JOURNAL OF BACTERIOLOGY, May 1997, p. 3378–3381 Vol. 179, No. 10
0021-9193/97/$04.00+0

DNA Sequencing and Expression of the Formyl Coenzyme A Transferase Gene, *frc*, from *Oxalobacter formigenes*

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Received 6 January 1997/Accepted 5 March 1997

Oxalic acid, a highly toxic by-product of metabolism, is catabolized by a limited number of bacterial species utilizing an activation-decarboxylation reaction which yields formate and CO_2. *frc*, the gene encoding formyl coenzyme A transferase, an enzyme which transfers a coenzyme A moiety to activate oxalic acid, was cloned from the bacterium *Oxalobacter formigenes*. DNA sequencing revealed a single open reading frame of 1,284 bp capable of encoding a 428-amino-acid protein. A presumed promoter region and a ρ-independent termination sequence suggest that this gene is part of a monocistronic operon. A PCR fragment containing the open reading frame, when overexpressed in *Escherichia coli*, produced a product exhibiting enzymatic activity similar to the purified native enzyme. With this, the two genes necessary for bacterial catabolism of oxalate, *frc* and *oxc*, have now been cloned, sequenced, and expressed.

Oxalic acid is one of the most highly oxidized organic compounds found in nature and acts as a strong chelator of cations, especially Ca^{2+} (12). These attributes limit the possibilities for its catabolism, reduce its efficacy as an energy source, and increase its toxicity for most forms of life, especially mammals. In high concentrations, oxalate can cause death in humans and animals due to its corrosive effects, while smaller amounts can cause various pathological disorders, including hyperoxaluria, pyridoxine deficiency, cardiomyopathy, cardiac conductance disorders, calcium oxalate stones (urolithiasis), renal failure, and possibly irritable bowel syndrome (15, 20, 25). Hyperoxaluria is commonly observed in patients with a variety of medical conditions, e.g., aspergillosis, Crohn's disease, steatorrhea, vestibulitis (vulvodynia), and cystic fibrosis, and in patients who have undergone ileal-bypass surgery (8, 13, 19).

A high rate of oxalate synthesis during metabolism by virtually every life form, coupled with the general inability of oxalate to be catabolized, can lead to its rapid accumulation. Depletion of oxalate from the biosphere involves several distinct mechanisms for oxalate catabolism (12, 14, 17, 22, 26). Two general mechanisms are decarboxylation to yield formic acid and CO_2 [$(COOH)_2 \rightarrow HCOOH + CO_2$] and oxidation to yield hydrogen peroxide and CO_2 [$(COOH)_2 + O_2 \rightarrow 2CO_2 + H_2O_2$]. Oxalate-catabolizing bacteria are known to use a modification of the decarboxylation mechanism in which coenzyme A (CoA) is coupled to an oxalate ion prior to decarboxylation. Aerobic soil bacteria, e.g., *Alcaligenes oxalaticus* and *Thiobacillus novellus*, are capable of converting oxalic acid to formic acid and CO_2 in a reaction that requires ATP, CoA, Mg^{2+}, thiamine pyrophosphate, and acetate (reviewed in reference 4). A similar mechanism appears to be typical of anaerobic bacteria, including species of *Pseudomonas*, *Clostridium*, and *Oxalobacter.*

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Oxalobacter formigenes is a recently identified bacterium colonizing the gastrointestinal tracts of vertebrates, including humans (2). The most unusual feature of this bacterium is its total dependence on oxalate as an energy source (5, 9). In order to catabolize oxalic acid, *O. formigenes* uses two proteins: (i) formyl-CoA transferase, an enzyme that activates an oxalate molecule by cycling a CoA moiety from formyl-CoA, and (ii) oxalyl-CoA decarboxylase, an enzyme that decarboxylates an activated oxalate molecule (oxalyl-CoA). For each molecule of oxalyl-CoA that is decarboxylated, one proton is consumed, resulting in a proton gradient that, coupled with a membrane potential established by the oxalate/formate transport, drives ATP synthesis. Formyl-CoA transferase is encoded by the *frc* gene, while oxalyl-CoA decarboxylase is encoded by the *oxc* gene (6, 18). A third protein important in this reaction is the $oxalate^{-2}/formate^{-1}$ antiporter, a membrane-bound transport protein that simultaneously imports oxalic acid into and exports formic acid out of *O. formigenes* (21).

