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Identification and Characterization of the *caiF* Gene Encoding a Potential Transcriptional Activator of Carnitine Metabolism in *Escherichia coli*

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Expression of the *Escherichia coli caiTABCDE* and *fixABCX* operons involved in carnitine metabolism is induced by both carnitine and anaerobiosis. When cloned into a multicopy plasmid, the 3′ region adjacent to the *caiTABCDE* operon was found to increase levels of carnitine dehydratase activity synthesized from the chromosomal *caiB* gene. The nucleotide sequence was determined, and it was shown to contain an open reading frame of 393 bp named *caiF* which is transcribed in the direction opposite that of the *cai* operon. This open reading frame encodes a protein of 131 amino acids with a predicted molecular mass of 15,438 Da which does not have any significant homology with proteins available in data libraries. In vivo overexpression consistently led to the synthesis of a 16-kDa protein. The *caiF* gene was transcribed as a monocistronic mRNA under anaerobiosis independently of the presence of carnitine. Primer extension analysis located the start site of transcription to position 82 upstream of the *caiF* initiation codon. It was preceded by a cyclic AMP receptor protein motif centered at position −41.5. Overproduction of CaiF resulted in the stimulation of transcription of the divergent *cai* and *fix* operons in the presence of carnitine. This suggested that CaiF by interacting with carnitine plays the role of an activator, thereby mediating induction of carnitine metabolism. Moreover, CaiF could complement in *trans* the regulatory defect of laboratory strain MC4100 impaired in the carnitine pathway. Expression of a *caiF-lacZ* operon fusion was subject to FNR regulator-mediated anaerobic induction and cyclic AMP receptor protein activation. The histone-like protein H-NS and the NarL (plus nitrate) regulator acted as repressors. Because of the multiple controls to which the *caiF* gene is subjected, it appears to be a key element in the regulation of carnitine metabolism.

Enterobacteria are facultative microorganisms that can use a large variety of electron acceptors under anaerobic growth conditions, depending on the particular environment. The availability of exogenous electron acceptors like nitrate, nitrite, dimethyl sulfoxide, trimethylamine-*N*-oxide, or fumarate ensures the induction of the corresponding terminal reductases. These enzymes participate in the formation of respiratory chains, allowing cellular energy generation via electron transport-linked phosphorylation reactions (for reviews, see references 13 and 14).

In the absence of the previously cited electron acceptors, the addition of L-(−)-carnitine has been shown to significantly stimulate anaerobic growth of *Escherichia coli* (29). L-(−)-Carnitine [*R*-(−)-3-hydroxy-4-trimethylaminobutyrate] is a compound widely distributed in nature (11). Found mainly in food of animal origin, it is present in the human intestine, where it can be metabolized by bacteria. Like other members of the family *Enterobacteriaceae*, *E. coli* is able, during anaerobic growth and in the presence of carbon and nitrogen sources, to catalyze the dehydration of L-(−)-carnitine into crotonobetaine and the subsequent reduction into γ-butyrobetaine as a final product (29, 30). Crotonobetaine is therefore thought to serve as an external electron acceptor. The two enzymes, carnitine dehydratase and crotonobetaine reductase, which catalyze this reaction pathway have been partially purified and characterized (15, 26).

The physiological function of L-(−)-carnitine is of considerable importance in eucaryotic cell metabolism, in which it serves as an essential factor in the transport of long-chain fatty acids through the inner mitochondrial membrane (2). Consequently, the increasing demand for this compound, particularly in medicine for the treatment of patients suffering from carnitine deficiency syndromes and various heart diseases, has incited the need for searches for microbial and enzymatic synthesis of L-(−)-carnitine (17).

Recently, we reported the isolation and characterization at the first minute on the *E. coli* chromosome of the *cai* and *fix* structural genes which are involved in the carnitine pathway (7–10). The *caiTABCDE* operon comprises six genes which have been demonstrated to code for carnitine dehydratase (*caiB*) (10), crotonobetaine reductase (*caiA*) (8), and carnitine racemase (*caiD*) (7, 18). It is also suggested that the *cai* operon directs the synthesis of transport system for carnitine (*caiT*), a crotonobetaine/carnitine coenzyme A ligase (*caiC*), and an enzyme involved in the synthesis or the activation of the still unknown cofactor required for carnitine metabolism activities (*caiE*). Transcribed in the orientation divergent from that of

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cai, the *fixABCX* operon contains four genes whose products have been postulated to facilitate the transfer of electrons to crotonobetaine reductase (CaiA) in order to reduce crotonobetaine into γ-butyrobetaine (9).

Regulation of expression of the *fix* operon (9) appears to be controlled by the same factors that influence the carnitine pathway enzymes (7, 19), indicating a physiological linkage between these two loci. Its induction requires the absence of external electron acceptors like oxygen or nitrate and the presence of carnitine or crotonobetaine. Its expression is also dependent on the global regulatory proteins mediating catabolic repression via cyclic AMP receptor protein (CRP), or anaerobiosis via fumarate nitrate reduction (FNR) transcriptional regulator, and the nucleoid-associated DNA-binding protein H-NS. Accordingly, the intergenic common regulatory region shared by *cai* and *fix* harbors putative motifs of recognition for the binding of CRP and H-NS (9). No typical consensus sequence, however, could be found for the binding of FNR. In addition, possible σ^{54}-dependent promoters were identified beside σ^{70} promoter sequence motifs (9). Activation of expression of all known −12/−24 RpoN-dependent promoters is mediated by the binding of a specific activator protein (22). In the case of the *cai* and *fix* operons, it can be hypothesized that such a *trans*-acting positive effector would bind the inducer, L-(−)-carnitine or crotonobetaine, and direct the transcription of both operons. This study deals with the molecular analysis of an open reading frame located downstream of the *cai* operon whose product was shown to act as a putative transcriptional activator in the regulation of carnitine metabolism.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. The *rpoN*::Tn*10* allele from strain YMC18 (35) was transferred into strain MC4100 by transduction with P1*cml*, selecting for tetracycline resistance and then screening for glutamine requirement. The *narL215*::Tn*10* and *rpoS*::*kan* mutations from strains RK5278 (32) and PHL503 were introduced into strain NM522, selecting for the appropriate antibiotic resistance and further scoring for colony color on MacConkey nitrate-glycerol agar under anaerobic conditions or for catalase activity, respectively. The *rpoS*$^+$ derivative of NM522 was constructed with the nearby *srlC300*::Tn*10* insertion from strain JC10240 (5). Plasmid pAB20 carrying the *caiT-lacZ* operon fusion was constructed by subcloning a 1.8-kb *Pst*I-*Bam*HI fragment harboring the *cai* promoter-operator region and part of the *caiT* gene in front of the promoterless *lacZ* gene of the monocopy fusion vector pJEL250 (36). Plasmid pRL40 was constructed in a similar manner by inserting a 0.86-kb *Eco*RV-*Mlu*I fragment containing the *caiF* promoter region and part of the *caiF* gene in pJEL250. To monitor expression of the *caiF-lacZ* fusion in a multicopy plasmid, this fusion was further amplified from pRL40 by PCR and introduced into the *Eco*RI site of pSU9, giving rise to plasmid pKE102. The *caiF* gene carried by plasmid pRL101 was inactivated by insertion of a 1,264-bp *Bam*HI Tn*903* (Km^r) cassette from pUCK (Pharmacia Biotech) into its unique *Bgl*II site, resulting in plasmid pAB101.

