JOURNAL OF BACTERIOLOGY, June 1999, p. 3716–3720 Vol. 181, No. 12
0021-9193/99/$04.00+0

Functioning of DcuC as the C_4-Dicarboxylate Carrier during Glucose Fermentation by *Escherichia coli*

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Received 21 December 1998/Accepted 7 April 1999

The *dcuC* gene of *Escherichia coli* encodes an alternative C_4-dicarboxylate carrier (DcuC) with low transport activity. The expression of *dcuC* was investigated. *dcuC* was expressed only under anaerobic conditions; nitrate and fumarate caused slight repression and stimulation of expression, respectively. Anaerobic induction depended mainly on the transcriptional regulator FNR. Fumarate stimulation was independent of the fumarate response regulator DcuR. The expression of *dcuC* was not significantly inhibited by glucose, assigning a role to DcuC during glucose fermentation. The inactivation of *dcuC* increased fumarate-succinate exchange and fumarate uptake by DcuA and DcuB, suggesting a preferential function of DcuC in succinate efflux during glucose fermentation. Upon overexpression in a *dcuC* promoter mutant (*dcuC), DcuC was able to compensate for DcuA and DcuB in fumarate-succinate exchange and fumarate uptake.**

Escherichia coli contains four different secondary carriers (DcuA, DcuB, DcuC, and DctA) for C_4-dicarboxylates (3, 4, 22, 26). DctA is used for aerobic growth on C_4-dicarboxylates (3, 12), whereas the Dcu carriers (encoded by the *dcuA*, *dcuB*, and *dcuC* genes) are used under anaerobic conditions and form a distinct family of carriers (4, 5, 18, 22, 25, 26). Each of the Dcu carriers is able to catalyze the uptake, antiport, and possibly also efflux of C_4-dicarboxylates. DcuB is the major C_4-dicarboxylate carrier for fumarate respiration with high fumarate-succinate exchange activity. It is synthesized only in the absence of oxygen and nitrate and in the presence of C_4-dicarboxylates (4, 6, 7, 27). DcuA is expressed constitutively in aerobic and anaerobic growth and can substitute for DcuB (7, 22). DcuC shows the same transport modes as DcuA and DcuB (exchange, uptake, and presumably efflux of C_4-dicarboxylates) (26), but the transport activities are significantly lower than for DcuA and DcuB. Thus, a mutant lacking DcuA and DcuB was severely inhibited for growth by fumarate respiration due to the limited transport activities of DcuC, whereas DcuA and DcuB were able to maintain full growth under these conditions (22, 26). These findings suggest a different physiological role for DcuC and use under different conditions. To obtain a clue as to the role of DcuC, the functions of Dcu and the conditions for Dcu synthesis were studied.

MATERIALS AND METHODS

Bacterial strains and growth conditions. For growth experiments and transport assays, the bacteria (Table 1) were grown under aerobic or anaerobic conditions in M9 mineral medium supplemented with acid-hydrolyzed casein (0.05%) and tryptophan (0.005%) (1, 24). Glucose (10 mM), glycerol (50 mM), sodium C_4-dicarboxylates such as fumarate or succinate (50 mM), and sodium nitrate (50 mM) were included as needed.

Genetic procedures and DNA manipulation. The *dcuC′-′lacZ* fusion strain (strain IMW240) was constructed by PCR amplification of the promoter region of *dcuC* from genomic DNA of strain AN387 (24) with primers dcuCBam (5′-CCC CAA TAA GGA TCC CAA TG), introducing a *Bam*HI site, and dcuCEco (5′-CCA GCG GTG AAT TCC AGA CC), introducing an *Eco*RI site. The resulting 1.1-kb fragment was cloned into the *Bam*HI and *Eco*RI sites of the protein fusion vector pJL29 (15), yielding pMW98. The corresponding *dcuC*′-′lacZ* fusion (strain IMW201) was made in the same way from genomic DNA of strain IMW152 with primers dcuCEcoV (5′-GCT ATC CAG GGA TAT CCG GG), introducing an *Eco*RV site, and primer dcuCBam. The resulting 0.5-kb DNA fragment was cloned into the *Sma*I and *Bam*HI sites of pJL29 to create plasmid pMW122. The gene fusions were transferred to the genome of *E. coli* MC4100 with phage λRZ5 (1, 17, 21). P1 transduction was performed as described previously (1) and checked by PCR and Southern blot analysis (22, 27).

