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The *fixA* and *fixB* Genes Are Necessary for Anaerobic Carnitine Reduction in *Escherichia coli*

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In *Escherichia coli*, the use of carnitine as a terminal electron acceptor depends on a functional *caiTABCDE* operon. It had been suggested that the adjacent but divergent *fixABCX* operon is also required for carnitine metabolism, perhaps to provide electrons for carnitine reduction. We have constructed *E. coli fixA* and *fixB* mutants and find that they are unable to reduce carnitine to γ-butyrobetaine under anaerobic conditions.

During anaerobic growth, *Escherichia coli* is able to synthesize a number of respiratory chains (6, 13). In the absence of alternative electron acceptors, such as nitrate, trimethylamine *N*-oxide, or fumarate, growth of anaerobic *E. coli* cultures can be stimulated by adding carnitine or its dehydration product, crotonobetaine (11). Seim and colleagues showed that *E. coli* cells reduce crotonobetaine to γ-butyrobetaine during anaerobic growth (9, 11, 12) and thus use carnitine as a terminal electron acceptor.

The carnitine metabolic pathway in *E. coli* involves the products of the *caiTABCDE* operon located at the first minute on the *E. coli* chromosome. *caiTABCDE* is induced under anaerobic conditions by the presence of carnitine or crotonobetaine. Transcription is inhibited by adding γ-butyrobetaine to cultures and is also repressed in the presence of "better" electron acceptors, such as nitrate (5, 6).

In vivo overexpression of the DNA region 5′ to the *cai* operon resulted in four polypeptides with amino acid sequence similarity to polypeptides encoded by the *Sinorhizobium meliloti fixABCX* operon (4, 6). The FixA and FixB proteins of both *E. coli* and *S. meliloti* resemble the β- and α-subunits of electron transfer flavoproteins (14), which typically transfer electrons from a dehydrogenase to a ubiquinone oxidoreductase. FixC has regions that are similar to the family of ubiquinone oxidoreductases. The fourth protein produced by the operon, FixX, is predicted to be a novel type of ferredoxin (1). In *S. meliloti*, it has been suggested that the four Fix proteins act together to transfer electrons from a carbon source to nitrogenase (4).

In *E. coli*, it has been shown (2, 6) that, during anaerobic growth, the divergent *fixABCX* and *caiTABCDE* operons are coregulated in the presence of carnitine. However, they did not link the *fix* genes directly to carnitine metabolism, since they were unable to obtain stable chromosomal *fix* gene mutations (6). They observed a high mortality rate and chromosomal rearrangements in the *fix* region when they attempted to make mutants and suggested this was due to a crucial role for the *fix* gene products in *E. coli* (6). In this study, we have created stable in-frame mutations in *E. coli fixA* and *fixB*. These mutations alter carnitine metabolism in a way that is consistent with the idea that carnitine can be used by *E. coli* as a terminal electron acceptor under anaerobic conditions.

Bacterial strains and growth conditions. The *E. coli* strains and plasmids used in this work are listed in Table 1. Cultures used for the growth experiments were grown aerobically in Luria-Bertani (LB) broth (10) at 37°C overnight. These cultures were then diluted 1:50 in 14-ml screw-top test tubes filled to the top with M9 broth (10) containing 0.2% glycerol as the sole carbon source and NH_4 as the sole nitrogen source. As indicated, carnitine was added at a final concentration of 10 mM, and sodium nitrate was added at a final concentration of 40 mM. Sodium molybdate was added to all tubes at a concentration of 2 μM. The cultures were incubated in stoppered glass tubes at 30°C and allowed to become anaerobic over a 48-h period before culture densities were determined.

