Regulation of the Carnitine Pathway in Escherichia coli: Investigation of the cai-fix Divergent Promoter Region

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The divergent structural operons caiTABCDE and fixABCX of Escherichia coli are required for anaerobic carnitine metabolism. Transcriptional monopy lacZ fusion studies showed that both operons are coexpressed during anaerobic growth in the presence of carnitine, respond to common environmental stimuli (like glucose and nitrate), and are modulated positively by the same general regulators, CRP and FNR, and negatively by H-NS. Overproduction of the CaiF specific regulatory protein mediating the carnitine signal restored induction in an fnr mutant, corresponding to its role as the primary target for anaerobiosis. Transcript analysis identified two divergent transcription start points initiating 289 bp apart. DNase I footprinting revealed three sites with various affinities for the binding of the cAMP-CRP complex inside this regulatory region. Site-directed mutagenesis experiments indicated that previously reported perfect CRP motif 1, centered at −41.5 of the cai transcriptional start site, plays a direct role in the sole cai activation. In contrast, mutation in CRP site 2, positioned at −69.5 of the fix promoter, caused only a threefold reduction in fix expression. Thus, the role of the third CRP site, located at −126.5 of fix, might be to reinforce the action of site 2. A critical 50-bp cis-acting sequence overlapping the fix mRNA start site was found, by deletion analysis, to be necessary for cai transcription. This region is thought to be involved in transduction of the signal mediated by the CaiF regulator.

To survive, the facultative anaerobe Escherichia coli is able to adapt to a wide variety of growth conditions by synthesizing the appropriate aerobic and anaerobic respiratory pathways. In the absence of oxygen, E. coli can still respire by using a number of terminal electron acceptors, such as nitrate, trimethyamine-N-oxide, or dimethyl sulfoxide (13). When these oxidants are absent, growth of the bacterium can be significantly enhanced in a complex medium supplemented with L-(-)-carnitine [R-(-)-3-hydroxy-4-trimethylaminobutyrate] or its dehydration product crotonobetaine (31). Carnitine is a ubiquitous compound which is mainly found in food of animal origin (6) and is present in the human intestine, where it can be metabolized by bacteria. In contrast to other bacterial species, such as Acinetobacter and Pseudomonas spp., which can utilize carnitine as the sole source of carbon or carbon and nitrogen, respectively (20), members of the family Enterobacteriaceae do not assimilate the carbon-and-nitrogen skeleton of L-carnitine. Rather, they are able to convert carnitine, via crotonobetaine, to γ-butyrobetaine during anaerobic growth in the presence of other substrates which act as carbon and nitrogen sources (32). The biological significance of this pathway is not clearly understood, but crotonobetaine could serve as an external electron acceptor in the absence of other electron acceptors.

Because of its critical role in mammals, where it ensures the transport of long-chain fatty acids through the inner mitochondrial membrane, L-carnitine is being used in a series of clinical and pharmaceutical applications (18). Therefore, increasing demand for this compound has stimulated a search for its stereospecific production using microbial and enzymatic processes.

The E. coli genes for carnitine metabolism have been recently cloned and sequenced. They comprise the two divergent caiTABCDE and fixABCX operons which are only induced under anaerobic conditions in the presence of l-carnitine (9, 10, 12). The cai operon encodes the carnitine dehydratase (caiB) and crotonobetaine reductase (caiA) activities which have been demonstrated to be involved in the two-step carnitine pathway (17, 28). It has been proposed that the remaining part of the cluster directs the synthesis of a carnitine transporter (caiT), a crotonobetaine-carnitine coenzyme A ligase (caiC), an enol hydratase-isomerase (caiD), and an enzyme involved in the formation of an active cofactor necessary for the carnitine pathway enzymes (caiE) (9). The fix operon was so named because it encodes four polypeptides with significant similarity to the corresponding gene products from diazotrophs involved in nitrogen fixation (10). Moreover, the homology of the E. coli FixA and FixB proteins to the small (β) and large (α) subunits of mammalian electron transfer flavoproteins (ETF) (10, 37), as well as similar regulation of fix genes with that of carnitine enzymes, suggests a role for the FixABCX proteins in a specific electron transfer related to carnitine transformation. Indeed, operon fusion studies have shown that the fix operon is repressed by the same effectors, in particular, oxygen, glucose, and nitrate, that repress levels of carnitine metabolism enzymes. Moreover, it is subject to global control by the same regulatory proteins which are known to modulate expression of carnitine metabolism, i.e., the cyclic AMP (cAMP) receptor protein (CRP) which mediates the activation of catabolic operons, the transcriptional regulator FNR that is responsible for anaerobic induction, and the DNA-binding protein H-NS (19, 10). In agreement with this, the intergenic region between the divergent cai and fix operons revealed important regulatory features, namely, potential recognition sequences for CRP and H-NS, but no binding site for FNR. In addition, fix operon expression was also markedly

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decreased in an rpoN mutant lacking the alternative RNA polymerase $\sigma^{54}$ factor, leading to the hypothesis that two presumptive $-12/-24$ RpoN-dependent promoters predicted in the fix direction could be functional (10; see Fig. 2A). In contrast, a putative $\sigma^{70}$ promoter sequence was postulated for cai (9; see Fig. 2A).

