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Organization and Nucleotide Sequences of the Genes Encoding the Biotin Carboxyl Carrier Protein and Biotin Carboxylase Protein of *Pseudomonas aeruginosa* Acetyl Coenzyme A Carboxylase

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The genetic organization of the *Pseudomonas aeruginosa* acetyl coenzyme A carboxylase (ACC) was investigated by cloning and characterizing a *P. aeruginosa* DNA fragment that complements an *Escherichia coli* strain with a conditional lethal mutation affecting the biotin carboxyl carrier protein (BCCP) subunit of ACC. DNA sequencing and RNA blot hybridization studies indicated that the *P. aeruginosa accB* (*fabE*) homolog, which encodes BCCP, is part of a 2-gene operon that includes *accC* (*fabG*), the structural gene for the biotin carboxylase subunit of ACC. *P. aeruginosa* homologs of the *E. coli accA* and *accD*, encoding the α and β subunits of the ACC carboxyltransferase, were identified by hybridization of *P. aeruginosa* genomic DNA with the *E. coli accA* and *accD*. Data are presented which suggest that *P. aeruginosa accA* and *accD* homologs are not located either immediately upstream or downstream of the *P. aeruginosa accBC* operon. In contrast to *E. coli*, where BCCP is the only biotinylated protein, *P. aeruginosa* was found to contain at least three biotinylated proteins.

The committed step in the biosynthesis of long-chain fatty acids in all prokaryotes is the ATP-dependent carboxylation of acetyl coenzyme A (CoA) to malonyl CoA by the enzyme acetyl CoA carboxylase (ACC) (1). This essential carboxylation reaction has been studied in *Escherichia coli* for over 20 years but has received little attention in other prokaryotes. In *E. coli*, the carboxylation proceeds through two distinct reactions and involves a protein that can be dissociated into four subunits (1, 27): biotin carboxyl carrier protein (BCCP), a dimer of two 16,700-Da subunits; biotin carboxylase (BC), composed of two 51,000-Da subunits; and carboxyltransferase (TC), a tetramer composed of two 33,000-Da β subunits and two 35,000-Da α subunits (13, 21, 22).

During the 1970s, Harder and coworkers (15) isolated several mutants affected in ACC, but the assignment of the lesions to specific subunits of ACC remained inconclusive until recently (21, 22, 23). Several independent laboratories (2, 5, 19, 21, 25, 26) recently cloned and sequenced the *accB* (*fabE*) and *accC* (*fabG*) genes encoding the BCCP and BC subunits of *E. coli* ACC. *accB* and *accC* were found to form a 2-gene operon located at min 72 on the Bachmann linkage map (4), with *accB* as the promoter-proximal gene (5, 21). The two genes that encode the α and β subunits of *E. coli* TC have also been cloned, sequenced, and mapped. Interestingly, neither *accD* (*dedB* [*usg*]), which encodes the β subunit of TC, nor *accA*, which encodes the TC α subunit, is linked to the *accBC* operon or to the other gene; *accA* and *accD* map at min 4 and 50, respectively (22, 23).

The *E. coli* ACC is the only biotinylated protein in *E. coli* (10), and the BC subunit of the *E. coli* enzyme will accept both free biotin and BCCP as substrates. For these reasons, the *E. coli* ACC has been used extensively as a model for studying the mechanism of biotin-dependent carboxylation. The four subunits of *E. coli* ACC participate in the carboxylation reaction in the following manner. In the first partial reaction, BC transfers a carboxyl group from bicarbonate to a carrier molecule, BCCP, which contains the protein's essential biotin cofactor. The final partial reaction, which is catalyzed by TC, involves the transfer of the carboxyl group from BCCP to an acceptor molecule, acetyl CoA, to form malonyl CoA, required for fatty acid chain elongation (1, 27).