Construction of *E. coli fixA* and *fixB* mutants. Since Eichler et al. had difficulties in constructing stable chromosomal insertion mutations in the *E. coli fix* genes (6), we decided to try to create in-frame mutations within the *fix* genes to eliminate possible negative polar effects of the mutations on downstream genes by using the PCR transformation method described by Datsenko and Wanner (3). Long primers containing sequences at the ends of the *fix* genes were used to amplify a kanamycin resistance DNA cassette. Primer 1 contained the 5′ *fix* DNA sequence and 5′ kanamycin cassette sequence, while primer 2 contained the 3′ *fix* DNA sequence and 3′ kanamycin cassette sequence (Table 1). PCR amplification of pKD4 with these primers generated PCR products with the 5′ and 3′ *fix* DNA sequences flanking the kanamycin cassette (3). These linear PCR products were electroporated into BW25113(pKD46) cells made competent as described previously (3). Mutants were selected by plating electroporated cells on LB agar containing 25 mg of kanamycin per liter and incubating them overnight at 37°C. Transformants were colony purified and tested for both the absence of pKD4 and the loss of the temperature-sensitive pKD46 plasmid by looking for penicillin sensitivity. The structure of the DNA in the *fix* region was examined by PCR to confirm replacement of the wild-type allele of the targeted *fix* gene with the kanamycin cassette (3). Constructs with *fixA* and *fixB* were successful, but despite sev-

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Genotype or description[a]	Source
Strains		
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	B. Wanner (3)
AW2A	BW25113 *fixA*	This work
AW1B	BW25113 *fixB*	This work
Plasmids		
pKD4	*oriR*[R6Kγ] Kan^r Amp^r	B. Wanner (3)
pKD46	Temperature-sensitive derivative of pSC101, Amp^r *araC*-P$_{araB}$ λ recombination genes (*gam*-β-*exo*)	B. Wanner (3)
Primers		
1A	5′-GCGTGATATCTGTAATTAACACCACCGATATGAACGAC**GTGTAGGCTGGAGCTGCTTC**-3′	This work
2A	5′-GAGAAAACGTGTTCATAGCATCCCCTGTAATTAAATGAC**CATATGAATATCCTCCTTAG**-3′	This work
1B	5′-CGCAAAGTCATTTAATTACAGGGGATGCTATGAACACG**GTGTAGGCTGGAGCTGCTTC**-3′	This work
2B	5′-GCCAAAATGCAGCCTTGCCAGAGTGGATCAACGCGCTAA**CATATGAATATCCTCCTTAG**-3′	This work

[a] In primers 1A and 2A, underlined sequence is identical to the *E. coli fixA* DNA sequences. In primers 1B and 2B, underlined sequence is identical to the *E. coli fixB* DNA sequences. Boldface sequence is identical to the kanamycin cassette DNA sequences on pKD4.

eral attempts, we were not able to construct the corresponding *fixC* or *fixX* mutants.

Growth of the *fixA* and *fixB* mutant cultures was not stimulated by the addition of carnitine. Seim et al.. (11) showed that addition of carnitine or its dehydration product, crotonobetaine, stimulates the anaerobic growth of *E. coli* O44 K74 cultures. We confirmed that cultures of wild-type BW25113 cells grew to a higher density in the presence of carnitine than in media containing no terminal electron acceptor (Fig. 1). Addition of nitrate or carnitine plus nitrate to BW25113 also increased cell density. Growth of BW25113 with carnitine as the sole terminal electron acceptor was similar to growth of BW25113 when nitrate was provided (Fig. 1), indicating that carnitine was as effective as nitrate in stimulating wild-type BW25113 *E. coli* cultures.

In contrast, growth of the *fixA* and *fixB* mutants, AW2A and AW1B, was similar in the presence of carnitine to growth of wild-type BW25113 cells grown in the absence of a terminal electron acceptor. Addition of nitrate or carnitine plus nitrate to the *fixA* or *fixB* cultures resulted in culture densities and growth rates comparable to those seen in wild-type cells grown with carnitine, nitrate, or carnitine plus nitrate in the media (Fig. 1). Thus, both mutants could use another electron acceptor, and the lack of growth by the mutants was not due to carnitine toxicity. We concluded that the *E. coli fixA* and *fixB* genes were necessary for the metabolism of carnitine to γ-butyrobetaine in *E. coli* and suggest that these mutants are unable to use carnitine as a terminal electron acceptor.