More recently, the caiF gene, located downstream of the cai operon and lying in the opposite orientation with respect to cai, was reported to enhance levels of cai and fix expression when cloned in multicopy (11). Based on the fact that inactivation of the caiF gene totally abolished transcription of cai and fix operons, it has been proposed that the 15-kDa CaiF protein acts as a specific transcriptional regulator for carnitine metabolism. Like cai and fix operons, the caiF gene appeared to be positively controlled by CRP and FNR and negatively controlled by H-NS and the NarL (plus nitrate) regulator. In contrast, its expression was independent of the presence of carnitine. Thus, the mechanism by which the carnitine pathway is regulated appears to be rather complex, since it involves a number of general and specific regulators acting at different levels.

In this study, we demonstrate that the structural cai and fix operons are transcribed in a strictly coordinated manner from two divergent promoters. We have attempted to identify the elements present in the 467-bp intergenic regulatory region which are required for transcription in each direction and ruled out the presumed direct role for the RNA polymerase $\sigma^{54}$ factor. We also show evidence that both promoters are directly activated by binding of the cAMP-CRP complex.

### MATERIALS AND METHODS

**Strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Plasmid pM-CFK was constructed in two steps. First, a 400-bp Stul-EcoRI fragment containing the coding sequence of caiF was amplified by PCR and introduced into the polylinker cloning sites of vector pMAL-c (New England Biolabs), downstream from the malE gene, resulting in the production of a maltose-binding protein fusion protein expressed in E. coli MG1655. Second, the Smal-digested uidA-Km$^r$ cassette from plasmid pUIDK3 (2) was inserted into the SacI site of the lacZ gene and its regulatory region 11

**DNA sequencing.** All PCR products, as well as insertions in pJEL250 (Table 1), were sequenced by using universal and reverse oligonucleotide primers with DNA sequencing. All PCR products, as well as insertions in pJEL250 (Table 1), were sequenced by using universal and reverse oligonucleotide primers with DNA sequencing. All PCR products, as well as insertions in pJEL250 (Table 1), were sequenced by using universal and reverse oligonucleotide primers with DNA sequencing.
The t7 sequencing kit, purchased from Pharmacia, and the chain termination procedure (30).

**Construction of lacZ fusions.** The list of pJEL250 recombinant plasmids used (from pXIC to pJBF) is given in Table 1. For the extension and orientation of fragments from the cai-fix intergenic region borne by the plasmids, see Fig. 2. Plasmids designated pE6E5 (C or F) were constructed by digesting plasmid pCTK with restriction enzymes E5 and E6, delimiting the insert (e.g., pEBF) was obtained by digesting pCTK with EcoRV (E) and BglII (B) (Table 1; see Fig. 2). The generated fragment harboring a part of the cai-fix regulatory region was filled in with Klenow polymerase and subcloned into the phosphorylated Smal site of pBluescript SK+. Further restrictions with EcoRI and BamHI allowed us to introduce the fragment, in either the pl(E5E6) or the flp (pE6E5) orientation, into the corresponding sites located in front of the promoterless gene of monocopy fusion vector pJEL250 (35). Plasmids called pKE6 were made in a similar manner but required additional steps. A SacI Tn903 (Kmr) cassette from vector pUC4-KISS (Pharmacia Biotech) was introduced into the SacI site of pBluescript SK+ containing the region of interest in order to provide fragments of a more suitable size for subsequent subcloning. After digestion by EcoRI and PstI, fragments were filled in with Klenow polymerase and introduced into the phosphorylated Smal site of pJEL250 (1). The two EcoRI sites flanking the Smal site were used to clone the fragments into the EcoRI site of pJEL250 in both orientations with an additional Kmr selection. When the Kmr cassette was located between the tested promoter and the lacZ gene, it was deleted by a BamHI digestion, giving rise to a pKE6 plasmid. In the other case, promoters of the kan gene and the lacZ gene were confirmed as being oriented in opposite directions in the resulting pKE6 plasmid. Construction of plasmids called pX(1,2)C and pKX(1,2)F differed only in the first step of cloning. In this case, fragments of interest were generated by PCR and then treated as described above.

**Primer extension analysis of transcript.** Total RNA was isolated from E. coli cells grown anaerobically in rich medium under inducing conditions. It was extracted by the frozen-phenol method described by Maes and Messens (25). RNA concentration was estimated spectrophotometrically and after electrophoresis on a formaldehyde-denaturing 1% agarose gel. Primer extension reactions were performed as described by Aebi et al. (1), with 40 μg of total RNA and about 4 × 10^6 cpm of [γ-32P]ATP end-labeled primers (Eurogentec), using the alkaline-denatured virus reverse transcription primer extension system kit from Promega. For cai transcript analysis, a 24-bp synthetic oligonucleotide, able to hybridize to the noncoding strand between nucleotides +31 and +54 (5′-CCGGGAAAGAAGACCCCTTGGTTC-3′) of the cai gene, was chosen. For detection of the transcriptional start site of the fix operon, a 24-bp primer (5′-CATCAGGCGACCAGCTTACAGAAG-3′) complementary to the region of the noncoding strand between nucleotides +19 and +42 of the fixA gene was used. Products of the primer extension reactions were separated on 6% polyacrylamide-urea sequencing gels in parallel with sequence reactions of pCTK performed by using the same oligonucleotide. A 2-week exposure was required for detection of direct signals.