Interest in *O. formigenes* has increased recently due to its possible role in regulating oxalate homeostasis in vertebrate animals both by scavenging oxalate in the gut from dietary sources and by maintaining a transepithelial gradient favorable for movement of oxalate from blood to the intestinal lumen (11). In fact, preliminary studies have found that individuals with enteric hyperoxaluria and recurrent kidney calcium oxalate stone formation lack intestinal colonization by *O. formigenes* (3, 10, 16). Despite the relative importance of oxalate anabolism and catabolism to the environment and the fact that the pathways for oxalate catabolism have been known for nearly 30 years, only recently has information begun to surface concerning the genetic elements controlling the proteins involved in oxalic acid degradation. In the present study, we describe the cloning and sequencing of the *frc* gene from *O. formigenes*.

Cloning and sequencing of the *frc* gene. N-terminal sequencing was carried out on both the purified, enzymatically active native formyl-CoA transferase protein (7) and a 30- to 40-kDa fragment generated following partial trypsinization of the native protein. Since the two sequences were distinct, it was concluded that a fragment was cleaved from the N-terminal

portion of the native protein by trypsin. By selecting a unique stretch of the amino acid sequence obtained from the trypsin-digested protein, a degenerate oligonucleotide (5′-GG[A/G] TTA[A/G][C/T]TC[C/T]TG[A/G/T]AT[A/G]TA[C/T]TC-3′) was synthesized (Interdisciplinary Center for Biotechnology Research [ICBR] Oligonucleotide Synthesis Laboratory, University of Florida, Gainesville) and used as a primer to sequence about 200 bp through the DNA region encoding the amino acid sequence. A homologous oligonucleotide (5′-TTC ATGTCCAGTTCAATCGAACG-3′) was synthesized from this DNA sequence and used in combination with a second degenerate oligonucleotide (5′-GA[C/T]TT[C/T]AC[A/G/C/T] CA[C/T]GT[A/G/C/T]GA[A/G]GC-3′), based on the N-terminal amino acid sequence of the native protein, as a primer in PCR with *Hin*dIII-digested genomic DNA prepared from *O. formigenes OxB* as a template. This resulted in a PCR product of approximately 200 bp that was cloned into the pCR2.1 vector system (Invitrogen, Inc., San Diego, Calif.). Sequencing of the PCR product revealed a 191-bp sequence of the *frc* gene that, when translated, contained overlapping portions of the amino acid sequences present in the N-terminal sequences. This permitted the synthesis of homologous oligonucleotides.

Southern blot analyses of *Hin*dIII-digested genomic DNA from *O. formigenes* with the homologous oligonucleotides as DNA probes identified the presence of the *frc* gene on a DNA fragment of approximately 2.5 to 3.0 kb. A subgenomic DNA library was constructed by ligating the 2.0- to 4.0-kb *Hin*dIII fragments of *O. formigenes OxB* genomic DNA into pUC18. Clones containing the *frc* gene were identified by colony filter hybridization and confirmed by Southern blotting with the homologous oligonucleotides labelled with digoxigenin (Boehringer-Mannheim, Indianapolis, Ind.). Both the positive and negative strands of one plasmid clone with a 3.0-kb insert containing the *frc* gene were sequenced completely with overlapping internal oligonucleotide primers and the Applied Biosystems, Inc., model 373A automated sequencer (ICBR DNA Sequencing Laboratory, University of Florida, Gainesville).

The complete nucleotide and deduced amino acid sequences of the *frc* gene are presented in Fig. 1. One open reading frame beginning with a methionine codon, as expected, extends for 1,284 bp and ends with a TGA stop codon. Assuming the ATG at position 1 to be the translation initiation codon, a region similar to the consensus prokaryotic ribosome-binding site, 5′-GAAATG, is found eight bases upstream, between −8 and −13. In addition, sequences resembling a putative TATA box, 5′-GAATAA, as well as a −35 RNA polymerase-binding site are present at −45 to −40 and −77 to −72 bp upstream, respectively. The TGA stop codon starting at position 1284 indicates the end of the coding region, while an 8-bp perfect inverted repeat located between positions 1311 and 1319 and between 1324 and 1331 followed by seven thymidines suggests a ρ-independent termination sequence (27). Similar ρ-independent termination signals are present in both the *oxc* (18) and *OxlT* (1) genes. Thus, despite the fact that coordinated expression of *frc* and *oxc* is essential for oxalate metabolism and production of energy in *O. formigenes*, the two genes are not part of a polycistronic operon: restriction enzyme mapping has indicated that they must reside at least 5 kb from one another on the chromosome (Fig. 2A), and both *frc* and *oxc* contain independent promoter regions (Fig. 2B) and termination signals. Sequence data of the entire 3-kb fragment indicated the presence of another open reading frame, but its identity remains unknown. This unknown gene was used to compare further the upstream genetic elements of regulation present in *O. formigenes* (Fig. 2B).

