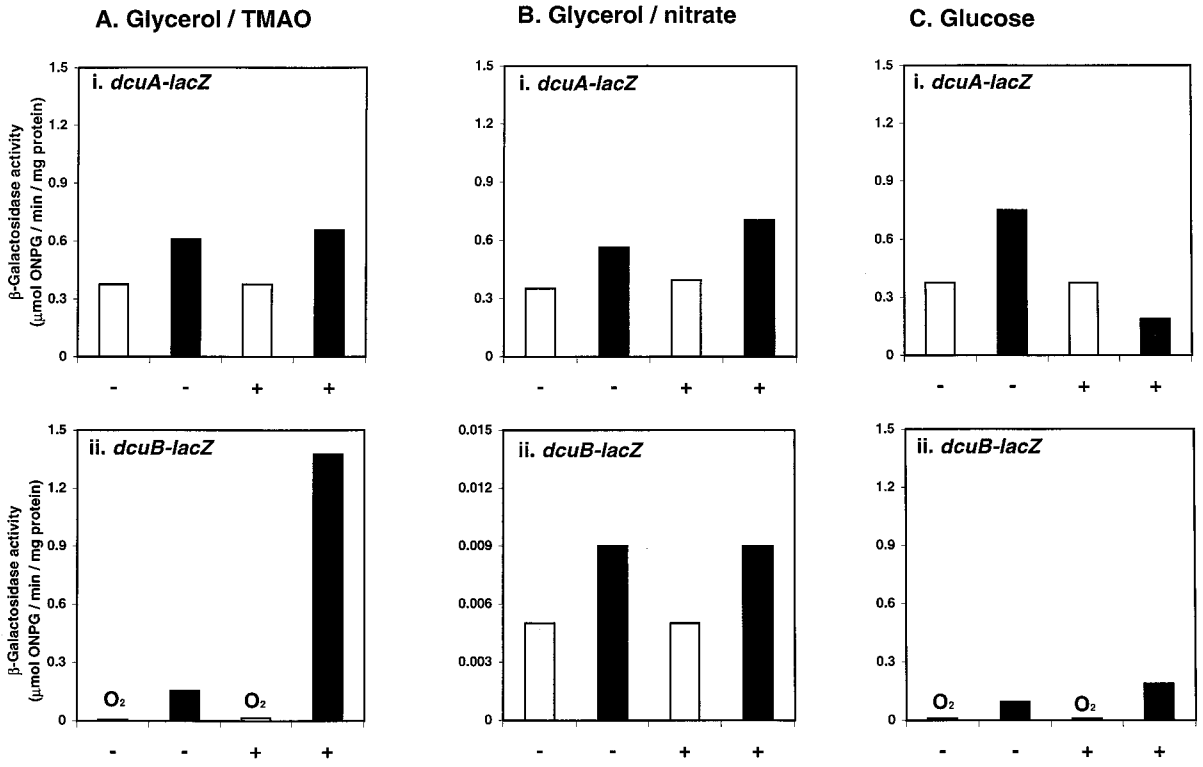

FIG. 3. Expression of the *dcuA*- and *dcuB-lacZ* fusions during aerobic (open bars) and anaerobic (closed bars) growth at 37°C in M9 minimal salts medium containing 0.05% Casamino Acids plus either 0.4% glycerol and 50 mM TMAO (A), 0.4% glycerol with 50 mM nitrate (B), or 0.4% glucose (C). The plus and minus signs indicate the presence and absence of 50 mM fumarate, respectively. β-Galactosidase activities were assayed in samples of mid-logarithmic to late logarithmic cultures of JRG3834 (*dcuA-lacZ*) and JRG3835 (*dcuB-lacZ*).

ArcA, NarP, and NarL. This was tested by measuring *dcuB-lacZ* (and *dcuA-lacZ*) expression in the appropriate regulatory mutants (Fig. 5). The anaerobic expression of *dcuB* in minimal medium containing glycerol plus TMAO and fumarate was 15-fold lower in a Δ*fnr* strain, JRG3839, than in the parental strain, JRG3835 (Fig. 5Aii). However, even in the absence of FNR, the anaerobic expression level was still 19-fold higher (0.094 μmol/min/mg) than the aerobic level (0.005 μmol/min/mg). These observations suggest that the anaerobic activation of *dcuB* transcription is mediated by FNR-dependent and FNR-independent mechanisms. Aerobic *dcuB* expression was only twofold lower in the Δ*fnr* strain (0.005 μmol/min/mg) than in the parental strain (0.009 μmol/min/mg), indicating that the FNR activation of *dcuB* expression is mainly an anaerobic process (Fig. 5A). Both the aerobic and anaerobic expression levels of *dcuB* were only ca. twofold lower in the *arcA* mutant (JRG3841), indicating that ArcA plays no more than a minor role in regulating *dcuB* expression in response to oxygen (Fig. 5B) and that ArcA is not responsible for the FNR-independent mechanism of anaerobic activation of *dcuB* transcription. The activation of *dcuB* expression was lowered ca. twofold by TMAO (from 2.5 to 1.4 μmol/min/mg Fig. 5Aii and Bii), although *dcuA* expression was unaffected by TMAO (Fig. 5Ai and Bi), suggesting that TMAO represses *dcuB* expression.