**SI mapping.** Total RNA was isolated as described for primer extension experiments. The transcriptional start site was obtained from Smal-endonuclease restriction with either BglII for cai or AvfI for fix, removal of the 5′ phosphate by alkaline phosphatase (Boehringer Mannheim), and 5′ labeling with [γ-32P]ATP and poly nucleotide kinase (Promega). A second digestion with EcoRV and BglII for cai and HpaI for fix generated fragments of 907 and 592 bp, respectively. The 5′ end label was blocked by incubation for 2 min at 30°C. After digestion by BpHI, filled in with Klenow enzyme, and subcloned in plasmid pBluescript KS+, introduction of pJEL250 was achieved as described above.

**Preparation of operator fragments for binding studies.** The regulatory region of the cai and fix operons was obtained by cutting plasmid pCTB with either EcoRV and BglII or BglII and HpaI for labeling of one or the other strand. DNA fragments were end labeled with [γ-32P]ATP (3,000 Ci/mmol) (Amer sham) in the presence of the Klenow fragment of DNA polymerase. These labeled fragments were further purified by using the Qiagen QIAquick extraction kit.

**Gel retardation assay.** Binding of CRP on the cai-fix regulatory region was performed as described by Søgaard-Andersen and Valentin-Hansen (34). CRP protein, purified by a procedure derived from that of Ghoshani et al. (14, 27), was a gift of W. Nasar (this laboratory). In general, the reaction was carried out in 20 μl containing 10 mM Tris-HCl (pH 7.8), 50 mM KCl, 1 mM dithiothreitol, 50 μM CAMP, 4 μg of acetylated bovine serum albumin, and 1 μg of poly(dI-dC) (dl-dC) (Pharmacia LKB) as bulk carrier DNA. After addition of the DNA probe, 50 μl of different amounts of purified CRP, the reaction mixtures were incubated for 30 min at 30°C, adjusted to 5% in glycerol, and then loaded onto an 8% nondenaturing polyacrylamide gel and electrophoresed in 10 mM Tris-HCl (pH 8) containing 50 μM CAMP. Gels were then dried and exposed to Amersham film.

**Footprinting with DNase I.** DNase I footprint analysis was performed by using a procedure modified from that of Ogilvie and Sanger (33). About 1 μg of DNA probe, labeled at one end, was incubated for 30 min with various concentrations of CRP and 50 μM CAMP in the buffer used for the mobility shift assay. The reaction mixtures were adjusted to 10 mM MgCl2, and 5 mM CaCl2. DNase I was then added (2.5 × 10^−7 U; Boehringer Mannheim), and the mixture was incubated for 2 min at 30°C. DNase I digestion was blocked by the addition of 25 μl of stop solution (100 mM EDTA, 0.4 mg of yeast tRNA/ml, pH 8). A 50-μl volume of ice-cold Tris-EDTA (pH 8) was then added. After phenol-chloroform extraction, DNA fragments were ethanol precipitated, resuspended in 5 μl of a formamide-dye mixture (1), and separated by electrophoresis on a 6% polyacrylamide sequencing gel. Bands were detected by autoradiography.

**Site-directed mutagenesis.** Oligonucleotide-mediated, site-specific mutagenesis was performed as described by Kunel et al. (22). Point mutations in the regulatory region of the cai-fix operons were introduced by using a two-step PCR achieved with Pro polymerase (Boehringer Mannheim). Primers generating the ends of the modified fragment were those used for primer extension experiments, and plasmid pCTB served as the DNA target. The end product was digested by BpHI, filled in with Klenow enzyme, and subcloned in plasmid pBluescript KS+. Introduction in pJEL250 was achieved as described above.

**DNase I footprint analysis.** For DNase I binding of 1′-2′ promoter consensus sequence (Fig. 2A, 2B, and 5 [562 bp]), and a 25-bp primer (5′-GGTGGTAAAAATAGACGTCTACTTGC-3′) was used to convert the −24/-12 promoter consensus sequence, GGNPyGC (23), to GTN-TG-N-GC. To test the functional importance of the CRP1 and CRP2 binding sites (see Fig. 5), mutations affecting the most conserved motif, 5′-TGTGA (36), were obtained using by a 31-bp primer (5′-TGAACACCAATTCAAGAATACGACTTC-3′) for CRP1 (cp101) and a 34-bp primer (5′- CTAGGTTTCTAACTTGAGGCAGGACAATAA-3′) for CRP2 (cp200). Alternatively, the CRP1 box was destroyed by changing the perfect consensus TGTGAA2-24/24 to TGTGAA-TACA in TGTGAA-TACA (15) (mutation cp100). This was achieved by using a 22-bp primer with the sequence 5′-TGTGACACTACAAAGAT-3′. All mutations were verified by sequencing.