Only a few prokaryotic ACCs have been studied, and some of these have a subunit structure that differs from that of the *E. coli* ACC. Enzymes with ACC activity have been isolated from two species of *Mycobacterium*. The enzyme from *Mycobacterium phlei* purifies as "an aggregated complex which does not separate into its constituent enzymes" (8). The enzyme from *Mycobacterium smegmatis* can be dissociated into two subunits: a 57,000-Da subunit and a 64,000-Da biotin-containing subunit (16). The ACCs isolated from *M. phlei* and *M. smegmatis* are also able to carboxylate propionyl CoA, an activity which is absent from the *E. coli* ACC (8, 16). The ACCs from two other microorganisms, *Streptomyces erythreus* and *Pseudomonas citronellolis*, have been described. The ACC from *S. erythreus*, like the enzyme from *M. smegmatis*, consists of two dissimilar subunits: a biotin-containing 67,000-Da subunit and a biotin-free 61,000-Da subunit (17). The *S. erythreus* enzyme carboxylates several acyl CoA acceptors: acetyl CoA, propionyl CoA, and butyryl CoA (17). The *P. citronellolis* ACC contains four protein subunits with molecular weights similar to those of the *E. coli* ACC (9). In addition, a protein fraction containing the *P. citronellolis* BCCP functions as an effective CO_2 carrier in an in vitro system containing *E. coli* BC and TC (9). None of the genes encoding the *P. citronellolis* ACC subunits have been cloned, and thus comparisons with the *E. coli* genes have not been possible.

We have been interested in the genetic organization of the *Pseudomonas aeruginosa* ACC and its structural similarity with other ACCs. In this report, we demonstrate that a DNA fragment containing the genes encoding the BCCP and BC subunits of the *P. aeruginosa* ACC will rescue an *E. coli* strain that expresses a defective BCCP subunit. In addition, we present the nucleotide sequence of an approximately 3.2-kb DNA fragment that includes the *P. aeruginosa accB* and *accC* genes and flanking sequences and demonstrate that the *P. aeruginosa accB* and *accC* genes form a 2-gene operon. Using a modification of the Western blotting (immunoblotting) tech-

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nique, we found that the *P. aeruginosa* BCCP is one of at least three soluble, biotin-containing proteins present in *P. aeruginosa*. DNA sequences from *P. aeruginosa* showing homology to the *E. coli accA* and *accD* genes are probably unlinked to the *P. aeruginosa accBC* operon or to each other.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. All of the bacterial strains used in this work were routinely cultured in LB broth or on LB agar (3). *P. aeruginosa* PAO1 (28) and *E. coli* DH5α (Bethesda Research Laboratories Inc., Gaithersburg, Md.), which was used as the host for DNA subcloning experiments and for storage of plasmid constructs, were cultured at 37°C. *E. coli* strains containing temperature-sensitive lesions in BCCP, *accB22*(Ts) [*fabE22*(Ts)], and *E. coli* LA1-6, which contains a temperature-sensitive lesion in the β subunit of ACC carboxyltransferase, *accD6*(Ts) (*fab-6*) (15, 23), were cultured at 30°C. *E. coli* EE451, which was used for library screening, was constructed in the following manner. First, a kanamycin-resistant transposon located near min 72 (*zhc-3170*::Tn*10kan*) of the CAG18606 (33) chromosome was transduced into L8 [*accB22*(Ts)] (21, 31) to create strain EE450 [*accB22*(Ts)... *zhc-3179*::Tn*10kan*]. A transducing lysate grown on EE450 was used to infect MM294 (14), and a kanamycin-resistant colony with a temperature-sensitive growth phenotype was saved as EE451. Transduction of the *accB22*(Ts) mutation into bacterial strains was done by using phage P1*vir* (32). When appropriate, media were supplemented with antibiotics at the following concentrations: penicillin, 300 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 20 μg/ml.

Cosmid cloning vector pMW1216 (38) was constructed by inserting the λ *cos* site from plasmid pVK102 (18) as a 1.7-kb *Bgl*II fragment into *Bgl*II-digested pRK310 (7).

Gene fragments used to probe the *P. aeruginosa* genome for sequences with homology to the *E. coli accA* and *accD* were amplified by the polymerase chain reaction (PCR) using *E. coli* DNA as template and synthetic primers based on the *E. coli accA* and *accD* DNA sequences (22).