Bacteria were grown aerobically in Luria broth (LB) at 30, 37, and 42°C, as indicated, or on plates with LB supplemented with 1.5% agar. Anaerobic growth took place in either tightly stoppered 250-ml bottles or 25-ml screw-cap tubes filled almost to the top with LB or buffered (pH 6.5) TYEP medium without glucose (9) supplemented with 2 μM ammonium molybdate and 2 μM sodium selenite, as described previously (37). For measurement of carnitine dehydratase activity in strain K38 harboring the pT7 derivatives, growth was achieved in a 2-liter Setric fermentor filled with 1.5 liters of TYEP medium supplemented with molybdate, selenite, and 20 mM DL-carnitine. Anaerobic conditions were ensured by saturating the culture medium with a stream of nitrogen gas. The minimal medium used was M9 (27). When required, antibiotics were added at the following final concentrations: ampicillin, 50 μg/ml; kanamycin, 20 μg/ml; chloramphenicol, 20 μg/ml; and rifampin, 200 μg/ml.

Standard molecular biology techniques. The following standard molecular biology techniques were all carried out as described by Sambrook et al. (27): DNA isolation, restriction analysis, construction of recombinant DNA, and transformation into *E. coli*. Northern (RNA) and Southern blotting analyses were performed as described previously (7, 10).

Overexpression. Segments of DNA containing the *caiF* gene were cloned downstream of the bacteriophage T7 ϕ10 promoter into expression vectors pT7-5 and pT7-6 used in the T7 RNA polymerase-promoter system (Fig. 1).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Source or reference
E. coli		
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301*	4
K38	HfrC *phoA4 pit-10 tonA22 ompF627 relA1 spoT1* λ$^+$	21
NM522	*supE thi* Δ(*lac-proAB*) Δ(*hsdMS-mcrB*)*5* (F′ *proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)	12
PHL502	MC4100 *hns*::Tn*10*	P. Lejeune
PHL503	MC4100 *rpoS*::*kan*	P. Lejeune
PHL504	MC4100 *hns*::Tn*10 rpoS*::*kan*	P. Lejeune
KE1005	PHL502 Δ*crp-45 cysG98*::Tn*5*	7
KE1356	MC4100 *rpoN*::Tn*10*	This study
KE4711	MC4100 Δ*crp-45 cysG98*::Tn*5*	7
MC4100NI1	MC4100 *fnr* (*nirR22*) *zcj-261*::Tn*10*	37
MAM100	NM522 *hns*::Tn*10*	9
MAM101	NM522 Δ*crp-45 cysG98*::Tn*5*	9
MAM102	NM522 *rpoN*::Tn*10*	9
MAM103	NM522 *fnr-22 zcj-261*::Tn*10*	9
MAM105	NM522 *narL215*::Tn*10*	This study
MAM106	NM522 *rpoS*::*kan*	This study
MAM107	NM522 *rpoS*$^+$	This study
Plasmids		
pUC18	Ap^r *lacIPOZ′*	38
pUC19	Ap^r *lacIPOZ′*	38
pSU9	Cm^r *lacIPOZ′*	1
pGP1-2	Km^r T7 gene 1 (RNA polymerase)	33
pT7-5	Ap^r T7 ϕ10	33
pT7-6	Ap^r T7 ϕ10	33
pJEL250	Ap^r *trpA′ lacZYA* λp_R λ*cI857*	36
pAB20	pJEL250 *caiT′-trpA′lacZYA*	This study
pAB30	pJEL250 *fixA′-trpA′lacZYA*	9
pRL40	pJEL250 *caiF′-trpA′ lacZYA*	This study
pRL101	pSU9 *caiF*$^+$	This study
pAB101	pRL101 *caiF*::Tn*903* Km^r	This study

Recombinant plasmids were transformed into strain K38 which harbors the compatible plasmid pGP1-2, which contains the gene coding for the T7 RNA polymerase under the control of the heat-inducible λp_L promoter. The proteins specifically expressed from the cloned genes were labelled with [^{35}S]methionine-cysteine (1,000 Ci/mmol; NEN DuPont) by the protocol of Tabor and Richardson (33), after heat induction of T7 RNA polymerase at 42°C and addition of rifampin to inhibit selectively *E. coli* RNA polymerase transcription. Cells were then lysed in a mixture of 60 mM Tris-HCl (pH 6.8), 1% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol by heating for 5 min at 95°C and finally loaded onto an SDS–17.5% polyacrylamide gel as described by Laemmli (20).

Primer extension analysis of transcript. Total RNA was isolated from cells grown in rich medium under inducing and noninducing conditions, as described previously. Primer extension reactions were performed with 1 μg of total RNA and 0.5 pmol of ^{32}P-end-labelled primer using the avian myeloblastosis virus reverse transcriptase primer extension system kit from Promega. The synthetic oligonucleotide complementary to the region from bp 674 to 655 of the noncoding strand (Fig. 2), 5′-CATCCATTCGGCGATTAACA-3′, was used for the determination of the transcriptional start site of *caiF* in strain NM522 carrying plasmid pKE101. Products of the primer extension reactions were separated on 8% polyacrylamide–urea sequencing gels in parallel with sequence reactions with pKE101 performed using the same labelled oligonucleotide.

Enzyme assays. Carnitine dehydratase was assayed on whole cells by following the method described by Jung et al. (15). One unit of enzyme activity was defined as the amount of enzyme which catalyzes the formation of 1 μmol of L-(−)-carnitine or crotonobetaine per minute. β-Galactosidase activity was measured with whole cells grown to the end of the exponential growth phase according to the method of Miller (23). Values are averages from at least three independent experiments performed in duplicate.

DNA sequencing. Fragments of the *caiF* gene from plasmid pKE101 (Fig. 1) were subcloned into vectors pUC18 and pUC19. With universal and reverse oligonucleotide primers, plasmid DNA was sequenced with the T7 sequencing kit purchased from Pharmacia according to the chain termination procedure (28). The complete sequences of both strands were obtained.