**RESULTS**

**Parallel expression of the cai and fix divergent operons during cell growth.** Transcription of both cai and fix operons is induced during anaerobic growth in the presence of n-carnitine (9, 10). To determine whether the corresponding promoters function in a coordinate manner, expression of cai-T7-lacZ and fixA-lacZ operon fusions borne by monocopy plasmids pAB20 and pAB30 was measured in wild-type strain NM522 along the growth curve. The pattern of cai-lacZ expression was essentially identical to that observed for fixA-lacZ throughout the entire cell growth period (Fig. 1). Expression from either fusion was induced from the mid-log phase, reached a maximum in the late exponential phase, and then remained constant for several hours in the stationary phase. The slightly higher level of β-galactosidase activity obtained with the fixA-lacZ fusion over that found with the cai-lacZ fusion might be due to differences in the construction of the fusions or may possibly reflect a small difference in the strength of the two promoters. The previous observation that carnine dehydrogenase activity is optimally synthesized at the end of the exponential phase (19) is in agreement with gene expression. This pattern may reflect the prerequisite synthesis of the CaiR regulatory protein, which controls the activation of both cai...
To document the role of each regulator in carnitine metabolism (10), we asked whether the various effectors tested displayed an effect on the expression of the caiF gene, which encodes the potential transcriptional activator of carnitine metabolism, designated CaiF, has been previously established (11). Expression of the caiF gene is dependent on the same general regulatory proteins, H-NS, CRP, FNR, and transcription factor RpoN (11), which control caiF and fix expression (Table 2) (10). In particular, lesions in the crp, fnr, and rpoN genes decreased expression of the caiF-lacZ fusion at various levels, the most dramatic effect being displayed by fnr. However, compared with that of caiF and fix expression of caiF was less severely affected. It was therefore interesting to examine the effect of overproduced CaiF by introducing either plasmid pSU9 pRL101 (genotype) or pAB20 (caiF-lacZ) or pAB30 (fixA-lacZ) in the presence of 20 mM DL-carnitine. Cells were harvested at the beginning of the stationary phase.

### Table 2. Expression of the caiT-lacZ fusion under various environmental conditions and in various genetic backgrounds

<table>
<thead>
<tr>
<th>Straina</th>
<th>Relevant genotype</th>
<th>Additionb</th>
<th>Oxygen</th>
<th>Effector</th>
<th>β-Galactosidase sp act (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM522</td>
<td>Wild type</td>
<td>+</td>
<td>DL-Carnitine</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>Nonec</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>DL-Carnitine</td>
<td>5,000</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>LCarnitine</td>
<td>5,500</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>Crotonobetaine</td>
<td>4,500</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>DCarnitine</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>γ-Butyrobetaine</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>Glucosec</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>Nitratec</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>Fumaratec</td>
<td>4,500</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>Trimethylamine-N-oxidec</td>
<td>1,500</td>
<td></td>
</tr>
<tr>
<td>MAM100</td>
<td>hns::Tn10</td>
<td>–</td>
<td>DL-Carnitine</td>
<td>15,000</td>
<td></td>
</tr>
<tr>
<td>MAM101</td>
<td>Δcrp-45</td>
<td>–</td>
<td>DL-Carnitine</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>MAM102</td>
<td>rpoN::Tn10</td>
<td>–</td>
<td>DL-Carnitine</td>
<td>900</td>
<td></td>
</tr>
<tr>
<td>MAM103</td>
<td>fnr-22</td>
<td>–</td>
<td>DL-Carnitine</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. Stimulation of expression of the caiT−lacZ and fixA−lacZ fusions in various mutants by the overexpressed regulatory gene caiF

<table>
<thead>
<tr>
<th>Fusion and strain (genotype)</th>
<th>β-Galactosidase sp act (U) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No plasmid</td>
</tr>
<tr>
<td>caiT-lacZ</td>
<td></td>
</tr>
<tr>
<td>NM522 (wild type)</td>
<td>5,000</td>
</tr>
<tr>
<td>MAM102 (pnuN)</td>
<td>900</td>
</tr>
<tr>
<td>MAM103 (fnr)</td>
<td>50</td>
</tr>
<tr>
<td>MAM101 (crp)</td>
<td>80</td>
</tr>
<tr>
<td>fixA-lacZ</td>
<td></td>
</tr>
<tr>
<td>NM522 (wild type)</td>
<td>5,500</td>
</tr>
<tr>
<td>MAM102 (pnuN)</td>
<td>800</td>
</tr>
<tr>
<td>MAM103 (fnr)</td>
<td>80</td>
</tr>
<tr>
<td>MAM101 (crp)</td>
<td>120</td>
</tr>
</tbody>
</table>

### Notes

a Cells transformed with monochromatidal plasmid pAB20 carrying the caiT-lacZ operon fusion were grown at 30°C, either aerobically or anaerobically, in TYEP medium supplemented as indicated. Cells were harvested at the beginning of the stationary phase.

Where indicated, DL-carnitine and glucose were added at 20 mM; t-carnitine, l-carnitine, crotonobetaine, and γ-butyrobetaine were added at 10 mM; and nitrate, fumarate, and trimethylamine-N-oxide were added at 40 mM.

This effector was tested in a medium supplemented with 20 mM t-carnitine.

### References

1. Regulation of expression of the cai operon. To examine more thoroughly the expression pattern of the cai operon in relation to the known regulation of carnitine metabolism (19) and the fix operon (10), β-galactosidase levels from the caiT-lacZ fusion were determined under various environmental conditions. As expected, caiT-lacZ expression was only observed in the absence of oxygen and was strongly induced by the presence of t-carnitine (Table 2). l-Carnitine and crotonobetaine were also able to induce transcription at the same level. In contrast, other intermediary compounds of the pathway, D-carnitine and γ-butyrobetaine (Table 2), as well as additional tested betaines, choline and glycine betaine (data not shown), had no effect. Addition of glucose led to total suppression of cai expression in accordance with catabolite repression of carnitine metabolism (32). Of the three anaerobic terminal electron acceptors tested, nitrate exerted a completely negative effect, whereas trimethylamine-N-oxide reduced cai expression by a factor of three and fumarate had no influence (Table 2). Therefore, the various effectors tested displayed an effect on cai expression similar to that previously observed on fix expression (10).