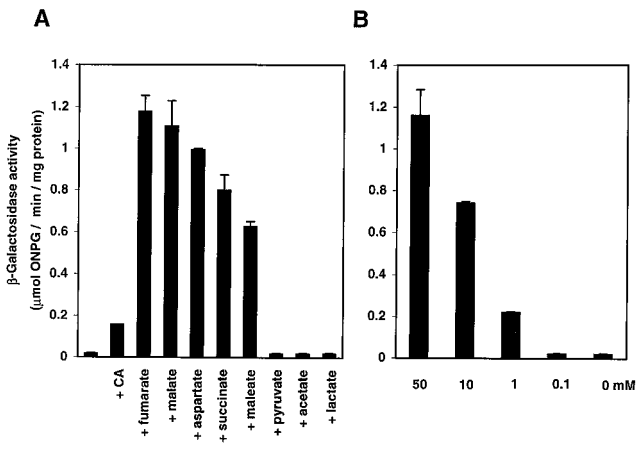

FIG. 4. Effects of carboxylates on expression of the *dcuB-lacZ* fusion during anaerobic growth in M9 salts medium containing 0.4% glycerol and 50 mM TMAO but lacking Casamino Acids (except where indicated). The alternative carboxylates (50 mM) or 0.5% Casamino Acids (CA) (A) and the fumarate concentrations (B) are indicated on the *x* axis. Other details are as described for Fig. 3.

During anaerobic growth in glycerol and fumarate, the activity of the *dcuB-lacZ* fusion (0.75 μmol/min/mg) in the Δ*crp* strain, JRG3846, was threefold lower than in the parental strain, JRG3837 (2.3 μmol/min/mg) (Fig. 5Ci). This indicates that the cyclic AMP (cAMP)-CRP complex weakly activates *dcuB* expression in the absence of glucose. In the absence of TMAO and in the presence of fumarate, the anaerobic expression of *dcuB* was repressed 14-fold by glucose (Fig. 5Ci and Cii), and this repression was relieved only slightly (to ~9-fold repression) by the *crp* mutation (Fig. 5Cii). These findings indicate that some other factor accounts for most of the 14-fold repression of *dcuB* by glucose.

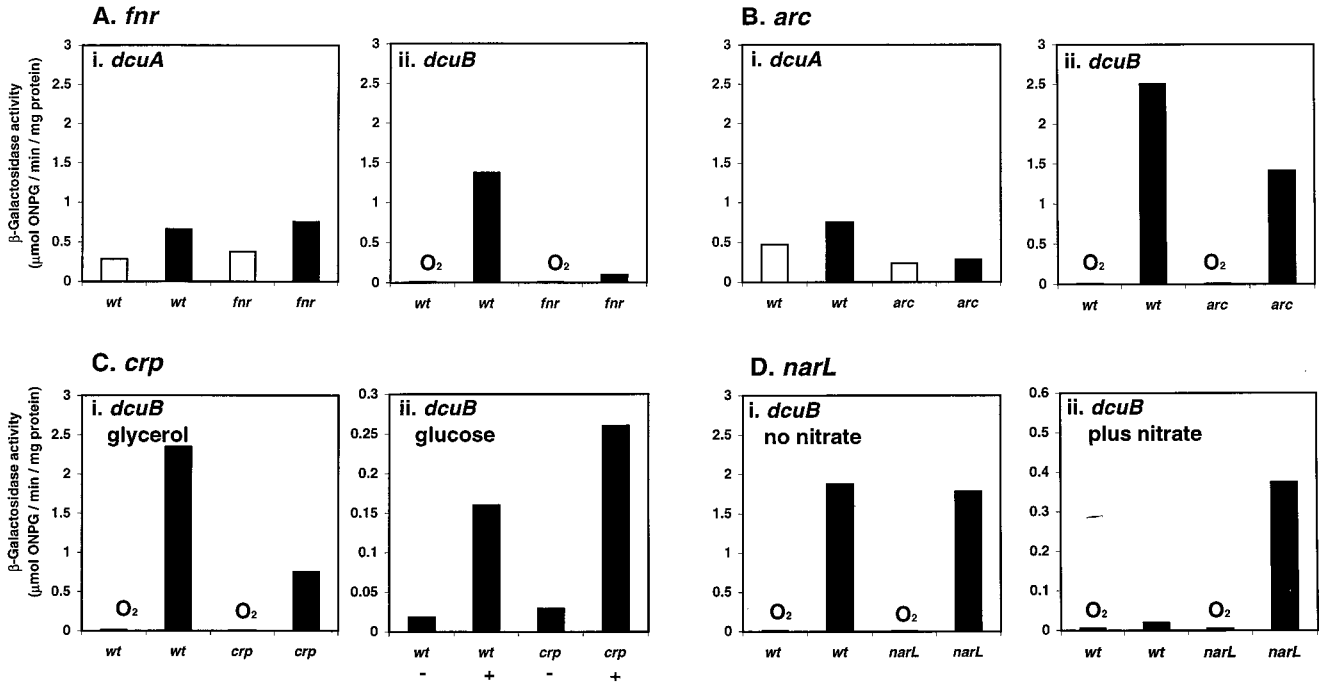

FIG. 5. Effect of *fnr* (A), *arcA* (B), *crp* (C), and *narL* (D) on *dcuA* and/or *dcuB* expression. Growth was performed under both aerobic (open bars) and anaerobic (closed bars) conditions in M9 minimal medium containing either 0.4% glycerol, 50 mM TMAO, 50 mM fumarate, and 0.05% Casamino Acids (A and D); 0.4% glycerol and 50 mM fumarate (B and Ci); and 0.4% glucose with (+) or without (−) 50 mM fumarate (Cii). The strains used were JRG3834 (Ai and Bi), JRG3835 (Aii, Bii, and D), JRG3837 and JRG3846 (C), JRG3838 (Ai), JRG3839 (Aii), JRG3840 (Bi), JRG3841 (Bii), and JRG3842 (D). Other details are as described for Fig. 3. wt, wild type.