Transport assays. Transport of C_4-dicarboxylates in cell suspensions of bacteria was measured by silicone oil centrifugation. For measurement of exchange, the bacteria were loaded with succinate and the uptake of [^{14}C]fumarate was measured by silicone oil centrifugation (5, 22, 26). Uptake was measured by adding [^{14}C]fumarate to energized bacteria and monitoring the increase in internal [^{14}C]fumarate levels by silicone oil centrifugation (22, 26).

RNA isolation and primer extension. Total RNA was isolated with the RNeasy mini kit (Qiagen). The 5′ end of the mRNA encoded by the *dcuC* or *dcuC** gene was mapped by primer extension with primer cpe2 (5′-GAG CTC AAT GAA TGT CAG CAT AAT TTT TCC-3′), which is complementary to positions 107 to 136 of *dcuC* in the transcript. The primer extension was performed at 37°C for 1 h with 20 U of Moloney murine leukemia virus reverse transcriptase and [γ-^{32}P]dATP. The extension products were purified by ethanol precipitation and subjected to denaturing polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

Expression of *dcuC′-′lacZ* in response to electron acceptors and the C source. The conditions for DcuC expression were studied with a *dcuC′-′lacZ* reporter gene fusion (Fig. 1). The *dcuC* gene was fused in frame to *′lacZ* to obtain a translational protein fusion. The fusion contained the complete promoter region up to position −971 and seven codons of *dcuC*. The *dcuC′-′lacZ* fusion was inserted with phage λRZ5 into the genome of *E. coli* MC4100, and monolysogenic strains were used (Table 2). During anaerobic growth, *dcuC* was expressed with relatively high activities, but the presence of O_2 caused complete repression irrespective of the growth substrate. During anaerobic growth, the addition of fumarate increased expression about twofold, whereas electron acceptors such as nitrate, dimethyl sulfoxide, and trimethylamine *N*-oxide (TMAO) caused slight repression (Table 2). Malate, aspartate,

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TABLE 1. Strains of *E. coli* used

Strain	Genotype	Reference or source
AN387	Parental	1, 24
MC4100	F$^-$ *araD138* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB530 deoC1 ptsF25 rbsR*	21
RM101	MC4100 but Δ*fnr*	19
RM313	MC4100 but *arcA1 zjj*::Tn*10*	19
RM600	MC4100 but *narL215*::Tn*10*	19
VJS4322	RK4353 but *narP253*::Tn*10d* Δ(*narXL*)*253*	13
IMW153	AN387 but *dcuA*::Spc^r *dcuB*::Kan^r *dcuC*::mini-Tn*10* (Cam^r)	26
IMW152	AN387 but *dcuA*::Spc^r *dcuB*::Kan^r *dcuC** (IS*5* upstream of *dcuC*)	This work
JRG2814	AN387 but *dcuA*::Spc^r *dcuB*::Kan^r	22
IMW240	MC4100 λ[Φ(*dcuC'-'lacZ*)]	This work
IMW256	MC4100 λ[Φ(*dcuC'-'lacZ*)] but Δ*arcA*	P1 (RM313) × IMW240
IMW257	MC4100 λ[Φ(*dcuC'-'lacZ*)] but *narL*::Tet^r	P1 (RM600) × IMW240
IMW258	MC4100 λ[Φ(*dcuC'-'lacZ*)] but *narP*::Cam^r	P1 (VJS4322) × IMW240
IMW255	MC4100 λ[Φ(*dcuC'-'lacZ*)] but Δ*fnr*	RM101 × λ*dcuC'-'lacZ*
IMW241	MC4100 λ[Φ(*dcuC'-'lacZ*)] but *dcuR*::Kan^r	P1 (IMW205) × IMW240
IMW201	MC4100 λ[Φ(*dcuC*'-'lacZ*)]	This work
IMW202	MC4100 λ[Φ(*dcuC*'-'lacZ*)] but Δ*fnr*	RM101 × λ*dcuC*'-'lacZ*
IMW271	MC4100 λ[Φ(*dcuC*'-'lacZ*)] but Δ*arcA*	RM313 × λ*dcuC*'-'lacZ*
IMW272	MC4100 λ[Φ(*dcuC*'-'lacZ*)] but Δ*narL*	RM600 × λ*dcuC*'-'lacZ*
IMW205	MC4100 but *dcuR*::Kan^r	27
IMW242	MC4100 λ[Φ(*dcuC'-'lacZ*)] but *citB*::Spc^r	P1 (IMW220) × IMW240
IMW272	MC4100 but *citB*::Spc^r	27