Global regulatory proteins CRP, FNR, and H-NS are involved in the synthesis of carnitine dehydratase activity (9, 11). To document the role of each regulator in cai operon expression, the caiT-lacZ fusion was transferred into isogenic strains that contained a mutation in one of the relevant regulatory genes. caiT-lacZ expression was dramatically abolished in crp and fnr mutants while it was increased threefold in the hns mutant (Table 2). Thus, CRP and FNR positively control cai expression and H-NS exerts a negative control. A similar pattern was previously reported for the control of fix operon expression (10). Surprisingly, mutation of the rpoN gene, which encodes the alternative RNA polymerase σ^54 factor, caused a fivefold diminution of cai expression, which was comparable to the reduction observed for fix expression (10). This was unexpected, since the only potential consensus sequences for binding of σ^54-associated RNA polymerase found in the cai-fix intergenic region were oriented in the same direction (10). This suggests indirect control by σ^54.

Expression of the caiT-lacZ and fixA-lacZ fusions in various mutants in the presence of a multicopy caiF gene. The existence of a potential transcriptional activator of carnitine metabolism, designated CaiF, has been previously established (11). Expression of the caiF gene is dependent on the same general regulatory proteins, H-NS, CRP, FNR, and transcription factor RpoN (11), which control caiF and fix expression (Table 2) (10). In particular, lesions in the crp, fnr, and rpoN genes decreased expression of the caiF-lacZ fusion at various levels, the most dramatic effect being displayed by fnr. However, compared with that of caiF and fix expression of caiF was less severely affected. It was therefore interesting to examine the effect of overproduced CaiF by introducing either plasmid pSU9 pRL101 (genotype) or pAB20 (caiF-lacZ) or pAB30 (fixA-lacZ) in the presence of 20 mM t-carnitine. Cells were harvested at the beginning of the stationary phase.
pRL101 or pM-CFK, which carries multicopies of the caiF gene into regulation mutants harboring the fusions. When plasmid pM-CFK was used, reduction or suppression of cai
and fix expression in the rpoN and fnr mutants, respectively, was completely restored to the enhanced level found in the wild-type strain (Table 3). Thus, control exerted by the RpoN
and FNR proteins on cai and fix expression appears to be mediated mainly via the caiF gene. Intermediate levels of stimulation were observed with plasmid pRL101, the fnr mutation
having the most limiting effect. This difference was attributed to the fact that the regulatory-promoter region of caiF was still present in plasmid pRL101 while it was missing in gene fusion
plasmid pM-CFK. As a consequence, caiF expressed from pRL101 was still partially dependent on its known regulatory proteins while it totally escaped their control when expressed
from the tac promoter of plasmid pM-CFK. In contrast, no enhancement of cai and fix expression by plasmid pRL101 was detected in the crp mutant, which strongly suggests that control
by CRP still operates at the level of the cai-fix intergenic regulatory region.

**Deletion analysis of the cai-fix intergenic regulatory region.**
Sequencing data had previously revealed the presence of putative divergent promoters for the cai and fix operons, as well as possible binding sites for the fixation of regulatory proteins (Fig. 2A). To localize the areas of interest, various fragments of the cai-fix intergenic region were subcloned into monocopy
operon fusion vector pJEL250 (35) in both orientations. The hybrid plasmids were introduced into wild-type strain NM522 and rpoN mutant MAM102, and β-galactosidase activity was measured after anaerobic growth in the presence of DL-carnitine. β-Galactosidase activities were expressed as nanomoles of o-nitrophenol produced per milligram (dry weight) of bacteria per minute. Each value represents the average of four independent experiments. nd, not determined.

![Image](http://jb.asm.org/)

**FIG. 2.** Effects of deletions in the cai-fix intergenic region on in vivo expression of the caiT-lacZ and fixA-lacZ fusions. (A) Schematic representation of the cai-fix divergent promoter region as previously suggested by Eichler et al. (9, 10). The first open reading frames of the fix and cai operons initiate at positions 324 and 792, respectively. Curved arrows indicate putative transcriptional start sites. Two proposed binding sites for the $\sigma^4$-associated RNA polymerase are indicated by open diamonds. Two remarkable inverted repeats, which might have a regulatory role, are designated 1 and 2. A perfect consensus sequence for the binding of CRP is shown by an oval. (B) Deletion analysis of the cai-fix intergenic region. The arrows show the extension and orientation of fragments linked to the lacZ reporter gene on monocopy plasmid pJEL250. The coordinates at the top correspond to the numbering of nucleotides in accordance with the previously published sequence (9) and give useful restriction sites. The table on the right shows the names of the fusion plasmids listed in Table 1 and the relevant reporter gene on monocopy plasmid pJEL250. The coordinates at the top correspond to the numbering of nucleotides at 30°C in TYEP medium in the presence of 20 mM DL-carnitine. β-Galactosidase activities were expressed as nanomoles of o-nitrophenol produced per milligram (dry weight) of bacteria per minute. Each value represents the average of four independent experiments. nd, not determined. (C) Schematic representation of the cai-fix divergent promoter region as deduced from the complete study reported in this paper (see also Fig. 5).
the control region of fix (Fig. 2A), namely, the CRP box and the putative σ^70-dependent transcription start site 2, are not involved in fix expression. The smallest fragment tested which conferred a significant level of cai transcription was the 316-bp EcoRV-MluI fragment harbored by plasmid pEMC (Fig. 2B). The absence of the predicted σ^70-dependent transcription start site in this construct (Fig. 2A) suggested that the cai promoter is located upstream. Indeed, removal of the upstream region including the CRP-binding site prevented any expression of the caiT-lacZ fusion in plasmid pEAC. Surprisingly, deletion of the 48-bp region upstream of fix (plasmid pX2C versus plasmid pEMC) led to complete loss of cai operon transcription, although no significant feature was evident in this area. This result was further substantiated by the absence of cai expression in construct pSSC, which contains an additional 109-bp deletion. The possible significance of this area is discussed later. When promoter-active constructs were tested in an rpoN mutant, a threefold reduction of β-galactosidase levels was observed relative to the wild-type strain (Fig. 2B). A similar diminution of cai and fix expression, as well as the absence of a properly oriented RpoN consensus sequence for cai (10), argues in favor of indirect control of both operons by RpoN. An improved schematic representation of the divergent cai-fix regulatory region, as deduced from this deletion analysis and from data reported below, is shown in Fig. 2C.