The effect of NarL (and NarP) on the expression of the *dcuB-lacZ* fusion was measured in the presence and absence of nitrate (Fig. 5D). Although the *narL* mutation had no effect on *dcuB* expression in the absence of nitrate (Fig. 5Di), the strong nitrate repression of *dcuB* expression in the *narL*$^+$ parental strain, JRG3835, was reduced 20-fold in the Δ*narL* strain, JRG3842. This demonstrates that NarL has the major role in nitrate-induced repression of *dcuB* expression (Fig. 5Dii). However, even in the absence of NarL, nitrate still caused a fourfold repression in *dcuB* expression. The reason for this is unclear, but since nitrate repression of *dcuB* expression was unaffected by a *narP* null mutation (data not shown), it appears that NarP is not involved in mediating the nitrate-induced repression of *dcuB*.

The twofold anaerobic induction of the *dcuA-lacZ* fusion was unaffected by the *fnr* deletion (data not shown), revealing that FNR has no role in *dcuA* expression. The Δ*arcA* mutation caused a ca. twofold reduction in *dcuA* expression, both aerobically and anaerobically (data not shown), suggesting a role for ArcA in the constitutive expression of *dcuA*. It is uncertain whether the minor effects of ArcA on *dcuA* and *dcuB* expression reflect a metabolic consequence of deregulation of the *arcA* regulon or a direct interaction of the *dcuA* and *dcuB* genes with the ArcA protein. The *fnr*, *crp*, and *narL* mutations had no effect on *dcuA* expression (data not shown).

Northern blot analysis of the *dcuA*, *dcuB*, *aspA*, and *fumB* transcripts. To determine whether the *lacZ* fusion analyses reflect transcript abundance and to examine the transcriptional organization of the *aspA-dcuA* and *dcuB-fumB* genes, Northern hybridization experiments were performed on the *dcuA*, *dcuB*, *aspA*, and *fumB* gene transcripts. Total RNA was extracted from MC4100 grown aerobically and anaerobically to mid-logarithmic phase in L broth containing glycerol plus fumarate. RNA was hybridized with labeled *dcuA*, *dcuB*, *aspA*, and *fumB* gene fragments (Fig. 1 and 6).

A major *dcuA*-hybridizing band, corresponding to a transcript of 1,400 nucleotides (nt), was detected under both aerobic and anaerobic conditions (Fig. 6A). Its size corresponds to that predicted for a *dcuA* monocistronic transcript initiating at the promoter defined in the *aspA-dcuA* intergenic region (bp 2060; see below) and terminating at an inverted repeat (bp 3440 to 3459) positioned just 10 bp downstream from the *dcuA* stop codon (bp 3430) (36). The transcript appears to be equally abundant in cells grown in aerobic and anaerobic conditions. The minor transcript of 3,000 nt corresponds in size to an *aspA-dcuA* cotranscript, whereas the minor 2,500-nt transcript could either be a degradation product of the *aspA-dcuA* cotranscript or a nonspecifically hybridizing species (such as 23s rRNA). The *aspA* hybridization revealed a major transcript of 1,600 nt, equally abundant in the aerobic and anaerobic samples (Fig. 6B). The size of this transcript matches that predicted for the *aspA* monocistronic transcript which initiates from the *aspA* promoter (bp 470; see below) and is presumed to terminate at an inverted repeat immediately downstream of *aspA* (bp 2063) (43). The minor 2,500- and 3,000-nt bands, which were detected by using the *dcuA* hybridization probe, were also detected with the *aspA* probe (Fig. 6A). This indicates that these transcripts correspond to cross-hybridizing *aspA-dcuA* cotranscripts, as suggested above. The *dcuA* hybridization results are consistent with the *dcuA-lacZ* fusion data and so confirm that *dcuA* is transcribed in both the presence and absence of oxygen. The *aspA* gene is, likewise, transcribed both aerobically and anaerobically. This supports a previous study showing that *aspA* is strongly expressed anaerobically and, in the absence of glucose, is also strongly expressed aerobically (44), but contrasts with the increased aspartase activity