Recombinant DNA techniques. Chromosomal and plasmid DNA isolation, gel electrophoresis, restriction endonuclease digestion, ligation reactions, lambda packaging and transfection, transformation of plasmid DNA, and DNA hybridization experiments were performed by standard methods (3, 24). DNA sequencing was done by the dideoxy chain termination method of Sanger et al. (29) with [α-^{35}S]dATP and the T7 DNA polymerase system (35) of U.S. Biochemical Corp. (Sequenase version 2.0). To ensure accuracy, both strands of DNA fragments were sequenced. DNA sequences were compiled and analyzed with computer software from IntelliGenetics, Inc. (Mountain View, Calif.) and the MacVector software from International Biotechnologies, Inc. (New Haven, Conn.). Oligonucleotide primers used for PCR and DNA sequencing were synthesized by the β-cyanoethyl phosphoramide method (3) with an Applied Biosystems Inc. oligonucleotide synthesizer.

RNA isolation and RNA (Northern) blot analysis. Total cellular RNA for RNA blot analysis and primer extension mapping experiments was prepared by the hot phenol extraction method from log-phase cultures of PAO1 (3). RNA (approximately 50 μg) was electrophoretically separated in a 1.2% (wt/vol) agarose gel containing 0.66 M formaldehyde and transferred to a nitrocellulose membrane (Schleicher and Schuell, Inc., Keene, N.H.) according to standard methods (24). *P. aeruginosa* DNA fragments used as hybridization probes were produced by PCR amplification and labeled with ^{32}P by the random-priming method (11). The DNA fragment used as the probe specific for *accB* contained the complete *accB* coding sequence. The probe specific for *accC* encoded the first 203 amino acids of that protein. Radiolabeled probes were hybridized to the immobilized RNA by standard procedures (24).

Enumeration of biotin-containing proteins. Soluble proteins were extracted from late-log-phase cultures of *P. aeruginosa* essentially as described by Silhavy and coworkers (32). Samples containing approximately 500 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) (12% polyacrylamide) (20) and electrophoretically transferred to nitrocellulose (36). The nitrocellulose filter was washed for 2 h with TTBS (50 mM Tris-Cl [pH 7.5], 200 mM NaCl, 0.5% Tween 20) containing 1% (wt/vol) skim milk to saturate nonspecific protein-binding sites. Streptavidin conjugated with alkaline phosphatase (Bethesda Research Laboratories Inc.) was added to a final concentration of 2 μg/ml, and the filter was incubated overnight at 4°C. The filter was washed four times with TTBS and once with substrate buffer (0.1 M Tris-Cl [pH 9.5], 0.1 M NaCl, 50 mM $MgCl_2$), and then it was incubated with substrate buffer containing 0.044 mg of nitroblue tetrazolium (Bethesda Research Laboratories Inc.) per ml and 0.033 mg of 5-bromo-4-chloro-3-indolylphosphate (Bethesda Research Laboratories Inc.) per ml to detect biotinylated proteins.

Nucleotide sequence accession number. The sequences of the *P. aeruginosa accB* and *accC* genes (see Fig. 2) have been deposited in the GenBank and EMBL sequence libraries under the accession number L14612.

RESULTS AND DISCUSSION

Cloning a *P. aeruginosa accB* homolog. A *P. aeruginosa* cosmid library was prepared by partially digesting chromosomal DNA from strain PAO1 with *Bam*HI, ligating the DNA with *Bam*HI-digested pMW1216, and then packaging the DNA into phage particles. The phage lysate was transfected into *E. coli* EE451, and cosmid clones capable of complementing the *accB22*(Ts) mutation in EE451 were selected on the basis of two criteria: growth at 42°C, the nonpermissive temperature for strain EE451; and resistance to tetracycline, conferred by the pMW1216 cosmid cloning vector.

Six cosmid clones ranging in size from 15 to 20 kb and satisfying the two selection criteria were examined in detail. ACC is thought to catalyze the committed step in fatty acid biosynthesis in both prokaryotes and eukaryotes, and subunits of the *E. coli* ACC are highly homologous to the ACCs of rats and chickens as well as to other biotin-dependent carboxylases (19, 21, 22). It therefore seemed likely that the BCCP sequence would be highly conserved between bacteria, especially in the region surrounding the Glu-Ala-Met-Lys-Met pentapeptide to which the essential biotin cofactor binds (34). Southern hybridization of *Bam*HI-digested *P. aeruginosa* chromosomal DNA with a gene fragment containing a 49-amino-acid portion of the *E. coli accB* gene, including the Glu-Ala-Met-Lys-Met sequence, revealed a single band of hybridization of approximately 3.3 kb. All six of the cosmid clones examined contained a 3.3-kb *Bam*HI fragment.