asparagine, and tartrate stimulated expression in a manner similar to that of fumarate (data not shown), whereas other carboxylic acids, including malonate, did not cause induction. When glucose was replaced during anaerobic growth by glycerol or other C sources, the expression of *dcuC* increased only negligibly, indicating that *dcuC* is not subject to glucose repression (Table 2).

Transcriptional regulators controlling *dcuC* expression. The expression of *dcuC'-'lacZ* in mutants deficient in regulators responding to electron acceptors was studied (Table 2). The *fnr* mutant, lacking the O_2-responsive regulator FNR, was completely devoid of *dcuC'-'lacZ* expression during aerobic and anaerobic growth. The *arcA* mutant, which is deficient in the O_2-responsive regulator ArcA (9), showed only a twofold decrease in *dcuC'-'lacZ* expression under anaerobic conditions. Therefore, anaerobic induction of *dcuC* is affected by both regulators, but FNR plays the major role. Inactivation of the *narL* and *narP* genes, encoding nitrate response regulators NarL and NarP, respectively (23), had only a weak effect on *dcuC* expression, in agreement with the marginal effects of nitrate. Fumarate regulation of anaerobic metabolism is mediated by the DcuSR two-component regulatory system of *E. coli* (6, 27). Mutants lacking the fumarate response regulator DcuR (*dcuR* gene) or the related CitB response regulator (*citB* gene) (27) showed the same fumarate stimulation of *dcuC* expression as the wild type. Thus fumarate stimulation of *dcuC* must be affected by a different regulatory system.

DcuC as the succinate efflux carrier for glucose fermentation? According to the results obtained here, the pattern of expression of *dcuC* differs clearly from that of *dcuA*, *dcuB*, and *dctA* (3, 7, 27). DcuC is synthesized only under anaerobic conditions, and synthesis is not or is only slightly repressed by glucose or nitrate, respectively, and is slightly stimulated by fumarate. The lack of glucose repression suggests that DcuC plays a role in glucose fermentation, e.g., succinate efflux, when only the (constitutive) DcuA carrier is produced as well. Accordingly, the low rates of transport of DcuC are sufficient for succinate export during fermentation (up to 0.2 mol of succinate/mol of glucose) but not for fumarate-succinate exchange in fumarate respiration. According to the presumed functioning of DcuC as an efflux carrier, the inactivation of *dcuC* significantly increased the uptake and exchange of C_4-dicarboxylates (Table 3). Table 3 compares the fumarate-succinate exchange and uptake activities in strains of *E. coli* containing different sets of Dcu carriers. When *dcuB* was inactivated in the strains, exchange and uptake activities for C_4-dicarboxylates decreased about two- to sixfold compared to those in the parental strains. This result is in agreement with the important role of DcuB in these transport reactions of anaerobically growing *E. coli*. However, when *dcuC* was inactivated, exchange and uptake activities increased compared to those in the parental strains. The surprising finding that inactivation of a carrier increased exchange and uptake activities for the same substrates can be explained by assuming that DcuC counteracts the exchange and uptake activities effected by DcuA and DcuB and that DcuC preferentially acts as an efflux carrier in *E. coli* cells. Direct measurement of efflux activities was obstructed by

TABLE 2. Expression of *dcuC'-'lacZ* as a function of electron acceptors and C_4-dicarboxylates

Growth condition[a]	*dcuC'-'lacZ* expression (β-galactosidase activity, in Miller units) of strain:			
	IMW240 (wild type)	IMW255 (Δ*fnr*)[b]	IMW256 (Δ*arcA*)[b]	IMW257 (*narL*::Tet^r)[b]
Glucose	52	3	26	63
Glucose[c] + O_2	4	3	6	ND[d]
Glucose + fumarate	99	3	42	87
Glucose + nitrate	38	ND	ND	51
Glucose + dimethyl sulfoxide	37	3	ND	ND
Glycerol + fumarate	109	NG[e]	ND	ND

[a] M9 mineral medium supplemented with acid-hydrolyzed casein was used. Aerobic growth and anaerobic growth were carried out as described previously (1, 24).
[b] Like IMW240 (MC4100 with *dcuC'-'lacZ*) but with the indicated mutation.
[c] Or glycerol or succinate.
[d] ND, not determined.
[e] NG, no growth.