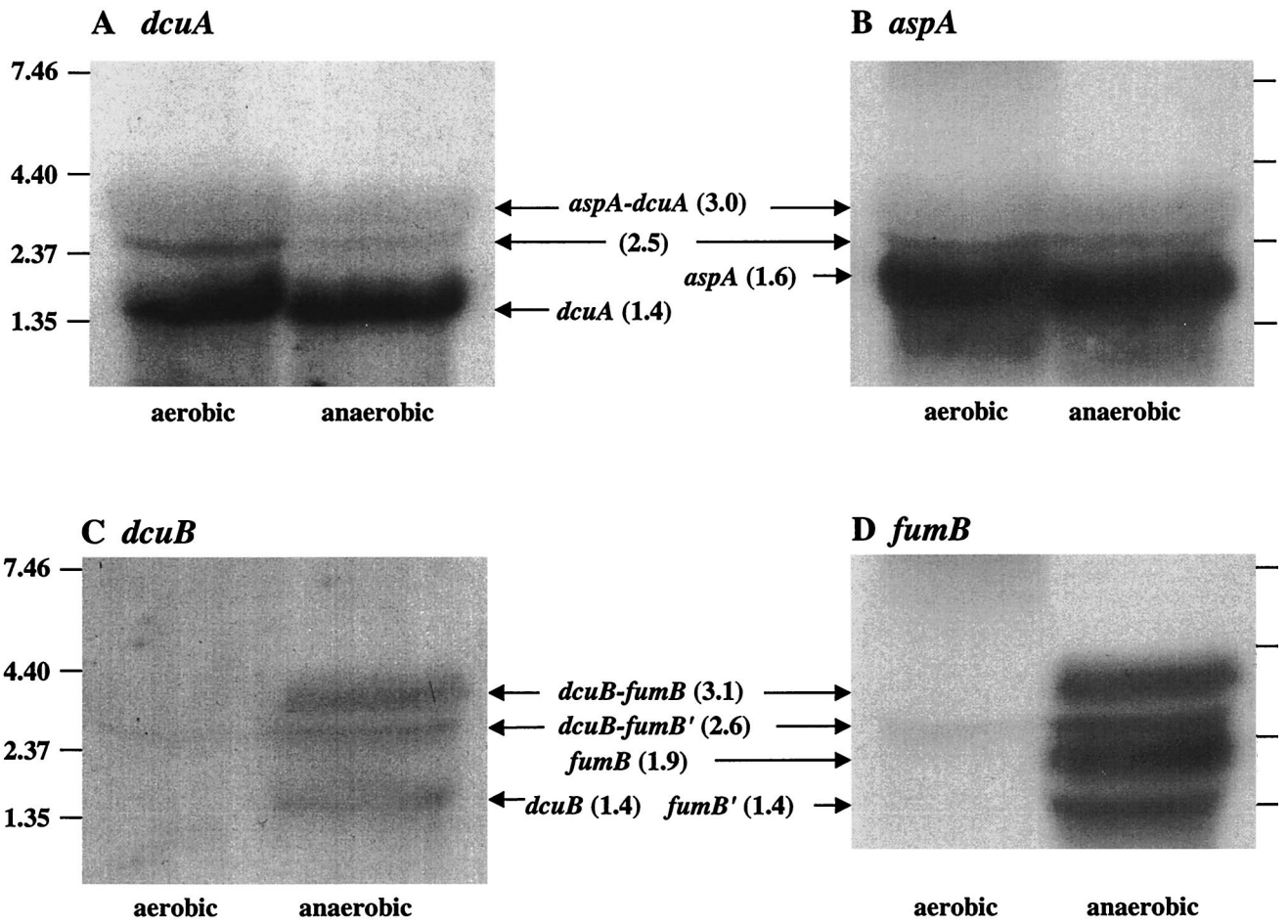

FIG. 6. Northern hybridization analysis of the *dcuA* (A), *aspA* (B), *dcuB* (C), and *fumB* (D) transcripts. Total RNA was extracted from MC4100 grown aerobically and anaerobically in L broth containing 0.4% glycerol and 50 mM fumarate and, after electrophoresis and capillary transfer, was hybridized with labeled *dcuA*, *aspA*, *dcuB*, and *fumB* gene fragments (see Materials and Methods). The positions of RNA molecular weight standards (in kilonucleotides) are indicated. The arrows mark hybridizing transcripts (sizes in kilonucleotides) and a minor band (2.5 kilonucleotides) possibly corresponding to nonspecifically hybridizing 23s rRNA.

under anaerobiosis reported by Jerlström et al. (16). This difference may relate to posttranslational effects on aspartase activity or to differences in the growth conditions used. Despite the obvious potential for cotranscription, the *dcuA* and *aspA* genes seem to be predominantly transcribed independently, at least under the conditions tested, as indicated by the hybridization data and the expression of the *dcuA-lacZ* fusion.

Three *dcuB*-hybridizing transcripts of 1,400, 2,600, and 3,100 nt were observed at a ratio of approximately 1:1:2 in RNA isolated from anaerobic cells (Fig. 6C). No major transcripts were detected in the aerobic samples. The predominant 3,100-nt transcript is likely to correspond to a *dcuB-fumB* cotranscript. This would be expected to initiate at bp 151 (see below) and terminate at an inverted repeat immediately downstream of *fumB* (bp 3251 to 3270) (3, 36), giving an overall size of ~3,100 nt. The 1,400-nt transcript is the expected size for a *dcuB* monocistronic transcript that initiates at bp 151 (see below) and presumably terminates at the stem-loop structure (bp 1520 to 1547) between the *dcuB* and *fumB* genes (36). The 2,600-nt transcript could be a *dcuB-fumB* degradation product lacking ~500 nt at the 3′ end (see below). Four anaerobic transcripts were detected with the *fumB* hybridization probe, but as for *dcuB*, none were present in the aerobic sample (Fig. 6D). The four transcripts were 1,400, 1,900, 2,600, and 3,100 nt in length and were present at a ratio of approximately 2:4:1:2 (Fig. 6D). The 3,100-nt transcript is likely to be identical to the *dcuB-fumB* cotranscript detected with the *dcuB* probe (Fig. 6C). The predominant 1,900-nt RNA probably corresponds to a *fumB* transcript, which should be at least 1,700 nt if it initiates at a *fumB* promoter located in the *dcuB-fumB* intergenic region (bp 1511 to 1587) and terminates at the inverted repeat (bp 3251 to 3270) downstream of *fumB* (36). The 1,400- and 2,600-nt RNA species could be derived from the 1,900-nt *fumB* and 3,100-nt *dcuB-fumB* transcripts, respectively, through endonucleolytic cleavage at a site ~500 nt from the 3′ ends of the corresponding transcripts (Fig. 1).