DNA from one cosmid clone was digested with *Bam*HI and ligated with *Bam*HI-digested Bluescript KS+ (Stratagene Cloning Systems, La Jolla, Calif.). A Bluescript KS+ clone containing a 3.3-kb fragment and capable of complementing the thermosensitive defect in strain EE451 was isolated and named pCGN3931 (Fig. 1). A second clone, pCGN3932, which contained the same 3.3-kb fragment cloned in the opposite

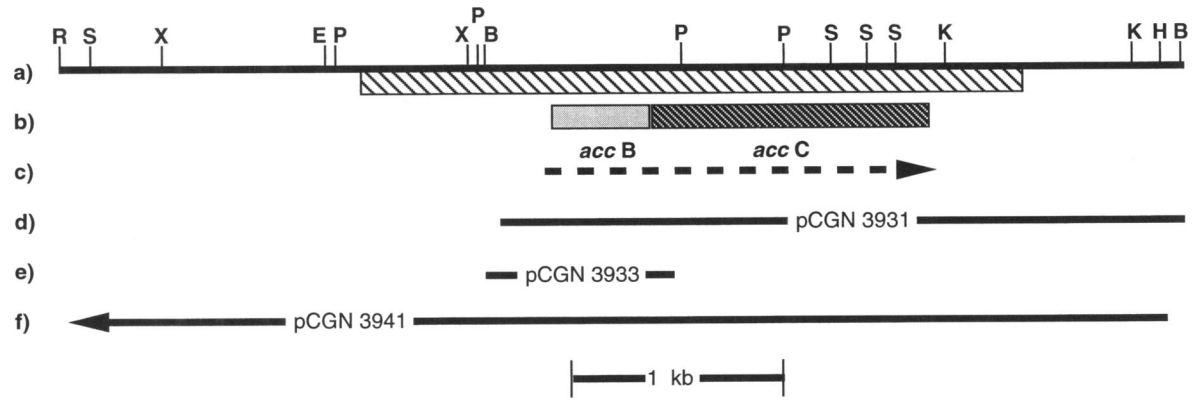

FIG. 1. Restriction map of the *P. aeruginosa* chromosomal DNA region containing the *accBC* operon. (a) Restriction map of a chromosomal segment containing *accB* and *accC* and extent of the DNA sequence reported (striped box). R, *Eco*RV; S, *Sal*I; X, *Xho*I; E, *Eco*RI; P, *Pst*I; B, *Bam*HI; K, *Kpn*I; H, *Hin*dIII. The extent of the coding regions for *accB* and *accC* (b), the approximate extent of the *accBC* transcript (c), the cloned inserts in pCGN3931 (d) and pCGN3933 (e), and the rightward 5.35 kb of the 11.2-kb insert in pCGN3941 (f) are also shown. All three plasmids listed above complement the *accB22*(Ts) lesion in EE451.

orientation from that in pCGN3931, was also found to complement strain EE451, suggesting that the *P. aeruginosa* gene responsible for complementation was expressed from its native promoter.

Hybridization of *Pst*I-digested pCGN3931 DNA with a restriction fragment containing a portion of the *E. coli accB* gene indicated that an approximately 800-bp *Pst*I fragment located close to the end of the 3.3-kb *Bam*HI fragment contained at least a portion of the *P. aeruginosa accB* homolog. The 800-bp *Pst*I fragment from pCGN3931 was subcloned into Bluescript KS+, and the resulting plasmid, pCGN3933, was capable of rescuing strain EE451 for growth at 42°C, suggesting that the 800-bp *Pst*I fragment contained the entire *P. aeruginosa accB* gene and that the *P. aeruginosa* BCCP can interact functionally with *E. coli* BC and TC.