Computer analysis of DNA and amino acid sequences was performed with

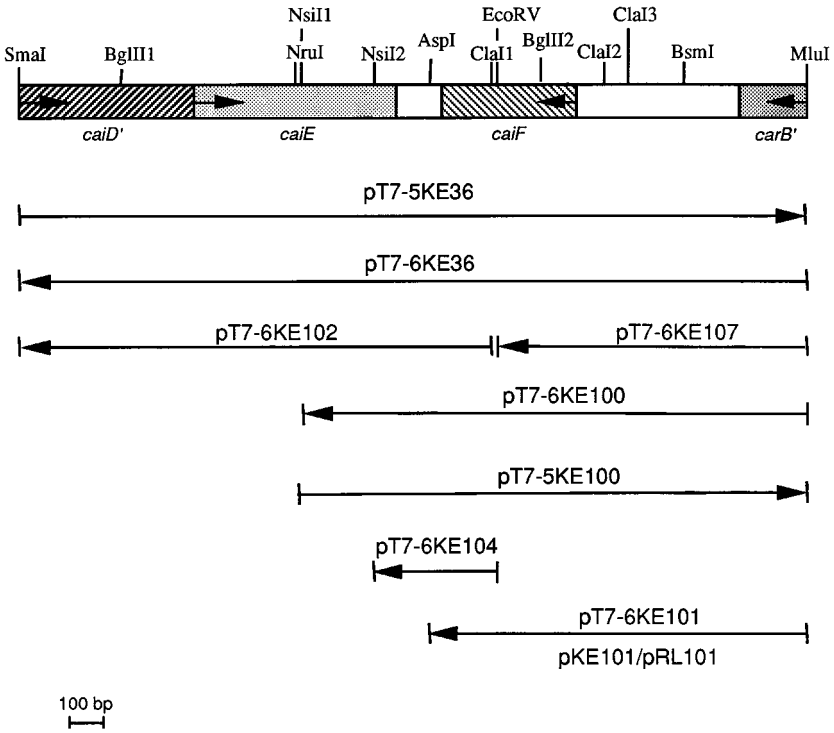

FIG. 1. Restriction map of the 3′ region of the *cai* operon from *E. coli* O44K74. Arrows in boxes indicate the direction of transcription of the genes. The extent and orientation of the DNA inserts cloned in pT7 expression vectors are represented by arrows. The designation of the resulting pT7 derivative plasmids is indicated above each DNA fragment. Plasmids pKE101 and pRL101 are derivatives of pUC19 and pSU9, respectively. *carB′* designates the end of the gene encoding one subunit of the carbamoylphosphate synthase.

Mac Molly Tetra from Soft Gene GmbH. The Swiss-Prot and NBRF protein databases were searched with the algorithm FASTA of Pearson and Lipman (24).

Nucleotide sequence accession number. The sequence of the *E. coli caiF* gene has been assigned EMBL accession number X80644.

RESULTS

Nucleotide sequence of a DNA region stimulating carnitine dehydratase activity. During the course of our cloning studies relevant to the *cai* operon (7), we noticed that strains harboring plasmids containing the 3′ region downstream of the *cai* operon could express enhanced levels of carnitine dehydratase activity, which is encoded by the *caiB* gene.

To further investigate the 3′ region of *cai* which could be involved in the regulatory pattern of carnitine metabolism, the 1-kb *Asp*I-*Mlu*I fragment (Fig. 1) was cloned in plasmid pUC19, giving rise to plasmid pKE101, and the nucleotide sequence was determined (Fig. 2). It contains one open reading frame of 393 bp which is separated from the *cai* operon by an intergenic region of 124 bp and is transcribed in the opposite direction. A putative Shine-Dalgarno sequence (GGAG) was found 6 bp upstream of the presumed ATG initiation codon for this open reading frame, which was named *caiF*. No terminator sequence was detected downstream of *caiF*. This gene encodes a putative polypeptide of 131 amino acids having a calculated molecular mass of 15,438 Da. To obtain further information about the function of CaiF, a protein search in the NBRF and Swiss-Prot databases was carried out with the algorithm of Pearson and Lipman (24), but no outstanding homology with sequences listed in the databases was detected.

Southern hybridization of the entire chromosomal DNA was carried out to determine the number of copies of the *caiF* gene on the chromosome of *E. coli* O44K74. Chromosomal DNA digested with *Mlu*I, *Eco*RV, and *Bam*HI was tested with probes directed against *caiF* (data not shown). One band, corresponding to the expected size already known from the nucleotide sequence (Fig. 1), was present for each sample. These results indicate that *caiF* is present as a single copy on the chromosome.

Overproduction of the CaiF protein. In order to confirm that the sequenced ORF directs the synthesis of a polypeptide, chromosomal fragments which contain the 3′ part of the *cai* operon and the adjacent *caiF* gene were subcloned into expression vectors pT7-5 and pT7-6, which contain the thermoinducible phage T7 ϕ10 promoter (33) (Fig. 1). Recombinant plasmids with both orientations of the insert with respect to the promoter were obtained. Plasmids pT7-6KE36, pT7-6KE100, and pT7-6KE101 (Fig. 3, lanes 1, 3, and 5) directed the synthesis of a protein with an apparent molecular mass of 16,000 Da, which was in good agreement with the protein size deduced from the nucleotide sequence of *caiF*. As expected, expression of this protein was not observed with plasmid pT7-5KE36, carrying the whole *Sma*I-*Mlu*I insert in the opposite orientation, and with plasmid pT7-6KE107, which harbors the truncated *caiF* gene (Fig. 3, lanes 2 and 4). A faint band of 6,500 Da which probably represents a nonspecific incorporation product was present in all lanes.

Effect of in vivo overproduction of CaiF on carnitine dehydratase activity. The initial observation that the presence of the region downstream to the *cai* operon led to the augmentation of carnitine dehydratase activity was further substantiated. To this end, the effect in *trans* of derivatives of plasmids pT7-5 and pT7-6, carrying the 3′ region adjacent to *cai* (Fig. 1), was measured in strain K38(pGP1-2), which overproduces the

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                                                                                                                    FNR
ACGCGTGGTG  GACCTGGCGG  CAAAACTGCT  GAAACAGGGC  TTCGAGCTGG  ATGCGACCCA  CGGCACGGCG  ATTGTGCTGG  GCGAAGCGGG  TATCAATCCG    100

                                                                                  NarL          FNR
CGTCTGGTAA ACAAGGTGCA TGAAGGCCGT  CCGCACATTC  AGGACCGTAT  CAAGAATGGC  GGAATATACC TACATCATCA ACACCACCTC  AGGCCGTCGT    200

                                                                                                    NarL
GCGATTGAAG  ACTCCCGCGT  GATCCGTCGC  AGTGCGCTGC  AATATAAAGT  GCATTATGAC  ACCACCCTGA  ACGGTGGTTT  CGCTACCGCG  ATGGCGCTGA    300

                                                    FNR                                                      FNR
ATGCCGATGC  GACTGAAAAA  GTAATTTCGG TGCAGGAAAT GCACGCACAG ATCAAATAATAGCGTGTCAT  GGCGGATATT  TTTCATCCGC  TAATTTGATC    400

                                                                                                FNR                 CRP
GAATAACTAA  TACGGTTCTC  TGATGAGGAC  CGTTTTTTTT  TGCCCATTAA  GTAAATCTTT  TGGGGAATCG  ATATTTTTGA TGACATAAGC  AGGATTTAGC    500

                        -10                  I→ +1
TCACACTTAT CGACGGTGAA  GTTGCATACT ATCGATATAT  CCACAATTTT  AATATGGCCT  TGTTTAATTG  CTTCAAAACG  AGTCATAGCC  AGACTTTTAA    600

    CRP       SD          M  C  E  G    Y  V  E    K  P  L    Y  L  L  I    A  E  W    M  M  A    E  N  R  W    V  I  A
TTTGTGAAAC TGGAGTTCGT  ATGTGTGAAG  GATATGTTGA  AAAACCACTC  TACTTGTTAA  TCGCCGAATG  GATGATGGCT  GAAAATCGGT  GGGTGATAGC    700