The *dcuB* Northern blotting results are consistent with the *dcuB-lacZ* expression data reported above and thus confirm that *dcuB* transcription is repressed by oxygen. Also, the absence of a *fumB* transcript in the aerobic sample is consistent with previous studies showing anaerobic induction of *fumB-lacZ* fusions of up to 5-fold and 3- to 10-fold reductions in anaerobic expression in the absence of FNR and/or ArcA (41, 44). The relative abundance of the *dcuB* and *fumB* transcripts is approximately 1:2, indicating that *fumB* is twofold more highly expressed than *dcuB*.

Determination of the transcriptional start sites for the *dcuA*, *dcuB*, and *aspA* genes. The transcriptional start sites of the

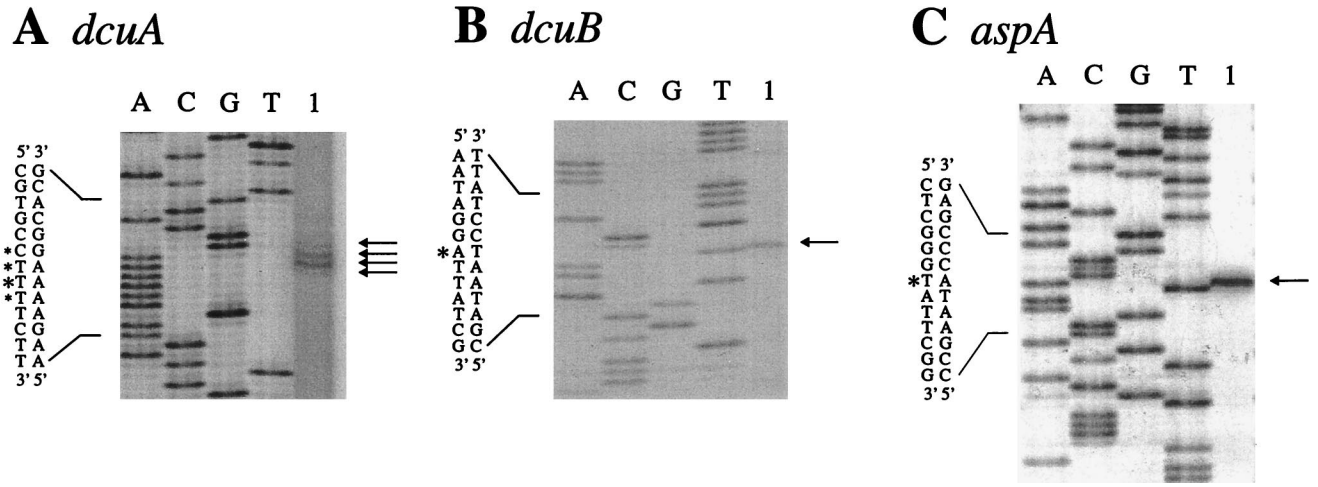

FIG. 7. Determination of the *dcuA* (A), *dcuB* (B), and *aspA* (C) transcriptional start sites by reverse transcriptase-mediated primer extension. Lanes 1 indicate primer extension products. Sequencing ladders (lanes A, C, G, and T) were generated by using the primers used for the reverse transcriptase reaction. The sequencing ladders and primer extension products shown in panels A, B, and C were generated by using, respectively, primers $P1_{dcuA}$, $P2_{dcuB}$, and $P2_{aspA}$ (see Materials and Methods). Similar results were obtained with primers $P2_{dcuA}$, $P1_{dcuB}$, and $P1_{aspA}$ (data not shown). The sequence, its complement, and the transcriptional start sites (indicated by asterisks) are shown. Horizontal arrows indicate primer extension products.

dcuA, *dcuB*, and *aspA* genes were determined by primer extension analysis (Fig. 7). Four primer extension products were obtained for the *dcuA* transcript; they correspond to transcriptional start sites at C-2058, T-2059, T-2060, and T-2061 (Fig. 7A and 8A). These sites are approximately 30 bp upstream of the +1 site predicted by Six et al. (36). The relative abundances of the four cDNA species were 1:3:6:1, respectively, indicating that of the four alternative transcriptional start sites, T-2060 is preferred. The latter is 70 bp upstream of the *dcuA* initiation codon, and suitably positioned and well predicted −10 and −35 sequences are centered at bp 2047.5 and bp 2025.5, respectively. No potential FNR or CRP binding site sequences were detected directly upstream of the *dcuA* promoter, which is consistent with the expression studies.