DNA sequence of *accB* and flanking regions. Synthetic oligonucleotide primers were used to sequence the *accB* gene contained on pCGN3933 and flanking sequences contained on pCGN3931 and pCGN3941 (described below). Analysis of the approximately 3.2-kb sequence revealed the two open reading frames (ORFs) shown in Fig. 2: a 471-nucleotide ORF extending from nucleotides 861 to 1331 and a 1,350-nucleotide ORF spanning nucleotides 1349 to 2698. Both of these ORFs are preceded by probable ribosome binding sites (Fig. 2). The 471-nucleotide ORF is predicted to encode a 156-amino-acid product with a molecular mass of 16.5 kDa. Comparison of the deduced amino acid sequence of this 471-nucleotide ORF with the *E. coli* BCCP reveals striking homology and identifies this *P. aeruginosa* ORF as the structural gene for BCCP, hereafter designated *accB*. The 1,350-nucleotide ORF is predicted to encode a 449-amino-acid protein with a molecular mass of 48.9 kDa. On the basis of its extensive homology with the 449-amino-acid *E. coli* BC subunit, we believe this 1,350-nucleotide *P. aeruginosa* ORF encodes *accC*. The fractional G+C contents of the *P. aeruginosa accB* and *accC* genes, 64.8 and 67.3%, respectively, are substantially higher than the G+C contents of the same genes in *E. coli* but consistent with the G+C content of the *P. aeruginosa* genome (67.2%).

The DNA sequence upstream of the *E. coli accB* contains a 98-bp AT-rich sequence which exhibits the characteristics of a "bent structure" (25). When subjected to PAGE, a fragment containing this 98-bp DNA sequence exhibits an unusually slow mobility (25). The importance of this AT-rich sequence in the expression of the *E. coli accBC* operon has not been established, but this sequence is not required for transcription (5). We note that the sequence upstream of the *P. aeruginosa accB* does not contain a sequence with homology to the 98-bp AT-rich region present in *E. coli* (Fig. 2).

A putative σ^{70}-requiring promoter for the *E. coli accBC* operon has been identified; this DNA sequence has limited homology to the σ^{70} consensus promoter (21). Our inspection of the sequence upstream of the *P. aeruginosa accBC* operon failed to reveal a good match to the *E. coli* σ^{70} promoter recognition sequence. However, our complementation data suggest that the promoter for the operon is located within the *Bam*HI fragment contained on plasmid pCGN3931.

Transcription of *accB* and *accC*. Our finding that *accB* and *accC* are separated by only 17 nucleotides suggests that these two genes are cotranscribed. To verify that *accB* and *accC* are cotranscribed and to determine whether these genes are part of a larger polycistronic message, we performed RNA blot hybridization studies. Hybridization studies performed with either a probe specific for *accB* or one specific for *accC* detected an mRNA species of approximately 1.8 kb (Fig. 3), indicating that *accB* and *accC* are cotranscribed and are the only two genes in that RNA transcript. Because the primers used were specific for *accB* or *accC*, the minor bands shown in both lanes A and B of Fig. 3 are almost certainly degradation products.

In agreement with our finding that *accB* and *accC* are part of a dicistronic message, we note that the DNA sequence following *accC* contains a region of hyphenated dyad symmetry (Fig. 2) that could potentially form an RNA stem-and-loop structure resembling a ρ-independent transcription terminator (6). Comparison of the loop region of this structure (5′-UCCGG-3′) with the loop structure found in the *E. coli* phage T4 terminator (5′-CUUCGG-3′), which has been shown to be important for maintaining the terminator structure (37), lends support to the idea that this structure has an important biological role in *P. aeruginosa*. These data suggest that the *P. aeruginosa accB* and *accC* genes are organized in a manner similar to the organization of the *E. coli accB* and *accC* genes, where *accB* is promoter proximal to *accC* and *accC* is separated from a downstream gene by a ρ-independent transcription terminator (19, 21).