***fixA* and *fixB* mutants do not metabolize carnitine to γ-butyrobetaine.** Supernatants from wild-type BW25113 and its *fixA* and *fixB* mutant derivatives grown in the presence of carnitine were analyzed by mass spectroscopy to determine whether carnitine was metabolized in the mutants. After incubation with carnitine, BW25113 supernatants showed only a small carnitine peak at 161.9 and a large peak of γ-butyrobetaine at 145.8, indicating almost complete conversion of carnitine to γ-butyrobetaine (Fig. 2B). In contrast, supernatants from *fixA* and *fixB* cultures contained no γ-butyrobetaine peak at 145.8, but still contained a large carnitine peak (Fig. 2C and D). The *E. coli fix* mutants were unable to metabolize carnitine to γ-butyrobetaine. Interestingly, two new peaks were observed in the mutants at 124.7 and 183.9. We are currently trying to determine the identities of these additional peaks.

Conclusion. Using a newly developed technique for chromosomal mutagenesis, we isolated *E. coli fixA* and *fixB* mutants and verified an earlier hypothesis that components of the *fixABCX* operon were necessary for anaerobic carnitine reduction. The instability of these mutants in the previous work may have been due to the method of mutagenesis used or to the different *E. coli* strains mutated. With these in-frame mutations in *E. coli fixA* and *fixB*, we have shown that the *fixA* and *fixB* mutants were unable to use carnitine anaerobically as the sole terminal electron acceptor and that these mutants were unable to metabolize carnitine to γ-butyrobetaine.

Under anaerobic conditions, *E. coli* does not assimilate the carbon and nitrogen of carnitine. It has been suggested that the primary function of the carnitine metabolic pathway in *E. coli* cells may be to use carnitine or crotonobetaine as electron acceptors during anaerobic growth in the absence of the preferred substrates (8, 9, 11, 12). Elssner et al. (7) have suggested that γ-butyrobetainyl-CoA and crotonobetainyl-CoA are substrates necessary for the conversion of carnitine to crotonobetaine and that this pathway proceeds at the CoA level, con-

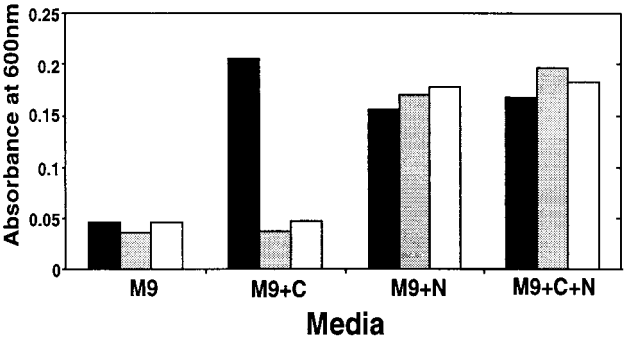

FIG. 1. Growth of *E. coli* cultures with various electron acceptors. Cultures were grown in M9 plus 0.2% glycerol under anaerobic conditions for 48 h in the presence of 10 mM carnitine, 40 mM nitrate, or carnitine plus nitrate as the available electron acceptors in the media. The data shown are representative of several experiments. Black bars, BW25113; gray bars; AW2A; open bars, AW1B.