A single, moderately abundant cDNA product corresponding to an initiation site at A-151, 20 bp upstream of the *dcuB* initiation codon (Fig. 7B and 8B) and just 3 bp upstream from that predicted by Six et al. (36), was observed for the *dcuB* transcript. A well-predicted −10 site is appropriately positioned at bp 138.5, but the associated −35 site at bp 115.5 is very poor. A well-predicted FNR site and a moderately predicted CRP site are centered at bp 103.5 (−45.5) and bp 43.5 bp (−106.5), respectively (Fig. 8B). These sites were identified by virtue of the high or moderate probability values obtained in a score matrix-based search (see Materials and Methods) and correspond to those previously predicted (36). The predicted FNR site matches the consensus FNR binding site at 7 of the 10 conserved positions, and the predicted CRP site matches the CRP binding site consensus sequence at 9 of a possible 12 positions. FNR sites are normally found just upstream of the −35 site, centered at around −41.5 (11, 20). The FNR site of the FNR-activated fumarate reductase (Frd) operon (*frdABCD*) is also centered at −45.5 (or −46.5), indicating that the position of the predicted *dcuB*-FNR site is appropriate to allow FNR-dependent transcriptional activation at the *dcuB* promoter (11). FNR-dependent promoters often possess weak −35 sites and good −10 sites, and in such cases the FNR site is compensatory in allowing FNR-dependent expression of otherwise weakly transcribed genes. This model for FNR induction is likely to apply to *dcuB* also. CRP is known to activate transcription by binding to sites located at various positions upstream of the −35 site (from −41.5 to −103) (11). The location of the CRP site at −106.5 is therefore appropriate to permit CRP-dependent activation. Several genes are known to be subject to dual FNR- and CRP-dependent activation (11). These include the *ansB* (asparaginase II) gene which contains FNR and CRP sites (at −41.5 and −91.5, respectively) with an organization resembling that of *dcuB* (15). Thus, like the *ansB* promoter, the *dcuB* promoter can be classified as class III (32).

Five sequences with similarity to the NarL or NarP binding site consensus sequence (7) were identified by using the score matrix approach. These heptameric sequences are centered at −109, −45, +1, +19, and +44 and are suitably positioned to interfere with the binding of FNR, CRP, and RNA polymerase and/or transcript extension. NarL or NarP sites are normally located, at multiple positions, upstream of the −35 site (7). The NarP heptameric binding sites possess a 7-2-7 organization, whereas NarL can recognize heptamers in various arrangements (7). The organization of the putative NarL sites in the *dcuB* operator-promoter region clearly does not conform to the preferred 7-2-7 arrangement, which is consistent with the observation that NarL, but not NarP, represses *dcuB* expression.

A single, relatively abundant cDNA product was detected in the primer extension analysis of the *aspA* transcript (Fig. 7C). The size of this product indicates a transcriptional initiation site at T-470, 105 bp upstream of the *aspA* initiation codon (Fig. 8C). A good −10 site (bp 461.5) and a weak −35 site (bp 436.5) are correctly positioned upstream of the *aspA* +1 site (Fig. 8C). A weakly predicted FNR site and a strongly predicted CRP binding site are centered at −40.5 and −89.5, respectively. Since *aspA* expression is reported to be induced 10- to 15-fold anaerobically by FNR (16, 44), a good FNR site would be expected. However, the Northern blot analysis showed no anaerobic induction of *aspA* expression under the growth conditions used here, and this is consistent with a weak FNR site. The strong CRP binding site is correctly positioned to function in *aspA* transcriptional activation by the cAMP-CRP complex. Such an interaction could compensate for the