 R  E  I    S  I  H  F    D  I  E    H  S  K    A  V  N  T    L  T  Y    I  L  S    E  V  T  E    I  S  C    E  V  K
AAGAGAGATC  TCTATTCATT  TCGATATTGA  ACACAGCAAG  GCGGTTAATA  CCCTGACTTA  TATTCTGTCG  GAAGTCACAG  AAATAAGCTG  CGAAGTTAAG    800

M  I  P  N    K  L  E    G  R  G    C  Q  C  Q    R  L  V    K  V  V    D  I  D  E    Q  I  Y    A  R  L    R  N  N  S
ATGATCCCTA  ATAAGCTGGA  AGGGCGGGGA  TGCCAGTGTC  AGCGACTGGT  TAAAGTGGTC  GATATCGATG  AGCAAATTTA  CGCGCGCCTG  CGCAATAACA    900

  R  E  K    L  V  G    V  R  K  T    P  R  I    P  A  V    P  L  T  E    L  N  R    E  Q  K    W  Q  M  M    L  S  K
GTCGGGAAAA  ATTAGTCGGC  GTAAGAAAGA  CACCGCGTAT  TCCTGCCGTT  CCGCTCACGG  AACTTAACCG  CGAGCAGAAG  TGGCAGATGA  TGTTGTCAAA   1000

 S  M  R    R STOP
GAGTATGCGT  CGTTAATGTC  ATTTCGATG   ATACCGTGCG  ACCTGGTCG                                                            1050
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FIG. 2. Nucleotide and deduced amino acid sequences of the *caiF* gene. The transcriptional start site of *caiF* is indicated by an arrow designated +1. The putative ribosome binding site (SD) and the Pribnow box upstream from *caiF* are underlined. Boldface letters indicate putative CRP and FNR half consensus sequences in addition to presumptive recognition sequences of the σ^{54} factor of RNA polymerase. Hypothetical NarL binding sites are also underlined. This sequence is available from the EMBL database under the accession number X80644.

T7 RNA polymerase after heat induction at 42°C (33). Growth was achieved at 30°C in a fermentor to monitor the level of carnitine dehydratase specific activity along the bacterial growth curve (data not shown). Maximal level was reached at the end of the exponential growth phase and was maintained in the early stationary phase. A temperature shift from 30 to 42°C, as recommended by the protocol of Tabor and Richardson (33), did not further increase the level of carnitine dehydratase activity, which implies that no expression from the T7 promoter was required to enhance carnitine dehydratase activity from this 3′ region. In addition, even when cloned in the opposite direction of transcription with respect to the ϕ10 promoter in plasmid pT7-5, the *caiF* gene significantly stimulated carnitine dehydratase activity, indicating that it was likely to be expressed from its own promoter-operator region (Table 2). Results clearly show that the 3′ region adjacent to the *cai* operon can stimulate levels of carnitine dehydratase activity synthesized from the chromosomal *caiB* gene in *trans*. An increase from 8 to 17 times over that found in strain K38 carrying the reference plasmid pT7-6 or pT7-5 was found, provided that the *caiF* gene was present (Table 2). Progressive deletions of the *caiE* gene and of the DNA sequence between *caiE* and *caiF* resulted in progressively reduced activities. As observed, *caiF* alone was able to significantly enhance carnitine dehydratase activity, which suggests that the cellular amount of CaiF is limiting in that strain.

Analysis of the *caiF* promoter region. A Northern hybrid-

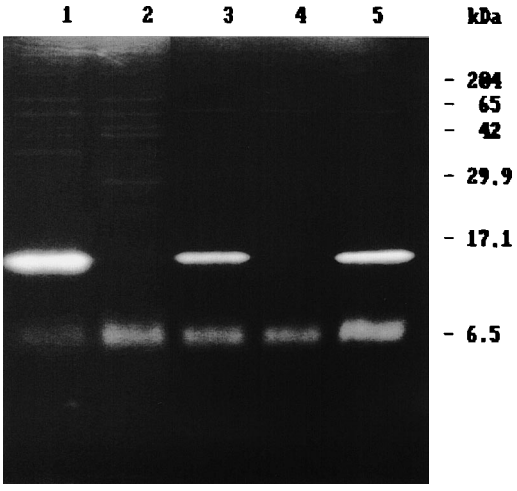

FIG. 3. Overexpression of the *caiF* gene with the T7 RNA polymerase-promoter system (33). The ^{35}S-labelled polypeptides were separated on a 17.5% polyacrylamide gel containing 0.1% SDS and detected by autoradiography. The radioactivity of each sample loaded onto the gel was approximately 10^6 cpm. Shown here are proteins expressed by pT7-6KE36 (lane 1), pT7-5KE36 (lane 2), pT7-6KE100 (lane 3), pT7-6KE107 (lane 4), and pT7-6KE101 (lane 5). Positions of molecular mass markers are shown in kilodaltons.

TABLE 2. Enhancement of carnitine dehydratase activity by the 3′ region downstream of *cai* supplied in *trans*

Plasmid[a]	Gene(s) carried by plasmid	Carnitine dehydratase activity (fold increase)[b]
pT7-5KE36	*caiE*, *caiF*	1.00 ± 0.04 (11)
pT7-6KE36	*caiE*, *caiF*	1.54 ± 0.04 (17)
pT7-5KE100	*caiF*	0.90 ± 0.04 (10)
pT7-6KE100	*caiF*	1.18 ± 0.04 (13)
pT7-6KE101	*caiF*	0.69 ± 0.03 (8)
pT7-6KE102	*caiE*	0.09 ± 0.02 (1)
pT7-6KE104	Truncated *caiF*	0.09 ± 0.02 (1)
pT7-6KE107	Truncated *caiF*	0.09 ± 0.02 (1)
pT7-5	—[c]	0.09 ± 0.02
pT7-6	—	0.09 ± 0.02

[a] Cells of K38(pGP1-2) carrying various pT7-6 and pT7-5 derivatives were grown anaerobically at 30°C in LB supplemented with 20 mM DL-carnitine.

[b] Enzymatic activities assayed on whole cells are expressed as units per milligram of bacteria (dry weight) ± standard deviation. The fold increase in activity (shown in parentheses) is as compared with activity with plasmid pT7-6 or pT7-5.

[c] —, no recombinant gene.