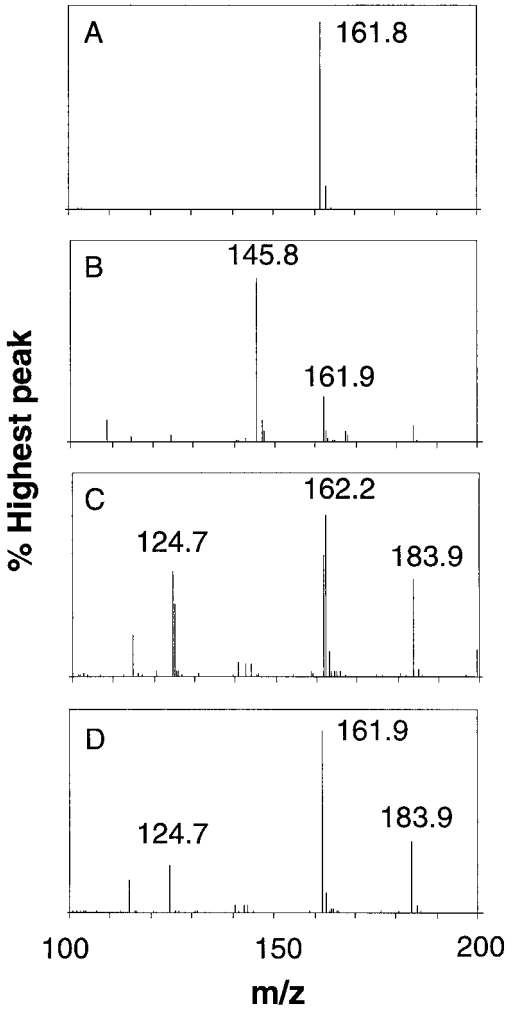

FIG. 2. Carnitine metabolism by wild-type and *fix* mutant strains. *E. coli* cultures were grown under anaerobic conditions for 48 h in the presence of 10 mM carnitine. Cultures were centrifuged to remove cells, and then supernatant samples were analyzed via mass spectroscopy. Positive ion atmospheric pressure ionization-electrospray mass spectroscopy was carried out with a Waters ZQ mass detector. The spray chamber parameters were optimized at 2.7 liters of nitrogen per min with a capillary voltage of 3.5 kV, cone voltage of 20 V, cone temperature of 520°C, and desolvation temperature of 125°C. Mass spectra were obtained from the medium control (A), BW25113 supernatant (B), AW2A supernatant (C), and AW1B supernatant (D).

cluding with the hydrolysis of γ-butyrobetainyl-CoA to give γ-butyrobetaine. This pathway, encoded by the *cai* operon, allows carnitine to be used as a terminal electron acceptor.

The divergent *fixABCX* operon is coregulated with the *cai* operon, and the products of the *fix* operon show homology to proteins found in known electron transport pathways (6, 14). Our results confirm that the *E. coli* FixA and FixB proteins are needed for carnitine reduction, and we hypothesize that they are involved in bringing reductant to CaiA, the postulated crotonobetainyl-CoA reductase (Fig. 3). Pathways that involve FixAB homologues have been shown to accept electrons from acyl-CoA dehydrogenases, pass these electrons to their FixC homologue, and finally donate the electrons to various respiratory chains through an electron transfer flavoprotein, ubiquinone oxidoreductase, homologous to FixC. If this is the mechanism of the FixABCX pathway in *E. coli*, we would expect FixX would interact with CaiA. It has been suggested that the FixABCX proteins in *S. meliloti* bring electrons to nitrogenase. In this case, it seems likely that reduced FixX is produced and is then able to transfer electrons to the nitrogenase proteins. However, FixAB homologues often interact with enoyl-CoA dehydrogenases, and we consider it possible that in *E. coli*, this pathway is run in the reverse direction, with the *E. coli* FixX protein being initially reduced and then passing electrons through the pathway to the electron transfer flavoprotein homologue encoded by the *fixAB* genes. Resolving how the Fix proteins move electrons to their substrates will require a more biochemical approach.

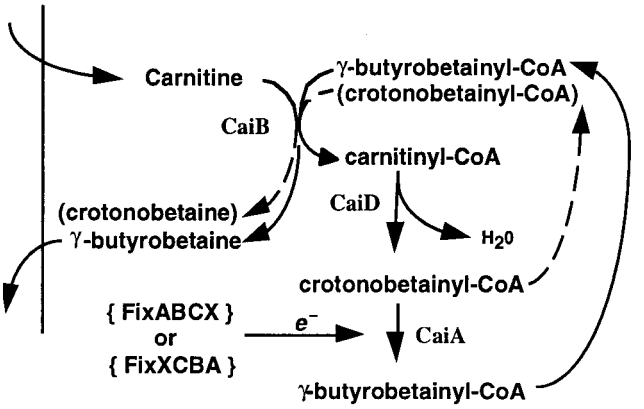

FIG. 3. Suggested pathway for the metabolism of carnitine to γ-butyrobetaine in anaerobic *E. coli* cultures. Carnitine imported into the cells is converted to its carnitinyl-CoA thioester by CaiB in a transfer reaction with γ-butyrobetainyl-CoA that releases γ-butyrobetaine. CaiD converts carnitinyl-CoA to crotonobetainyl-CoA in a dehydration reaction, and the latter is reduced to γ-butyrobetainyl-CoA by CaiA. Electrons for this reaction are postulated to be contributed by the FixABCX proteins, which are reduced during the oxidation of other substrates available to the cell.

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