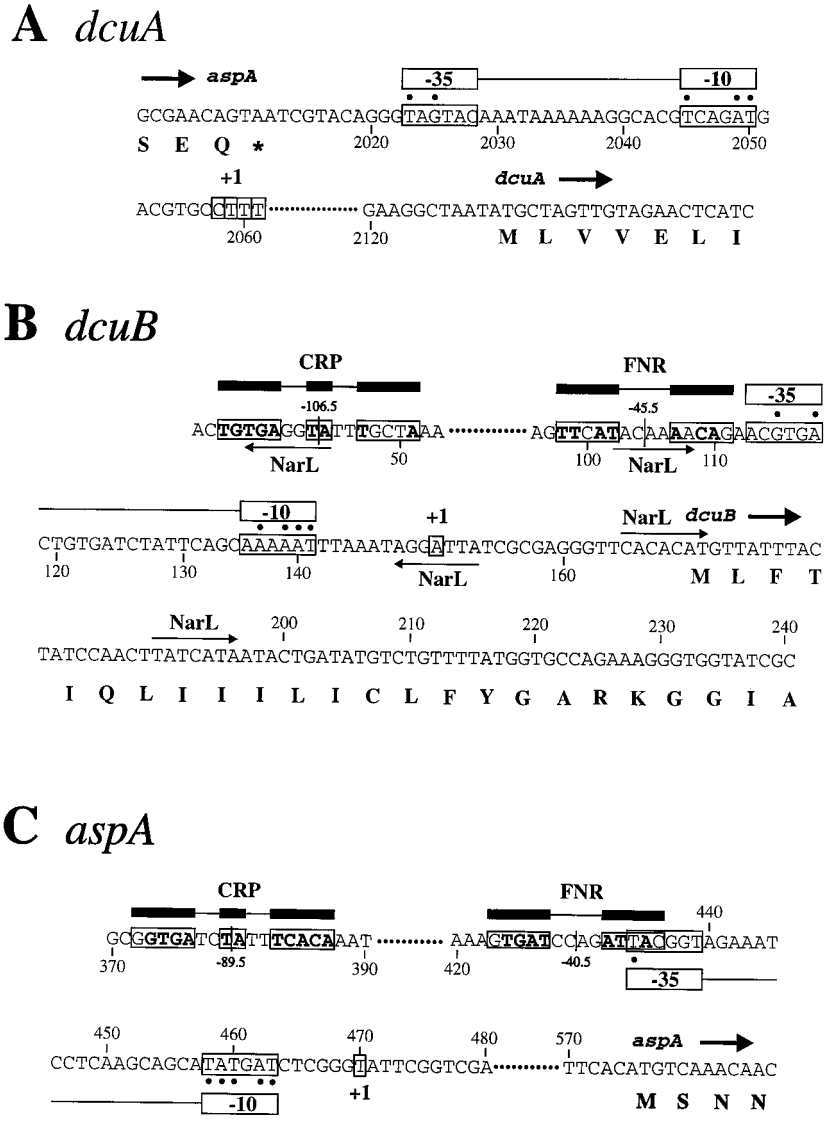

FIG. 8. Nucleotide sequence of the *dcuA* (A), *dcuB* (B), and *aspA* (C) promoter regions. Coordinates for the *aspA* gene are from the work of Woods et al. (43), and coordinates for the *dcuA* and *dcuB* gene are from the work of Six et al. (36). The experimentally determined +1 sites are boxed and labeled, as are the deduced −35 and −10 sites and predicted CRP and FNR sites. Putative NarL binding sites are indicated by horizontal arrows: rightward arrows indicate that the binding site is on the coding strand, while leftward arrows indicate that the binding site is on the noncoding strand. Residues matching the corresponding consensus sequence residues for the FNR and CRP sites are in boldface type. Closed circles indicate residues matching the corresponding consensus sequence residues for the −35 and −10 sites. The positions of the predicted FNR and CRP sites with respect to the +1 sites are indicated.

weak −35 site and could also account for the fivefold repression of *aspA* by glucose (44).

No primer extension products were observed in the analysis of the *fumB* transcript. The reason for this is uncertain. The Northern blot analysis indicated a ~1,900-nt *fumB* transcript. If *fumB* transcription terminates, as expected, at the stem-loop structure at bp 3250, then this would place the transcriptional initiation site ~250 bp upstream of the *fumB* initiation codon, well within the *dcuB* structural gene. This is far further upstream than anticipated (36) and might explain the failure to detect a primer extension product for the *fumB* transcript. Previous analyses of *fumB* expression utilizing *fumB′-lacZ* fusions suggest that the *fumB* gene possesses an independent promoter and associated operator region located within the 800-bp region directly upstream of the *fumB* initiation codon (41, 44).

DISCUSSION

This study shows that the *dcuA* and *dcuB* genes of *E. coli* are differentially regulated. The *dcuB* gene is expressed exclusively under anaerobic conditions in a manner that is largely FNR dependent, is repressed by nitrate through a mechanism that is mostly NarL mediated, and is strongly induced by C_4-dicarboxylates anaerobically. Expression of *dcuB* is also repressed by glucose, is slightly repressed by TMAO, and in the absence of glucose is threefold induced by CRP. In agreement with the expression data, the *dcuB* promoter region contains well-predicted binding sites for FNR, NarL, and CRP (Fig. 8). ArcA has no significant role in *dcuB* regulation, although it appears that a factor other than FNR also contributes to anaerobic induction of *dcuB*. Furthermore, in the absence of NarL, *dcuB* is repressed fourfold by nitrate in a manner that appears to be

NarP independent, and, in addition, *dcuB* expression is reduced by glucose in a fashion that is mostly CRP independent. Thus, factors other than FNR, NarL, and CRP also appear to contribute to the regulation of *dcuB* in response to oxygen, nitrate, and glucose. In contrast to the strong regulation of *dcuB*, expression of the *dcuA* gene is virtually unaffected by the environmental and regulatory factors tested. The *dcuB* gene is ca. fourfold more strongly expressed than *dcuA* during fumarate respiration (Fig. 3A, 5Bii, and 5Ci). This supports the results of transport experiments with *dcu* mutants showing that DcuB is the dominant Dcu carrier during fumarate respiration (45). However, the *dcuA* gene is more strongly expressed than *dcuB* under most of the other growth conditions examined, and this is concordant with the better codon usage of *dcuA* relative to that of *dcuB* (36).

The expression and transport data are consistent with each other insofar as Dcu transport activity and the combined *dcuA*- and *dcuB-lacZ* activities are activated anaerobically by FNR, repressed anaerobically in the presence of nitrate by NarL, increased by fumarate or succinate, and weakly affected by ArcA (8, 9). The factors affecting Dcu transport activity described above clearly reflect those influencing *dcuB* expression. This is because *dcuB* expression, but not *dcuA* expression, is strongly modulated by these factors and also because *dcuB* expression exceeds that of *dcuA* during fumarate respiration. The slight repression effect (greater than twofold) of TMAO on *dcuB* expression is consistent with the lack of effect of TMAO on Dcu transport activity. It is possible that the TMAO effect on *dcuB* expression is mediated by the TMAO-responsive two-component sensor-regulator system, TorS-TorR (18, 34), although this has not been tested.