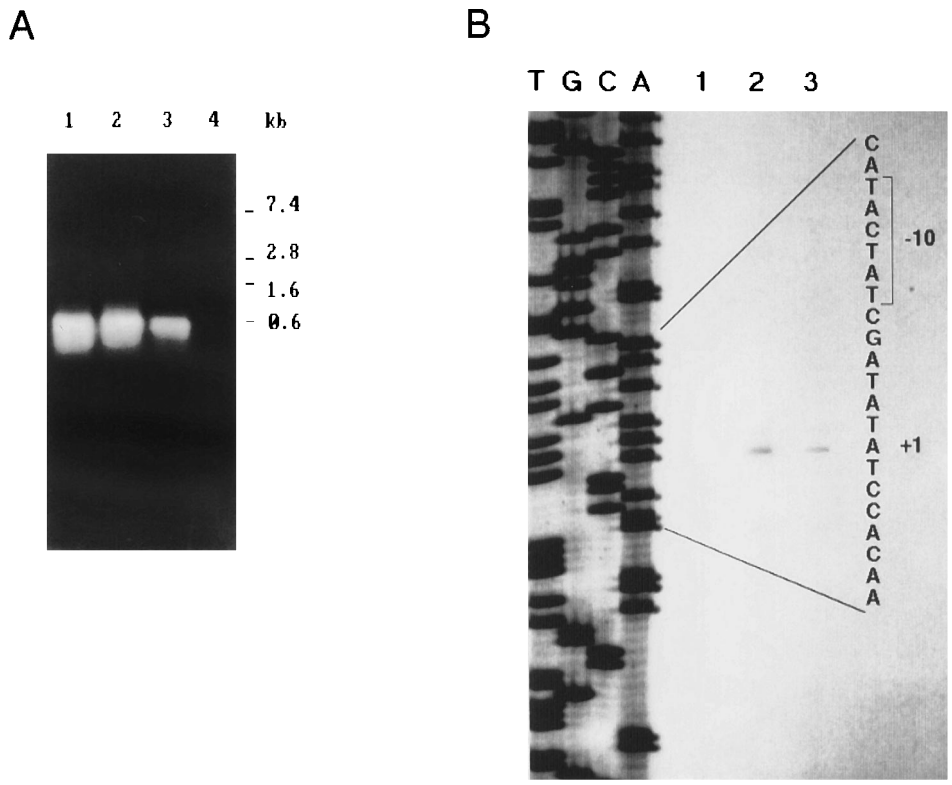

FIG. 4. Northern analysis and determination of the *caiF* transcription start site. (A) Luminograph of a Northern blot of RNA extracted from *E. coli* NM522 harboring pKE101 (Fig. 1) grown anaerobically in the presence (lane 1) or absence (lane 2) of 20 mM DL-carnitine as well as under semiaerobic (lane 3) and strictly aerobic (lane 4) conditions. The mRNAs were probed with an internal fragment derived from *caiF* labelled with digoxigenin-dUTP and detected by the DIG Luminescent Detection Kit (Boehringer Mannheim). (B) Transcription start point of *caiF*. The first four lanes give a DNA sequencing reaction of plasmid pKE101. Lane 1 corresponds to the control experiment in the absence of RNA. In lanes 2 and 3 transcripts from NM522/pKE101 were separated: lane 2, anaerobic growth in the presence of 20 mM DL-carnitine; lane 3, anaerobic growth in the absence of DL-carnitine. The −10 region of the *caiF* promoter and the *caiF* transcription initiation site are indicated on the right.

ization was first performed in order to determine whether the *caiF* gene is transcribed as a monocistronic mRNA and to determine the effects of the oxygen status on transcription. A band of about 0.6 kb was detected in the presence and absence of 20 mM DL-carnitine under anaerobic growth conditions with an RNA probe derived from the *caiF* gene (Fig. 4A, lanes 1 and 2). In contrast, a less strong hybridization signal was observed under semiaerobic growth conditions (Fig. 4A, lane 3), and no specific mRNA was found from a strongly aerated culture harvested at mid-exponential growth phase (Fig. 4A, lane 4). Although analysis of the *caiF* transcript was performed with a plasmid-encoded copy of *caiF* and therefore outside its chromosomal context, the results suggest that the *caiF* gene is expressed as a single transcriptional unit subjected to the control of oxygen and insensitive to the effect of carnitine. These observations are consistent with the pattern of expression of the *caiF-lacZ* fusion reported below.

In addition, primer extension was used to locate the 5′ end of the *caiF* gene in cultures grown anaerobically with or without DL-carnitine. In both cases, the transcription is initiated at position A-539 (Fig. 4B, lanes 2 and 3). This allowed the tentative identification of the −10 (TACTAT) region and of a CRP-binding sequence (GA-6 bp-TCACA) (6) centered at position −41.5 (Fig. 2). Another putative half consensus sequence for CRP was detected at 4 bp from the Shine-Dalgarno sequence of *caiF*.

Expression of a *caiF-lacZ* operon fusion. To gain further insight into the conditions of expression of the *caiF* gene, we constructed a *caiF-lacZ* operon fusion. This was achieved by cloning the 0.86-kb *Eco*RV-*Mlu*I fragment (Fig. 1) in front of the promoterless *lacZ* gene of plasmid pJEL250 (36), yielding the low-copy-number plasmid pRL40. This plasmid was then transferred into the wild-type strain NM522 and a series of derivatives carrying mutations in known regulatory genes. Expression of the *caiF-lacZ* fusion was repressed 20-fold in the presence of oxygen, and it was not induced by DL-carnitine even under anaerobic conditions (Table 3). This observation perfectly corroborates the pattern of transcription deduced from the Northern blot data (Fig. 4). Addition of nitrate or glucose resulted in the significant reduction of the levels of β-galactosidase activity. Lesion in the *hns* gene led to a slight enhancement of *caiF-lacZ* expression. Introduction of an *rpoS*::*kan* mutated allele did not affect β-galactosidase activity, in accordance with the fact that strain NM522 itself behaves as an *rpoS* mutant. However, an *rpoS*$^+$ derivative exhibited reliable slightly reduced *caiF-lacZ* expression. In contrast, *rpoN*, *crp*, and *fnr* mutations decreased expression of the *caiF-lacZ* fusion at various levels, the most dramatic effect being displayed by *fnr*. Furthermore, in a *narL* mutant that failed to produce protein NarL, one of the regulator components belonging to the two sensor-regulator systems responding to nitrate (31), the *caiF-lacZ* fusion was expressed at a reliable higher level in the presence of nitrate than in the parental strain (50 versus 25%). Moreover, when the *caiF-lacZ* fusion was introduced into strain MC4100, which is thought to be a *caiF* mutant (see below), comparable results were obtained,

TABLE 3. Expression of the *caiF-lacZ* fusion in various genetic and environmental conditions

Strain[a]	Relevant genotype	Addition		β-Galactosidase activity (Miller units)
		Oxygen	Effector	
NM522	Wild type	+		60 ± 10 (500 ± 50)[b]
		+	DL-Carnitine	50 ± 10
		−		1,200 ± 200 (3,500 ± 500)
		−	DL-Carnitine	1,250 ± 200 (3,600 ± 600)
		−	Glucose	800 ± 100 (2,100 ± 300)
		−	Nitrate	300 ± 50 (1,600 ± 200)
MAM100	*hns*::Tn*10*	−		1,600 ± 150
MAM101	Δ*crp-45*	−		450 ± 50
MAM102	*rpoN*::Tn*10*	−		800 ± 100
MAM103	*fnr-22*	−		200 ± 20
MAM105	*narL215*	−		1,000 ± 100
		−	Nitrate	500 ± 50
MAM106	*rpoS*::*kan*	−		1,200 ± 100
MAM107	*rpoS*$^+$	−		950 ± 100

[a] Cells transformed with the monocopy plasmid pRL40 carrying the *caiF-lacZ* operon fusion were grown at 30°C either aerobically or anaerobically in TYEP medium supplemented as indicated. DL-Carnitine was added at 20 mM, nitrate was added at 40 mM, and glucose was added at 20 mM.