**Identification of cai and fix transcriptional start sites.** To identify the promoters responsible for cai and fix transcription, total RNA isolated from wild-type strain NM522 and its rpoN derivative, with or without plasmid pCTK (which carries multicopies of the entire intergenic cai-fix region), was subjected to primer extension analysis and S1 nuclease mapping. A major extension product of 115 bp was detected for the cai-fix 10 region and the transcription start point (+1) of the cai and fix operons are indicated on the right. (C) S1 nuclease mapping of the 5' end of the cai transcript. RNA was isolated as described for primer extension assays. The TGCA sequence is the same as in panel B and serves as a size marker. Lanes: 1, MAM102 (rpoN)/pCTK; 2, NM522/pCTK; 3, NM522. The +10 region and the transcription start point (+1) of the cai and fix operons are indicated on the right. (C) S1 nuclease mapping of the 5' end of the cai transcript. RNA was isolated as described for primer extension assays. The TGCA sequence is the same as in panel B and serves as a size marker. Lanes: 1, MAM102 (rpoN)/pCTK; 2, NM522/pCTK; 3, probe without S1 nuclease digestion. The sizes of the detected bands are on the left. The start site (+1) of the cai operon and coordinates (in parentheses) relative to Fig. 2 are on the right.

When a primer which specifically hybridized within the caiT coding sequence was used, a single extension product was seen, corresponding to an initiation at the G-686 residue located 106 bp upstream of the caiT start codon, as well as S1 nuclease mapping of the 5' end, did not allow us to identify other extension products (data not shown), and (ii) partial deletion mutagenesis of the σ^70 consensus sequence 1 (see Materials and Methods) had no effect on fix expression. Determination of the transcription start site for fix confirms deletion analysis data which indicate that the 50-bp surrounding region is critical for significant expression (Fig. 2, pEBF versus pKX2F).

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Interaction between CRP and the cai-fix regulatory region.

To assess the involvement of CRP in the activation of transcription of cai and fix operons (Tables 2 and 3), the binding specificity of purified CRP to the cai-fix intergenic promoter region was tested in vitro by performing gel mobility shift assays. As shown in Fig. 4A, three types of complexes were observed, depending on the concentration of CRP, which may indicate that multiple CRP-binding sites are present in the cai-fix promoter-regulatory region or that oligomerization of CRP bound to one or more sites might occur. To localize the potential CRP-binding sites more precisely, DNase I footprinting was carried out for both strands of the DNA-regulatory

FIG. 4. Specific binding of CRP in the cai-fix regulatory region. (A) Gel mobility shift assay of the cai-fix intercistronic region with purified CRP. The α-32P-end-labeled 396-bp BspHI-HpaI DNA insert of plasmid pCTB was incubated in the presence of 1 μg of poly(dI-dC)-(dI-dC) without CRP (lane 1) or with 6.17 nM (lane 2), 123 nM (lane 3), or 1.23 μM (lane 4) CRP. The reactions were carried out in the presence of 50 μM cAMP. Free (F) and CRP-bound (B1, B2, and B3) DNA bands are indicated. (B and C) DNase I footprinting of the CRP-DNA interactions in the cai and fix promoter region. Labeling was performed on both strands independently, from either the BglII end of the 507-bp EcoRV-BglII fragment corresponding to the coding strand of cai (B) or the BspHI end of the 396-bp BspHI-HpaI fragment corresponding to the coding strand of fix (C). Protected regions are indicated by vertical brackets and marked 1 through 3. The CRP concentrations are as follows: lane 1, 0; lane 2, 6.17 nM; lane 3, 123 nM; lane 4, 1.23 μM. The values to the left of panels B and C are coordinates relative to the transcription start sites of cai and fix, respectively.