Surprisingly, the correspondence between transport and expression activities is not maintained under all conditions examined. Previous studies showed that Dcu activity is only slightly reduced by glucose and is unaffected by cAMP or a *cya* mutation, suggesting that CRP does not regulate Dcu synthesis (8, 9, 45). However, clear glucose and CRP effects on *dcuB* expression were observed in the studies reported here, although the CRP effect was relatively weak. The reason for these discrepancies is unclear, but they could reflect posttranscriptional effects or a lack of specificity when transport activity mediated by at least three alternative systems is measured.

The 70-fold induction of *dcuB* expression by C_4-dicarboxylates (Fig. 4) is likely to be mediated by an undefined C_4-dicarboxylate-dependent regulator able to sense and respond to exogenous C_4-dicarboxylates. A good candidate for such a regulator is a putative two-component regulatory system composed of a response regulator and a membrane-associated histidine kinase sensor encoded by the *yjdHG* genes located just 570 bp upstream of *dcuB*. The corresponding proteins have strong sequence similarity to the *Klebsiella* CitB and CitA proteins involved in the regulation of anaerobic citrate metabolism (5, 25). The possible involvement of these genes in C_4-dicarboxylate-responsive gene regulation is being investigated. Other *E. coli* genes are also known to be induced, albeit weakly, by C_4-dicarboxylates. These are the *frdABCD* and *nuo* operons which are 1.5-fold induced by fumarate and ~2.5-fold induced by fumarate or succinate, respectively (4, 17). The mechanism governing this regulation is unknown.

The pattern of *dcuA* and *dcuB* expression provides important clues for the likely physiological functions of the homologous and functionally related DcuA and DcuB proteins. The profile for *dcuB* expression is consistent with a role for DcuB in the provision of substrate and export of product for the reaction catalyzed by Frd during fumarate respiration. Indeed, the expression profile of *dcuB* resembles that of the *frdABCD* operon. Appropriately, both are anaerobically induced by FNR and repressed by NarL in response to nitrate (14, 17), thus ensuring that oxygen and nitrate are utilized in preference to fumarate as terminal electron acceptors. The *fumB* gene, which is adjacent to *dcuB*, encodes the enzyme fumarase B, which acts as a malate dehydratase in the conversion of malate to fumarate. Fumarase B thus provides substrate for Frd during anaerobic fumarate respiration. Therefore, DcuB and fumarase B are both involved in feeding substrate to Frd and constitute consecutive steps in the anaerobic transport and metabolism of malate. The *dcuB* and *fumB* genes would therefore be expected to be expressed in a coordinated fashion, as is suggested by the Northern blotting analysis showing strong anaerobic induction and partial cotranscription (Fig. 6).

The expression of *dcuA* under both aerobic and anaerobic conditions is inconsistent with the previously proposed, anaerobic function for DcuA (36, 45) and suggests that DcuA has an aerobic function in addition to contributing to anaerobic C_4-dicarboxylate uptake. However, transport studies failed to demonstrate any Dcu activity, attributable to DcuA, under aerobic conditions, possibly because the Dcu systems are inactivated by oxygen (8, 9). Therefore, it is possible that the aerobic expression of *dcuA* produces an inactive DcuA protein. However, inactivation of Dcu activity by transient exposure to oxygen (or other oxidants) can be reversed by subsequent treatment with reducing agents (8). This offers the possibility that constitutive expression of *dcuA* allows *E. coli* to respond rapidly to transitions from aerobic to anaerobic conditions through the activation of presynthesized DcuA.

Aspartase, the product of the *aspA* gene located upstream of *dcuA*, converts L-aspartate to fumarate. Together with the constitutive aspartate aminotransferase (encoded by *aspC*), it is thought to provide an alternative mechanism for converting oxaloacetate to fumarate as part of the reductive branch of the noncyclic form of the citric acid cycle (6). A previous *lacZ* fusion analysis (44) and the Northern blot analysis reported above suggest that, like *aspC* and *dcuA*, the *aspA* gene is well expressed under both aerobic and anaerobic conditions, indicating that DcuA and aspartase have aerobic as well as anaerobic functions. However, it should be stressed that this is inconsistent with aspartase activity measurements that show that aspartase is anaerobically induced (16). Aspartase may also have a role, together with the anaerobically induced periplasmic asparaginase II, in the utilization of exogenous asparagine (24) and in the degradation of aspartate for use as a carbon source (29). Furthermore, aspartase is required for regenerating oxaloacetate in the aerobic and anaerobic utilization of glutamate (38). Whether the DcuA protein assists in any of these processes is uncertain, but the colocations of the *aspA* and *dcuA* genes and the related substrate specificities of their products are certainly suggestive of linked functions.

ACKNOWLEDGMENTS

We thank the BBSRC for a project grant (S.C.A. and J.R.G.) and for an Advanced Fellowship (S.C.A.).

ADDENDUM IN PROOF

A recent publication (E. Zientz, J. Bongaerts, and G. Unden, J. Bacteriol. **180:**5421–5425, 1998) as well as our own unpublished results show that the *yjdHG* (*dcuSR*) genes do indeed encode a sensor-regulator system responsible for the C_4-dicarboxylate-dependent regulation of *dcuB* (and other genes).

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