[b] Values shown in parentheses correspond to the expression of the multicopy *caiF-lacZ* operon fusion cloned into plasmid pSU9, resulting in plasmid pKE102.

with an overall decrease of β-galactosidase activity of 30%, which may reflect the fact that strain MC4100 carries the *rpoS*$^+$ allele. As a consequence, the *caiF* gene does not seem to autoregulate its own synthesis.

Assays of the multicopy *caiF-lacZ* fusion cloned into plasmid pSU9 yielded a threefold stimulation of β-galactosidase activity under anaerobic conditions in the presence or absence of inducer. However, repression in the presence of oxygen or nitrate was significantly reduced with respect to the monocopy fusion, which suggests that an increase in the copy number of *caiF* renders transcription of the gene less dependent on the anaerobic regulators FNR and NarL.

Synthesis of carnitine dehydratase activity in various mutants harboring a multicopy *caiF* gene. In the course of our investigation we noticed that levels of carnitine dehydratase activities displayed by laboratory strains varied in a wide range. For example, strain MC4100 exhibited a far lower carnitine dehydratase activity (Table 4) than the reference strain O44K74 from which the *cai* operon was cloned (0.15 U mg of bacteria [dry weight]$^{-1}$). In contrast, strain NM522 displayed a fairly high level of carnitine dehydratase activity (0.3 U mg of bacteria [dry weight]$^{-1}$). Since the low level of carnitine dehydratase activity detected in strain MC4100 could be enhanced in *hns* and *rpoS* backgrounds (Table 4) (7), this strain is likely to contain a fully functional *caiB* gene. Thus, we suspected that strain MC4100 is impaired in the regulation of carnitine metabolism. In agreement with this hypothesis, carnitine dehydratase activity of MC4100 was strongly stimulated by introducing plasmid pRL101 (Fig. 1), which carries multicopies of the *caiF* gene (Table 4). The effect of this plasmid was further examined after transformation into a set of mutants altered in regulatory functions. Introduction of plasmid pRL101 in the *hns*, *rpoS*, and *rpoN* mutants was associated with a significant stimulation of carnitine dehydratase activity. In contrast, the total absence of enzymatic activity found in the *crp* and *fnr* mutants was not relieved by multiple copies of *caiF*. It is, however, worth noticing that the presence of the multicopy *caiF* plasmid precluded additional enhancement of carnitine dehydratase activity in the *hns* or *rpoS* mutants with respect to the parental strain MC4100 which carries pRL101. This was in striking contrast to the situation encountered in the absence of plasmid.

Induction of transcription of the *cai* and *fix* operons by *caiF*. In order to distinguish between the influence of *caiF* on transcription and on enzyme activity and to confirm the initial observation that the *cai* and *fix* operons are subject to the same regulatory control (9), we analyzed the effect of plasmid pRL101 on the behavior of *lacZ* operon fusions to *cai* and *fix* borne by the monocopy plasmids pAB20 and pAB30, respectively. Levels of expression of the *caiT-lacZ* and *fixA-lacZ* fusions were compared in both strains NM522 and MC4100. Absolutely no expression of these fusions could be detected in MC4100 in the presence of DL-carnitine, in contrast to NM522, which exhibited fairly high levels of β-galactosidase activity (Table 5). This finding supported the notion that regulation of carnitine metabolism is deficient in strain MC4100, as suggested above. Introduction of plasmid pRL101 carrying the *caiF* gene led to a 15-fold enhancement of the *caiT-lacZ* and *fixA-lacZ* expression in strain NM522 over the expression found in the presence of the control vector pSU9. This stimulation largely exceeds what would be expected from the threefold enhancement of expression of the multicopy *caiF-lacZ* fusion in plasmid pKE102 (Table 3). It might result from differences in the sizes of the two plasmids used (3.5 kb for pRL101 versus 6.7 kb for pKE102) and in the orientation of the cloned *caiF* genes (in the same orientation as *cat* in pRL101, which could allow a readthrough, and in the orientation opposite that of *cat* in pKE102); both observations support a better expression of the *caiF* gene harbored by pRL101. It should be also mentioned that the significantly reduced level of expression of the fusions in strain NM522 carrying pSU9 in

TABLE 4. Effect of multicopy plasmid-borne *caiF* on the carnitine dehydratase activity in different regulatory mutants

Strain[a]	Relevant genotype	Carnitine dehydratase activity (fold increase)[b]	
		Without pRL101	With pRL101 (*caiF*)
MC4100	Wild-type	0.01 ± 0.005	0.66 ± 0.05
PHL502	*hns*::Tn*10*	0.22 ± 0.04 (22)	0.80 ± 0.04 (1.2)
PHL503	*rpoS*::*kan*	0.12 ± 0.03 (12)	1.00 ± 0.04 (1.5)
PHL504	*hns*::Tn*10 rpoS*::*kan*	0.50 ± 0.05 (50)	0.80 ± 0.05 (1.2)
KE4711	Δ*crp-45*	<0.005	<0.005
KE1005	Δ*crp-45 hns*::Tn*10*	<0.005	<0.005
KE1356	*rpoN*::Tn*10*	0.01 ± 0.005 (1)	0.50 ± 0.06 (0.75)
MC4100NI1	*fnr-22*	<0.005	<0.005

[a] Cells were grown anaerobically at 37°C in LB supplemented with 20 mM DL-carnitine.

[b] Enzymatic activities assayed on whole cells are expressed as unit per milligram of bacteria (dry weight) ± standard deviation. The fold increase in activity (shown in parentheses) is as compared with that of the wild-type strain MC4100 without or with the *caiF* plasmid.

TABLE 5. Effect of multicopy plasmid-borne *caiF* on the expression of the *caiT-lacZ* and *fixA-lacZ* fusions

Strain[a]	Plasmid	β-Galactosidase activity (Miller units) with:		
		pJEL250	pAB20 (*caiT-lacZ*)	pAB30 (*fixA-lacZ*)
NM522	None	30 ± 5	5,000 ± 1,000	5,500 ± 1,000
	pSU9	30 ± 5	900 ± 200	700 ± 150
	pRL101 (*caiF*$^+$)	35 ± 10	14,000 ± 1,500	16,000 ± 1,500
	pAB101 (*caiF*::*kan*)	35 ± 5	1,800 ± 200	1,700 ± 200
MC4100	None	20 ± 5	25 ± 5	10 ± 5
	pSU9	50 ± 10	—[b]	150 ± 50
	pRL101 (*caiF*$^+$)	45 ± 10	14,000 ± 1,500	17,000 ± 1,500
	pAB101 (*caiF*::*kan*)	50 ± 10	—	140 ± 40

[a] Cells were grown at 30°C in TYEP medium under anaerobic conditions in the presence of 20 mM DL-carnitine. Both strains NM522 and MC4100 harboring the monocopy fusion plasmids exhibited low levels of β-galactosidase activity (30 Miller units) in the absence of DL-carnitine (see Table 6).
[b] —, not determined.

comparison with that in NM522 without plasmid may be accounted for by the derivation of cell metabolism to the benefit of plasmid replication. Moreover, a striking maximal expression of *caiT-lacZ* and *fixA-lacZ* fusions was restored in strain MC4100 when transformed by plasmid pRL101 (Table 5). This suggests that the defect of MC4100 is complemented by the *caiF* gene. To demonstrate that the *caiF* gene was actually responsible for complementation of the lesion in MC4100, *caiF* was inactivated by insertion of a kanamycin resistance cassette into the unique *Bgl*II site of plasmid pRL101, resulting in plasmid pAB101. MC4100(pAB101) expressed a low level of the *fixA-lacZ* fusion similar to that detected in MC4100(pSU9), indicating that MC4100 is likely to be mutated for *caiF*. As expected, NM522(pAB101) displayed expression of the *caiT-lacZ* and *fixA-lacZ* fusions of the same order of magnitude as that of NM522(pSU9). As a whole, these findings strongly suggest that the *caiF* gene codes for an activator protein required for the transcription of the structural genes of carnitine metabolism.