FIG. 5. Locations of the sequences protected by CRP from cleavage by DNase I. The protected sequences, termed CRP1 to CRP3, are represented by horizontal brackets. The locations of the transcription initiation sites of fixA and caiT are indicated by arrows designated +1. The putative −10 and −35 boxes are underlined (for fixA) or overlined (for caiT), and two inverted repeats are highlighted by converging dashed arrows. The start codons of fixA and caiT are in boldface. Useful restriction sites are also noted.
TABLE 4. Effects of crp mutations on caiT-lacZ and fixA-lacZ expression

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sequence</th>
<th>β-Galactosidase sp act&lt;sup&gt;α&lt;/sup&gt; (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>4.200</td>
</tr>
<tr>
<td>crp-100</td>
<td>TTTGGCAGTACACA</td>
<td>150</td>
</tr>
<tr>
<td>crp-101</td>
<td>TTTGGCAGTACACA</td>
<td>40</td>
</tr>
<tr>
<td>crp-200</td>
<td>GGTGA-N6-AAAAAC GTTGGGCAATTACACA</td>
<td>4.700</td>
</tr>
</tbody>
</table>

<sup>α</sup> The 456-bp BspHII fragment (Fig. 2), harboring or not harboring the indicated mutations (crp-100 and crp-101 for site 1 and crp-200 for site 2), was cloned into monocopy plasmid pJEL250 in either the cai or the fix transcription direction as described in Materials and Methods. Strain NM522 transformed with the different constructs was grown anaerobically at 30°C in TYEP medium in the presence of 20 mM L-carnitine.

<sup>β</sup> Activity was determined on cells harvested at the beginning of the stationary phase.

region. The exact locations of the footprints are presented in Fig. 5. It is clear from Fig. 4 that CRP has a higher affinity for site 1 than for the other two sites, since occupancy of site 1 occurred at a very low CRP concentration (6.17 nM) (Fig. 4B and C), shifting 100% of the free DNA (Fig. 4A). Moreover, this protected region corresponds to the binding of CRP on the perfect consensus sequence situated at position −41.5 of the transcription start site for cai. Site 2 was largely occupied in the presence of an intermediate concentration of CRP (123 nM) (Fig. 4, compare lanes 3 in panels B and C with lane 3 in panel A). It determines the presence of a region (GGTGA-N6-TAACA) that shows homology with the consensus CRP-binding site and is centered at position −69.5 relative to the fix transcription site, which is compatible with a direct role in activation of transcription (8). A deeper examination of footprints with the fix coding strand revealed the location of a third region observed only at a high concentration of CRP (1.23 μM) (Fig. 4, compare lanes 4 in panels C and A). It contains a GGTGA-N6-AAAAAC sequence centered at position −126.5 versus the fix transcription start site.

The hypothesis of a direct role of CRP sites 1 and 2 in the activation of transcription of cai and fix, respectively, was tested by examining the effect of alterations in the two sites on expression of caiT-lacZ and fixA-lacZ fusions. Changes in the affinity of CRP for the different mutated sites was tested by gel retardation assays and DNase I footprinting experiments. No binding of CRP on mutated site 1 was observed, whatever the protein concentration used. In contrast, CRP was still able to bind to similarly mutated site 2 but with decreased affinity (data not shown). These in vitro observations are in good correlation with the in vivo results presented in Table 4, which shows that mutations in site 1 resulted in total suppression of the activity of the cai promoter, whereas introduction of a mutation in site 2 only partially decreased transcription from the fix promoter. In both cases, mutations of CRP sites had no effect on the corresponding distal promoters; i.e., the level of fix transcription remained unaffected in the absence of CRP fixation on site 1, and cai was normally expressed in the presence of a mutated site 2.

DISCUSSION

The present investigation clearly demonstrates that the two divergent cai and fix structural operons involved in the carnitine pathway are simultaneously expressed, under anaerobic conditions, in the presence of L-carnitine and controlled in parallel in response to a number of effectors and regulatory genes (Fig. 1; Tables 2 and 3). Genetic studies provide evidence that activation of both operons depends on the action of the two structurally and functionally related global activators CRP and FNR and on the specific activator CaiF, which mediates the carnitine signal. It is particularly noteworthy that the relative strengths of the two promoters, measured by both the in vivo monocopy lacZ fusion technique (Fig. 1) and in vitro mRNA analysis (Fig. 3), are very similar, supporting strictly controlled coordinate expression of the two gene clusters.

The occurrence of divergent operons is relatively frequent in bacteria (3), but it does not systematically imply a physiological linkage (24) or a common regulatory pattern (5). Deletion analysis of the cai-fix intercistronic region (Fig. 2) and determination of the exact transcription start site of each cai or fix operon (Fig. 3) allowed us to conclude that the two promoters are in the back-to-back arrangement of divergent promoters defined by Beck and Warren (3) and that they are separated by a 289-bp region which comprises binding sites for regulatory proteins. Few functionally related divergent structural operons of this category have been described. One of the best-characterized examples is represented by the malEp-malKp divergent regulatory region, which directs expression of malEFG and malK-lamB-malM, the operons which encode components of the maltodextrin transport system. In this model, several features relating to the cai-fix coordinated control could be outlined: (i) malEp and malKp promoter activity depends on the synergistic action of the global regulator CRP and the specific activator of the maltose regulon, MalT, (ii) the two promoters have a 271-bp regulatory region in common that is located between their transcription sites, and (iii) they are relatively similar in strength (36).