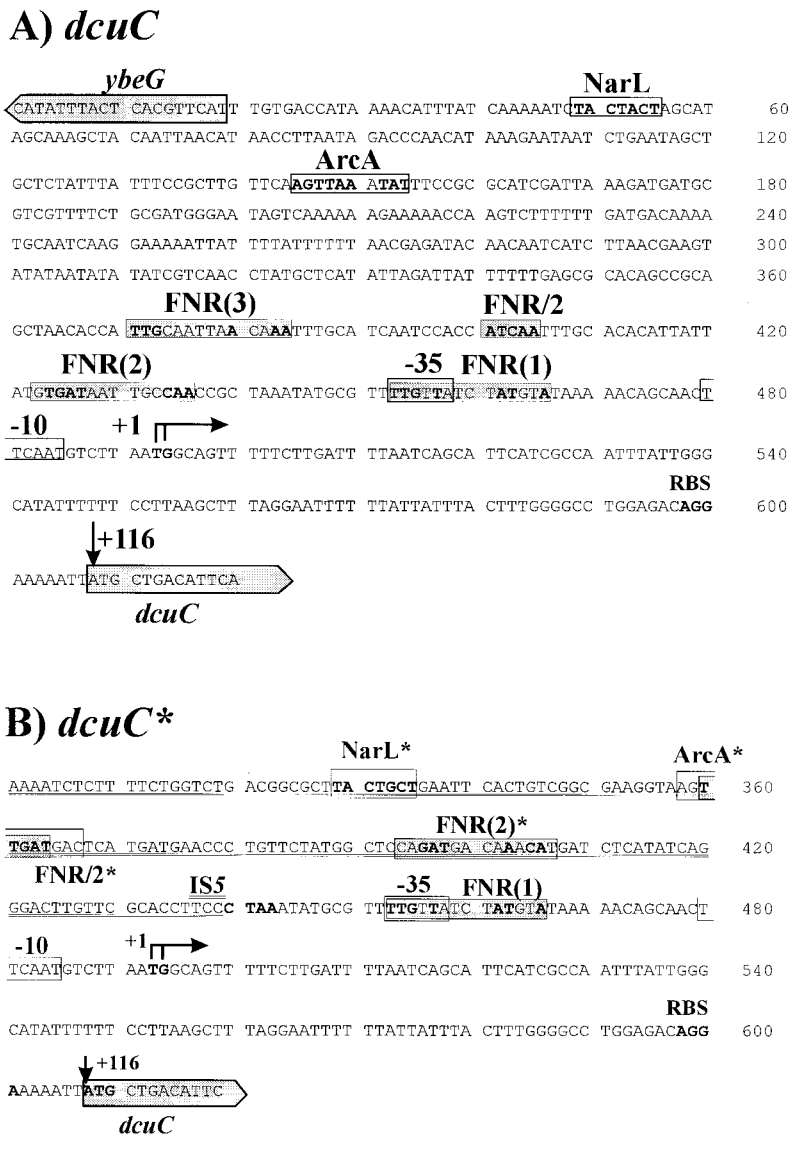

FIG. 1. Promoter regions of *dcuC* (*E. coli* AN387) (A) and *dcuC** (*E. coli* IMW152) (B). The putative promoter region (positions −10 and −35) is boxed, and the transcriptional start sites (+1) and putative ribosome binding sites (RBS) are shown in bold. FNR consensus sites [FNR(1) to FNR(3)] and half-sites (FNR/2) with ≥6 conserved residues (bold letters) of the FNR consensus sequence (TTGAT----ATCAA) (8, 11) are shaded. ArcA (WGT TAA TTA W, with W = A or T) (16) and NarL (TACYYMT, with Y = C or T and M = A or C) (13) consensus sequences are boxed, and the conserved residues are shown in bold. In panel B, the site of IS*5* insertion is indicated (CTAA, shown in bold), and the sequence corresponding to IS*5* is underlined. Sites derived from IS*5* are indicated by asterisks. For the ArcA* site, the conserved residues are not indicated.

the high rates of diffusion of C_4-dicarboxylates through the membranes under the respective conditions (10).