It has been previously shown that γ-butyrobetaine, the final product of the carnitine pathway, represses carnitine dehydratase activity when cells are grown anaerobically in the presence of L-(−)-carnitine (19). In perfect correlation with these observations, increasing amounts of γ-butyrobetaine, in the presence of either L-(−)-carnitine or crotonobetaine as an inducer, were found to decrease the levels of *cai* and *fix* expression significantly (Table 6). Interestingly, repression by γ-butyrobetaine occurred at a far lower concentration when crotonobetaine instead of L-(−)-carnitine was used as the inducer.

DISCUSSION

The results presented in this paper show that the *caiF* gene located downstream of the *cai* operon at the first minute of the *E. coli* chromosome (7) and transcribed in the opposite direction to *cai* (Fig. 3) could play the role of an activator protein mediating the induction of carnitine metabolism. The arguments in favor of a regulatory role for CaiF are that the cloned gene complements the regulatory defect of the expression of both the *cai* and *fix* operons in the laboratory strain MC4100 in *trans* and that it restores their normal regulatory pattern under anaerobic conditions. Furthermore, introduction of a multicopy *caiF* gene significantly augments the expression of the *cai* and *fix* genes governed by the single chromosomal *caiF* copy in strain NM522 (Table 5), resulting in an elevation of carnitine dehydratase activity (Tables 2 and 4). Mutation of *caiF* by insertion of a kanamycin resistance cassette showed unambiguously that this *caiF* gene was responsible for promoting transcription of the *cai* and *fix* operons in response to carnitine under anaerobic conditions. It thus appears likely that CaiF exerts a transcriptional effect on the expression of the *cai* and *fix* operons rather than displaying a protein effect stimulating carnitine dehydratase activity.

However, a thorough examination of the deduced amino acid sequence of the 15-kDa protein CaiF did not yield any significant homology with known proteins in data libraries. Moreover, we found no evidence for the existence of a typical helix-turn-helix motif which could be involved in binding to the intergenic promoter-operator region of *cai* and *fix* operons. In addition, mobility shift experiments failed to show any specific

TABLE 6. Effect of trimethylammonium compounds on expression of the *caiT-lacZ* and *fixA-lacZ* fusions carried by strain NM522

Addition(s)[a]	β-Galactosidase activity (Miller units) with:		
	pJEL250	pAB20 (*caiT-lacZ*)	pAB30 (*fixA-lacZ*)
None	30 ± 5	20 ± 5	30 ± 5
L-(−)-Carnitine	30 ± 5	5,000 ± 1,000	5,500 ± 1,000
Crotonobetaine	30 ± 5	4,500 ± 1,000	5,000 ± 1,000
γ-Butyrobetaine	30 ± 5	10 ± 5	15 ± 5
L-(−)-Carnitine, γ-butyrobetaine	—[b]	4,600 ± 1,000	4,500 ± 1,000
L-(−)-Carnitine, 50 mM γ-butyrobetaine	—	1,000 ± 150	1,000 ± 150
Crotonobetaine, 20 mM γ-butyrobetaine	—	900 ± 150	800 ± 150
Crotonobetaine, γ-butyrobetaine	—	500 ± 100	400 ± 100

[a] Cells were grown anaerobically at 30°C in TYEP medium supplemented as indicated. Supplements were all added at 10 mM unless otherwise indicated.
[b] —, not determined.

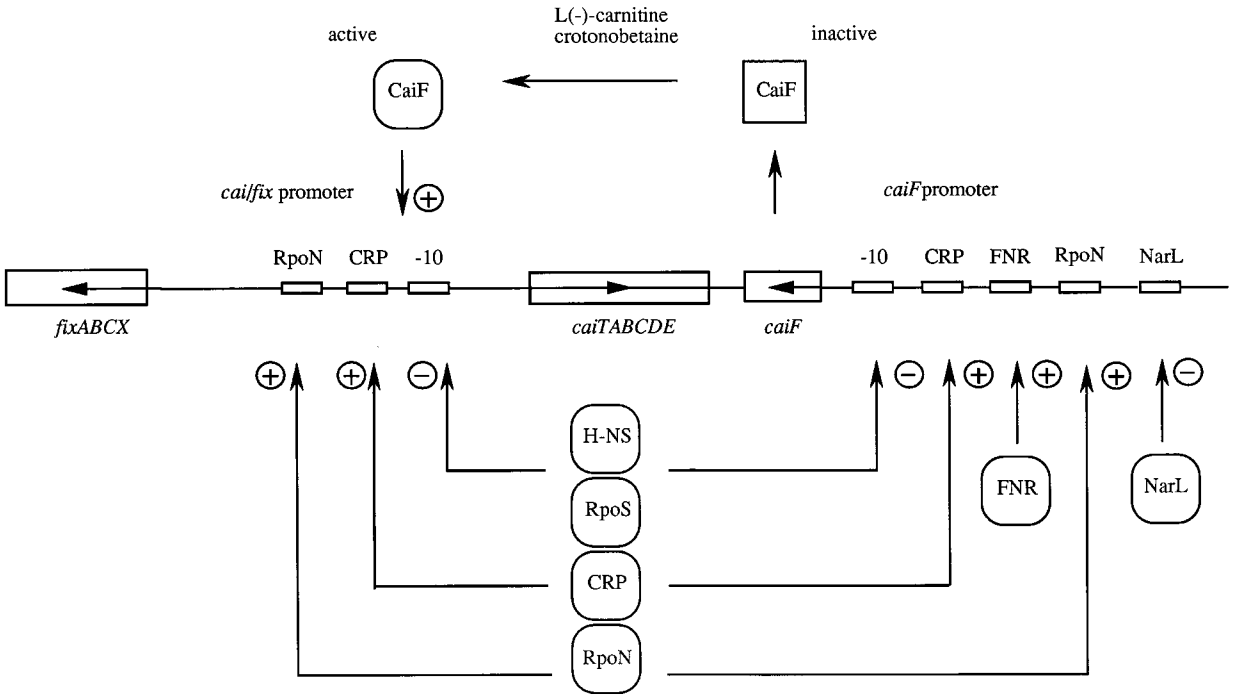

FIG. 5. Model for the regulation of carnitine metabolism in *E. coli*. The *cai* and *fix* operons and the *caiF* gene are diagrammed schematically (not to scale). Arrows indicate their direction of transcription. +, positive regulation (activation); −, negative regulation (repression). Small open boxes in the promoter regions represent the putative consensus sequences for the binding of regulatory proteins as deduced from the nucleotide sequences and the expression pattern of the fusions (9) (Fig. 2 and 3).

formation of a DNA-CaiF complex between the *cai-fix* promoter region and either cell extracts from cultures carrying the plasmid-borne *caiF* gene or a partially purified MalE-CaiF fusion protein (3). Consequently, this could argue in favor of the possibility that CaiF modulates transcription of the *cai* and *fix* operons through an indirect mechanism. It could, for instance, activate in cascade the function of a yet unknown regulatory protein which will in turn control expression of the structural components of carnitine metabolism. It could alternatively enhance transcription of genes after association with a regulatory component which will directly bind to the promoter region.