The formyl-CoA transferase enzyme has a reported molecular mass of 45 kDa and a pI of 4.7 as determined by isoelectric focusing (7). The deduced amino acid composition of the *frc* gene would give the formyl-CoA transferase protein a calculated molecular mass of 47,331 Da and a pI of 5.2. Comparison of the number of lysine plus arginine residues (total of 45) with the number of aspartic acid plus glutamic acid residues (total of 58) reveals a significant excess of acidic amino acids, which is in line with the isoelectric point observed for the native protein.

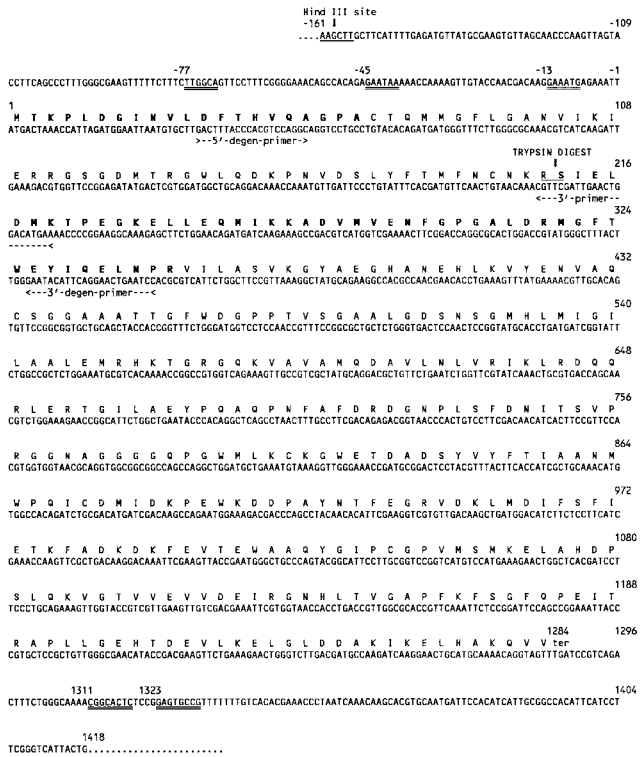

FIG. 1. Nucleotide and translated amino acid sequences of the *frc* gene and flanking regions. Bold letters (amino acid residues 2 to 21 and 69 to 118) represent amino acid residues determined by N-terminal protein sequencing. Double-underlined bases indicate the putative ribosome-binding site (−13 to −8), TATA box (−45 to −40), polymerase-binding site (−77 to −72), and the ρ-independent termination sequence (+1311 to +1319 and +1324 to +1331) shown upstream and downstream of the open reading frame, respectively.

Overexpression of the *frc* gene in *Escherichia coli*. To express the cloned *frc* gene, a PCR-amplified product of the open reading frame was cloned into the prokaryotic expression vector pKK223-3 (Pharmacia Biotech, Piscataway, N.J.), and the recombinant vector was grown in *E. coli* JM109. Two 32-mer oligonucleotides, ACACAC*GAATTC*ATGACTAAACCATT AGATGG (5′ primer) and ATATAT*GAATTC*GGGCAGTT TATGGCAGTAGTG (3′ primer), each containing a single *Eco*RI site (shown in italics) plus a sequence homologous to the 5′ or 3′ end of the *frc* gene, were used in PCR to amplify the open reading frame of the gene from the *Hin*dIII-digested *O. formigenes* genomic DNA preparation. The PCR product was digested with *Eco*RI and ligated into pKK223-3 downstream of the *Taq* promoter. Recombinant plasmids were used to transform *E. coli* JM109. Transformants were selected by ampicillin resistance, and the orientation of each insert was verified by restriction enzyme map analysis. Two recombinant plasmids, referred to as pKK-Frc^{f} and pKK-Frc^{r}, showed a positive and reverse orientation of the inserts, respectively. *E. coli* transformed with either pKK223-3 or one of the recombi-