Isolation of an IS*5* promoter mutation of *dcuC* (*dcuC).** The *dcuA dcuB* double mutant grows only slowly on glycerol plus fumarate (22, 26). From the double mutant, a spontaneous mutant which had regained full anaerobic growth on glycerol plus fumarate was obtained. The nucleotide sequence of *dcuC* was the same in the mutant as in the wild type (26), except that an IS*5* element was inserted upstream of the coding region (Fig. 1). Southern blotting and PCR analysis confirmed that *dcuA* and *dcuB* were still inactivated by the inserted resistance cassettes. The expression of *dcuC* in the mutant (*dcuC**) was determined with a *dcuC*'-'lacZ* fusion. The expression of *dcuC*'-'lacZ* was increased by a factor of about 2.2 compared to that in the wild type, but the responses to oxygen, nitrate, and the regulators FNR, NarL, and NarP were comparable to those in the wild type (data not shown).

Functional replacement of *dcuB* by overexpression of *dcuC* in the *dcuC mutant.** In wild-type *E. coli*, DcuC supports only slow growth by fumarate respiration (26). By using the *dcuC** mutant, we tested whether this finding was due to restricted functioning of DcuC in the antiport mode or to limiting transport rates. In Fig. 2, the fumarate-succinate antiport activities in strains containing only *dcuC* or *dcuC** are related to the rates of growth on glycerol plus fumarate. The increase in fumarate-succinate antiport in the *dcuC** strain (about twofold) compared to that in the strain containing only *dcuC* was similar to the increase in *dcuC* or *dcuC** expression and was paralleled by a similar increase in the rate of growth on glycerol plus fumarate. A further increase in fumarate-succinate

TABLE 3. Effects of DcuC and DcuB inactivation on exchange and uptake activities in strains with various *dcu* gene compositions

Activity measured	Strain (genotype)	Carriers present	Transport activity (μmol/min/mg dry wt)	Change in activity (fold) with inactive	
				DcuC[a]	DcuB[b]
Exchange [^{14}C]fumarate-succinate	AN387 (parental)	DcuA DcuB DcuC	21.8	1.8	0.49
	JRG2821 (*dcuA*)	DcuB DcuC	29.5	1.4	0.18
	JRG2813 (*dcuB*)	DcuA DcuC	10.6	1.5	NA[c]
	IMW157 (*dcuC*)	DcuA DcuB	39.7	NA	0.47
Uptake of [^{14}C]fumarate	AN387 (parental)	DcuA DcuB DcuC	13.9	1.2	0.39
	JRG2821 (*dcuA*)	DcuB DcuC	11.6	1.4	0.16
	JRG2813 (*dcuB*)	DcuA DcuC	5.4	1.5	NA
	IMW157 (*dcuC*)	DcuA DcuB	16.7	NA	0.47

[a] Same as the reference strain but *dcuC*::mini-Tn*10*.
[b] Same as the reference strain but *dcuB*::Kan^r.
[c] NA, not applicable.

antiport in strains also containing DcuA and/or DcuB caused no further increase in the growth rate. This result indicates that antiport rates of ≥10 U/mg of dry weight are sufficient to support full growth by fumarate respiration and that DcuC, in addition to its preferred function as an efflux carrier, is able to operate as a fumarate-succinate antiporter and to replace DcuB, if it is required and produced in sufficient amounts.

Transcriptional start sites at the *dcuC* and *dcuC promoters.** The transcriptional start sites of *dcuC* and *dcuC** were determined with mRNA isolated from strains carrying either *dcuC* or *dcuC**. In primer extension experiments, transcripts of the same length were produced from the mRNAs of both strains (Fig. 3) and started 116 bp upstream of the supposed translational start sites (26). In *dcuC*, the transcriptional start site is preceded by two potential FNR consensus sites and one half-site between positions −33.5 and −115.5. Therefore, *dcuC* lacks a typical FNR binding site at position −41.5 (class II site) (8, 11). In *dcuC**, the promoter region at positions −35 and −10 of *dcuC* is retained, since the insertion site for IS*5* is located at position −53 (Fig. 2). However, regulatory sites upstream of position −53, including two of the FNR consensus sites, are replaced by IS*5* sequences. The IS*5* element supplies two FNR consensus sites at positions −92.5 and −131 which could serve as the FNR regulatory sites of *dcuC**.