Interestingly, the high level of carnitine dehydratase activity conferred by plasmid pT7-6KE36 was shown to be progressively reduced by successively removing the *caiE* gene and the DNA sequence between *caiE* and *caiF* (Table 2). Indeed, stimulation of carnitine dehydratase activity in plasmid pT7-6KE36 could be attributed to three overlapping effects: first, the induction of transcription of the *cai* operon, including the *caiB* gene for carnitine dehydratase, under the control of the *caiF* gene; second, synthesis of the CaiE protein, which has been postulated to be involved in the synthesis or activation of the cofactor required for carnitine dehydratase activity and which has been demonstrated to contribute to the enhancement of carnitine dehydratase activity (7); and third, the possible presence of a structure in the intergenic region between the *cai* operon and *caiF* which may act in regulation by stabilizing the overall region. Suppression of this region decreased carnitine dehydratase activity by 40% (pT7-6KE101 versus pT7-6KE100 in Table 2). A computer search revealed the hypothetical existence of a highly stabilized RNA, but its possible implication is speculative since we did not find evidence that it is actually transcribed.

Northern blot data (Fig. 4A) were confirmed by analysis of expression of a monocopy *caiF-lacZ* fusion (Table 3) which showed that the *caiF* gene is transcribed under anaerobic conditions, disregarding the presence of DL-carnitine in the growth medium. This was in striking contrast to the *cai* and *fix* operons whose expression was strongly dependent on the availability of carnitine in the growth medium (7, 9) (Table 6). The dependence of the transcription of *caiT-lacZ* and *fixA-lacZ* operon fusions by plasmid pRL101 (*caiF*$^+$) on the presence of carnitine (Table 5) indicates that carnitine or a metabolite of it is the biochemical effector for modulation of the CaiF protein. Crotonobetaine was also able to induce transcription to the same extent (Table 6), and since it could be converted into L-(−)-carnitine via the reverse reaction catalyzed by carnitine dehydratase (15), we could not rule out that the inducing effect of crotonobetaine may be indirect. In contrast to this, γ-butyrobetaine, the final product of the carnitine pathway, appeared as a potent inhibitor of transcription of the *cai* and *fix* operons, whose impact is in relation to the quality of the potential inducer used, L-(−)-carnitine or crotonobetaine (Table 6). Clearly, L-(−)-carnitine was a far more efficient inducer than crotonobetaine in the presence of γ-butyrobetaine. This would reflect a competitive mechanism between inducer and inhibitor molecules occurring at the level of either a common transport system allowing their penetration into the cell (16) or a specific regulatory protein, possibly CaiF, which will become activated upon binding. In each case, the relative affinity of L-(−)-carnitine for the receptor protein will be higher than that of crotonobetaine. Purification of CaiF and binding experiments should resolve this issue.

Primer extension analysis identified the transcription start site of *caiF* at an 82-bp distance from the ATG start codon of this gene (Fig. 2). One hexanucleotide, TACTAT, that

matches four of the six bases in the −10 (TATAAT) consensus sequence for the σ^{70} promoter was found at the right distance upstream from transcription initiation site. A CRP recognition sequence (6) centered at −41.5 is preceded by a half site binding motif for the anaerobic regulator FNR (13) at position −57. The presence of FNR and CRP consensus sequences upstream of *caiF* is consistent with the activation of transcription of a *caiF-lacZ* fusion by these two proteins (Table 3). FNR is known to stimulate transcription of target gene promoters by binding to a DNA sequence centered between 41 and 42 bp upstream of the transcription site (13). Although CRP sites appear from different distances upstream to cognate promoters, maximal activation is obtained when CRP is located at bp −61.5 or −41.5 upstream of the transcription start site (reference 25 and citations therein). Therefore, the presence of a CRP motif at position −41.5 in the promoter of *caiF* strongly suggests that it is of physiological significance. Indeed, preliminary in vitro binding assays showed that purified CRP binds to the *caiF* promoter region (data not shown). Whether FNR binds at the vicinity of CRP and acts in competition with CRP or on its own will require a more thorough investigation of the 5′ regulatory region of *caiF*. In addition, it should be noted that four other putative half sites for FNR were detected more upstream in the sequence, as was one half site for CRP at 4 bp from the Shine-Dalgarno sequence of *caiF*. However, the functionality of these elements is puzzling because of their location with respect to the identified transcription start site.

Further inspection of the 5′ nontranslated potential regulatory region of *caiF* revealed three potential −24/−12 consensus sequences common to all RpoN-dependent promoters (22) and two presumptive NarL boxes (34). This may correlate with the reduction of transcription of the *caiF-lacZ* fusion in an *rpoN* mutant and with the partial relief of the repression by nitrate in a *narL* mutant (Table 3). Clearly, the regulation of transcription of *caiF* appears to be complex, reflecting a key role for protein CaiF in the induction of carnitine metabolism. Elucidation of the mechanisms involved poses the intriguing problem of how the various regulatory proteins interact with each other.

Our data demonstrated that the *hns* and *rpoS* genes exert a cooperative effect to repress carnitine dehydratase activity (Table 4). When the CaiF protein was overproduced, the pattern of induction of carnitine dehydratase activity was shown to escape from the control of the histone-like protein H-NS and the alternate sigma transcription factor σ^S and to rely primarily on the stimulatory effect of CaiF. This finding argues in favor of the fact that both H-NS and σ^S control carnitine metabolism mainly via their effect on the *caiF* gene.

Our results are in good agreement with the initial finding that carnitine dehydratase activity is suppressed by glucose, oxygen, and nitrate (19). From this work and a previous study (17) it appears very likely that the glucose repressive effect is mediated via the interaction of CRP with both the regulatory regions of *caiF* (Fig. 2, Table 3, and data not shown) and the *cai* and *fix* operons (7). In support of this idea is the observation that carnitine dehydratase activity is still lacking in a *crp* mutant that is transformed by plasmid pRL101 which expresses multicopies of *caiF* (Table 4). A similar pattern of carnitine dehydratase activity is also observed in an *fnr* mutant; however, the same kind of argument cannot apply since, in contrast to the regulatory region of *caiF* (Fig. 2), the intergenic region between the *cai* and *fix* operons does not contain an FNR consensus sequence (7, 9). This would imply that another still unknown gene or operon implicated in the synthesis of carnitine dehydratase is controlled by FNR. A working model for the regulation of carnitine metabolism is presented in Fig. 5. On the basis of the present work it should now be possible to dissect the regulatory features of the *cai* system.

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