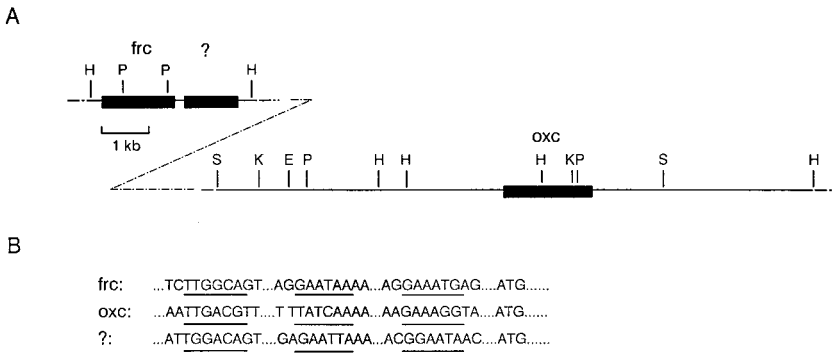

FIG. 2. (A) Restriction enzyme map of the chromosomal regions surrounding the *frc* and *oxc* genes and (B) comparison of the genetic elements within the upstream promoter regions of *frc* (present study), *oxc* (18), and an unidentified gene (indicated by a question mark) residing 3′ to *frc* (unpublished data). The dashed line in panel A indicates an unknown relationship and distance between *frc* and *oxc*. In panel B, from left to right, the putative ribosome-binding site, the TATA box, and polymerase-binding sites are shown underlined. The sequence of the unidentified gene indicates it is not the *OxlT* gene encoding the oxalate/formate transporter.

nant plasmids, pKK-Frc^f and pKK-Frc^r, were grown to early log phase and then induced with 4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Expression of protein was monitored every 30 min for 2 h. Bacterial cultures were harvested by centrifugation, the bacteria were sonicated, and their lysates were size separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 3, purified formyl-CoA transferase (lane 1) ran as a 45-kDa protein under these conditions. Lysates of pKK-Frc^f showed significantly increased expression over time of a protein with a similar molecular mass following IPTG induction. No induction of this protein occurred in *E. coli* transformed with the parental pKK223-3 vector (Fig. 3, lanes 6 and 7) or with pKK-Frc^r (data not shown).

Enzymatic activity of the cloned *frc* gene product. To confirm that the *frc* gene product expressed in *E. coli* could participate in the enzymatic degradation of oxalic acid in a manner similar to native formyl-CoA transferase, enzymatic reactions were performed. Formyl-CoA transferase activity was determined by estimating formate production by measuring the NAD reduction in the presence of formate dehydrogenase. As shown in Fig. 4, both the native and cloned forms of formyl-CoA transferase proved capable of promoting catabolism of oxalate to formate in the presence of oxalyl-CoA, oxalyl-CoA decarboxylase, and appropriate cofactors. No enzymatic activity of the *frc* gene product was observed when oxalyl-CoA was replaced with succinyl-CoA in the reaction. In addition, *E. coli* lysates transformed with pKK223-3 or with pKK-Frc^r lacked enzymatic activity.

Conclusions. Formyl-CoA transferase, like oxalyl-CoA decarboxylase, is a major cytosolic protein of *O. formigenes*. Together these two enzymes constitute up to 20% of the total protein of the cell (6, 7), a point underlying the absolute requirement for oxalate catabolism for the survival of this bacterium (4, 6, 7). The presence in *O. formigenes* of formyl-CoA transferase, which activates oxalic acid to oxalyl-CoA prior to its decarboxylation by oxalyl-CoA decarboxylase, permits a significant conservation of energy in a system which is limited in its ability to derive energy (7). While both formyl-CoA transferase and oxalyl-CoA decarboxylase are required for *O.*

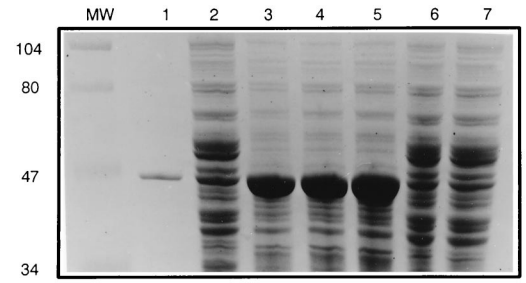

FIG. 3. SDS-PAGE analysis of cloned formyl-CoA transferase in *E. coli*. Sonicated cell lysates of *E. coli* JM109 transformed with pKK-Frc^f were size fractionated by SDS-PAGE prior to (lane 2) and following 60, 90, and 120 min of (lanes 3 to 5) IPTG induction and were visualized by Coomassie blue staining. Purified native enzyme (lane 1) and *E. coli* JM109 transformed with the vector system pKK223-3 prior to (lane 6) and 2 h after (lane 7) IPTG induction are also shown. MW, molecular weight standards; numbers at left are molecular weights, in thousands.