Due to this situation, the FNR (1) consensus site (Fig. 1) could be used for FNR-dependent regulation of *dcuC* and *dcuC** as well. In this case, the IS*5* element would cause increased *dcuC** expression by indirect effects, e.g., topological changes at the *dcuC** promoter. Alternatively, other (upstream) FNR regulatory sites could be used in *dcuC* and *dcuC**. This would mean that the IS*5* element is able to provide FNR regulatory sites if inserted at appropriate positions. In any case, insertion of an IS*5* element is able to increase FNR-dependent expression or to place genes under FNR control. IS*5* elements frequently have been identified in or at promoters with altered expression of anaerobic pathways genes in *E. coli* (2, 14, 20). IS*5* therefore could be important for the evolution of anaerobic pathways, either by inserting new reg-

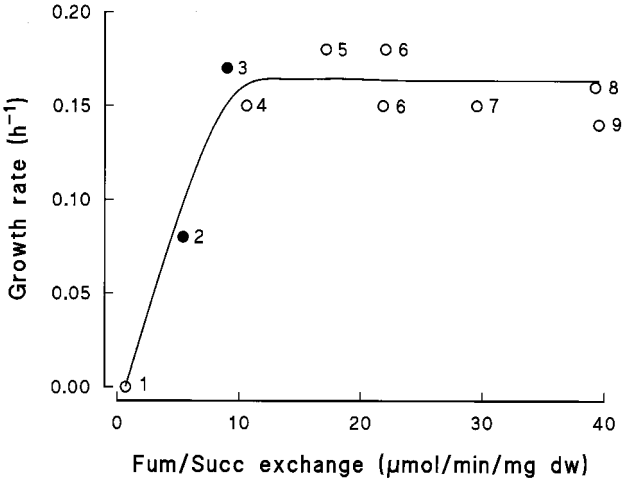

FIG. 2. Rates of anaerobic growth on glycerol plus fumarate and fumarate-succinate (Fum/Succ) exchange activities for *E. coli* strains containing *dcuC* or *dcuC** as the only *dcu* gene (●) or strains with different combinations of *dcu* genes (○). The strains are isogenic except for the presence of the *dcu* genes. 1, IMW153 (*dcuA dcuB dcuC*); 2, JRG2814 (*dcuA dcuB*); 3, IMW152 (*dcuA dcuB* with *dcuC**); 4, JRG2813 (*dcuB*); 5, IMW158 (*dcuB dcuC*); 6, AN387 (wild type, parental strain); 7, JRG2821 (*dcuA*); 8, IMW159 (*dcuA dcuC*); and 9, IMW157 (*dcuC*). Growth was determined with supplemented M9 mineral medium; fumarate-succinate exchange was determined with cell suspensions of the bacteria. dw, dry weight.

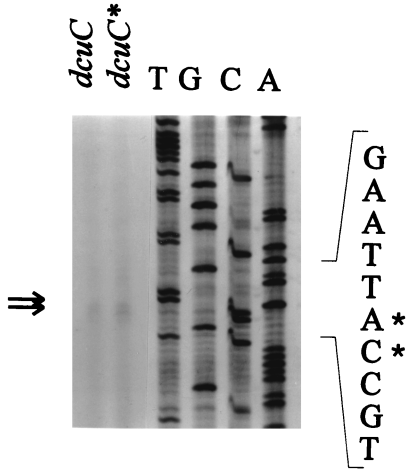

FIG. 3. Determination of the transcriptional start sites of the *dcuC* and *dcuC** genes in strains AN387 and IMW152 by primer extension. mRNA was isolated from strains AN387 (*dcuC*$^+$) and IMW152 (*dcuC**) grown anaerobically on glucose plus fumarate in supplemented M9 mineral medium to an A_{578} of 0.5. The primer extension products (arrows) were obtained with primer cpe2. The sequencing reactions (T, G, C, and A) were performed with the same primer and pMW98 DNA. The nucleotides corresponding to the transcriptional start sites are labelled with asterisks.

ulatory sites or by changing the quality of adjacent promoters by affecting DNA topology.

ACKNOWLEDGMENTS

The work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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