Both cai and fix promoters were found to possess features typical for activation by σ<sub>70</sub>-associated RNA polymerase. This finding was as expected for cai after inspection of the DNA sequence (9), and the data presented here permitted us to appropriately position the deduced −10 region (Fig. 3 and 5). In contrast, because of its homology with the σ<sub>70</sub>-dependent fix genes of members of the family Rhizobiaceae and the detection of potential −12/−24 elements in the DNA sequence, the E. coli fix operon was suspected of being transcribed under the control of the σ<sub>70</sub>-associated RNA polymerase (10). However, based on the presence of relatively well-conserved −10 and −35 hexamer sequences with respect to the transcription start point of fix (Fig. 5), it seems reasonable to suggest that the fix operon may depend on the σ<sub>70</sub> transcription factor for its expression. This result is of particular relevance since the cai and fix operons appear to be coregulated in the presence of carnitine. This observation is consistent with the classification of these E. coli fix gene products in the ETF-like protein family, which, in contrast to housekeeping ETs linked to the fatty acid degradation pathway, are synthesized only under specific conditions (37).

Evidence is presented that CRP is directly involved in the expression of the cai and fix genes and plays a crucial role in the activation of carnitine metabolism. The regulatory effect of CRP was first inferred from the absence of induction of caiT-
lacZ and fixA-lacZ fusions in a crp mutant (Tables 2 and 3). Investigation of the ability of purified CRP to bind to the cai-fix promoter-regulatory region revealed that the protein is, in fact, able to bind to three distinct sites with different affinities (Fig. 4 and 5). Moreover, the location of each binding site is characteristic of one of the three classes of CRP-dependent promoters which have been described by Ebright (8). Occupancy of the perfect consensus sequence with the highest affinity at position −41.5 relative to the cai transcription start point (Fig. 5) corresponds to a class II promoter in which the DNA-binding site for CRP overlaps the DNA-binding site for RNA polymerase. In agreement with this, no −35 determinant was detected. In contrast, the lower-affinity binding site, located at position −69.5 with respect to the fix transcription start point, designated the fix promoter as belonging to class 1, in which CRP would promote fix transcription by interacting with the α subunit of RNA polymerase. Accordingly, well-conserved −10 and −35 boxes for the binding of RNA polymerase were found (Fig. 5). The third binding site detected for CRP displayed the lowest affinity and was the most distal upstream from the cai and fix transcription start points, at positions −162.5 and −126.5, respectively (Fig. 5). Site-directed mutagenesis of CRP sites 1 and 2 clearly demonstrated that site 1 is required to activate only transcription of the cai operon whereas site 2 only plays a role in the activation of the fix operon. CRP is considered to behave as a simple transcription activator when it binds closer to the promoter start, i.e., at positions −41.5, −61.5, and −71.5 (8, 21). In these cases, CRP-cAMP alone is sufficient to activate initiation of transcription by directly contacting the DNA polymerase. This situation might occur in the case of the cai promoter. For the fix promoter, one could imagine that the partial action of CRP primary binding site 2 might be reinforced by that of distal site 3, which will therefore contribute to the modulation or coordination of fix expression in a cooperative manner. It would be interesting to know if the particular localization of CRP2 and CRP3 boxes, which are arranged symmetrically in the spacing determined by the succession of inverted repeats 1 and 2 (Fig. 5), is of functional significance. The mechanism of activation by CRP is clearly different from that occurring at the divergent mauEp-malKp promoters, which are simultaneously controlled by an array of three binding sites for CRP, all located at distal positions in between (36), and forming a high-order structure responsible for activation, assisting direct activation by the ManI promoter regulator.

In addition to the effect of CRP, activation of cai and fix promoters has been shown to be subject to the essential function of CaiF, the specific activator of carnitine metabolism (11). Interestingly, control by anaerobiosis of the carnitine pathway is shown here to be exerted exclusively through the transacting CaiF protein. Consistent with this idea is the observation that maximal levels of lacZ fusions reflecting cai or fix expression can be reached in an fnr mutant, provided that a constitutively synthesized CaiF protein is present (Table 3). This is in striking contrast to the action of CRP, which plays a direct role in the promoters of the cai and fix operons (Fig. 4 and 5), in addition to modulating caiF expression (11). As pointed out above (Fig. 2), minimal promoter fragments still exhibiting transcription ability were all induced by carnitine, indicating that transduction of the signal was mediated by these DNA sequences. Surprisingly, removal of the 48 bp surrounding the transcription initiation site of fix led to total suppression of cai expression (plasmid pX2C versus plasmid pEMC), indicating that this area, corresponding to the RNA polymerase binding site for fix transcription, was also necessary for cai transcription activation. Primer extension and S1 mapping experiments (Fig. 3) have clearly shown that the cai operon is transcribed from a unique mRNA start site located 106 bp from the caiT coding sequence, thus, around 260 bp away from the short deleted region. Moreover, the assignment of CRP sites 1 and 2 to the respective activation of cai and fix (Table 4) further substantiates the fact that the two promoter regions are distinct from each other. The in vivo inducing role of the caiF gene in the presence of carnitine (11) would be better understood after a thorough investigation of the in vitro binding of the purified CaiF protein to the divergent regulatory region. Very preliminary data suggest that CaiF could bind upstream of the −35 region of the fix promoter. In this context, deletion of the adjacent region reported in plasmid pXC2 might severely affect the stability of activator binding. Further studies will be aimed at analyzing the interaction of CaiF with the cai-fix divergent regulatory region and examining its possible synergistic action with CRP in the coactivation process.

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