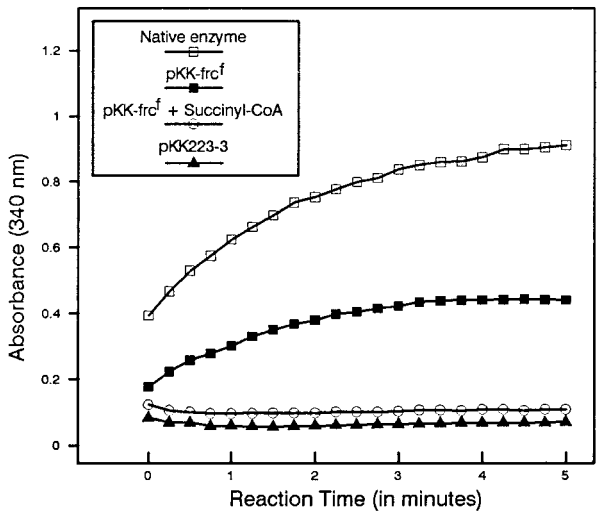

FIG. 4. Enzymatic activity of the cloned formyl-CoA transferase gene product. Sonicated cell lysates of *E. coli* JM109 transformed with pKK-Frc^f or pKK223-3 (negative control) were tested for enzymatic activity in a reaction mixture containing 50 mM KH_2PO_4 (pH 6.7), 5 mM $MgCl_2$, 2 mM thiamine pyrophosphate, 10 mM potassium oxalate, 0.375 mM oxalyl-CoA, 0.05 U of oxalyl-CoA decarboxylase, 0.25 IU of formate dehydrogenase, and 1.0 mM β-NAD. Reactions were initiated by the addition of formyl-CoA transferase (10 μg of purified enzyme containing 0.03 U of activity or 20 μg of protein of the sonicated lysate). Changes in A_{340} were measured continuously at 37°C in a thermal cuvette holder. Purified native formyl-CoA transferase (0.03 U) was used as a positive control. Succinyl-CoA was substituted for oxalyl-CoA in one reaction with lysates of *E. coli* JM109 transformed with pKK-Frc^f.

formigenes to derive energy, the present study indicates that their respective genes, *frc* and *oxc*, are not part of a polycistronic operon (because the genes are not located in close proximity) and are apparently not part of a regulon (because repeat elements upstream of the *oxc* gene promoter region are absent from the *frc* gene), and thus, they are not coregulated. However, cloning and sequencing of the *frc* gene (present study), the *oxc* gene (18), and the *OxlT* gene (1) have revealed that all three genes have single open reading frames with consensus prokaryotic promoter elements and ρ-independent termination sequences. It will be interesting to discover if other bacteria which utilize similar enzymes to catabolize oxalate, e.g., the soil bacterium *A. oxalaticus*, have similar genetics.

N-terminal sequencing of the native formyl-CoA transferase revealed the first amino acid to be threonine, strongly suggesting that its N-terminal methionine is cleaved by methionine aminopeptidase. Virtually all bacterial proteins contain an *N*-formylmethionine residue at the N terminus when translation is initiated. Many long-lived bacterial proteins contain an alanine, serine, or threonine at position 2. However, the presence of a deformylase enzyme, which cleaves the formyl group from the methionine residue, plus methionine aminopeptidase, which subsequently cleaves the N-terminal Met, results in proteins starting with Ala, Ser, or Thr at position 1 (24). Formyl-CoA transferase appears to be one of these modified proteins.

Formyl-CoA transferase and oxalyl-CoA decarboxylase are especially important enzymes because of their possible involvement in detoxification of oxalate in the intestinal tracts of animals and humans. The possibility of expressing biologically active enzymes from cloned genes can offer a unique opportunity to provide the oxalate-degrading capacity to individuals suffering from an increased body burden of oxalate and from the resulting disorders. Clinical studies emphasizing the role of *O. formigenes* in the pathogenesis of enteric hyperoxaluria and recurrent calcium oxalate urolithiasis (10, 16) further illustrate the importance of these oxalate-degrading enzymes and their potential application in preventing such disorders. Working towards this goal, we have successfully used unique regions of the *frc* and *oxc* gene sequences in our laboratory to develop probes and primers for DNA-based detection of *O. formigenes* bacteria in human fecal samples (23). Such rapid, sensitive, and specific tests facilitate identification of hyperoxaluric patients, who lack this normal intestinal oxalate-degrading bacterium.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the GenBank TM/EMBL database under accession no. U82167.

We thank Savita Shankar for assistance in sequencing the *frc* gene and Janet Cornelius and Vincent Lopez for expert technical assistance and help in preparing the manuscript.

This work was supported in part by PHS grant DK20586 and a research grant from Ixion Biotechnology, Inc., Alachua, Fla. H. Sidhu was supported by a research fellowship to the University of Florida from Ixion Biotechnology, Inc.

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