Thus, four pieces of data suggest that *accB* and *accC* form a

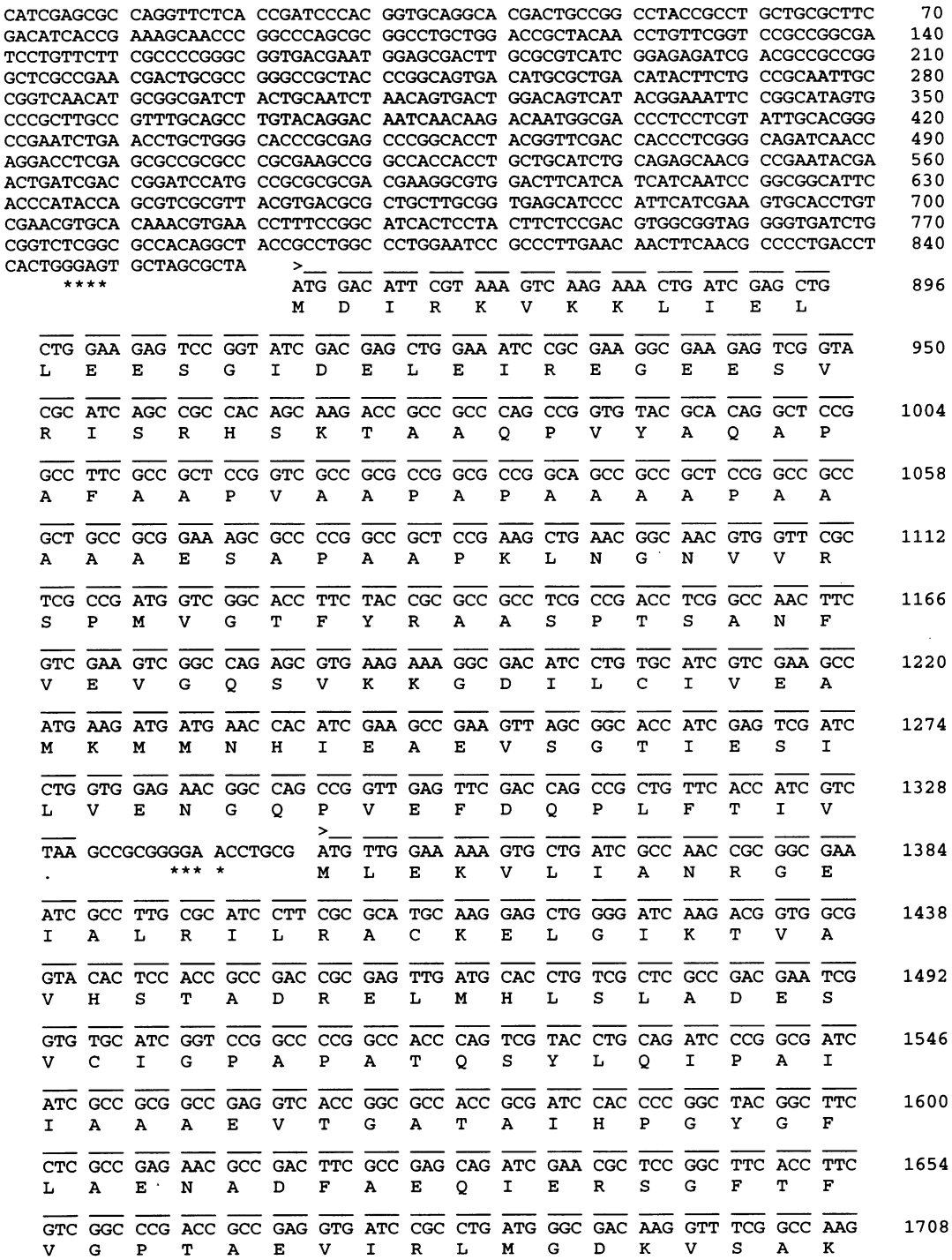

FIG. 2. Nucleotide sequence of the *accBC* operon and flanking sequences. The predicted amino acid sequences of the AccB and AccC proteins are written in one-letter abbreviations below the nucleotide sequences. Nucleotides 861 to 1331 and 1349 to 2698 compose the *accB* and *accC* structural genes, respectively. A region of hyphenated dyad symmetry that resembles a ρ-independent transcription terminator is underlined. Putative ribosome binding sites are indicated by asterisks.

2-gene operon in *P. aeruginosa*. (i) RNA hybridization experiments suggest that *accB* and *accC* are encoded by the same 1.8-kb message. (ii) DNA sequence analysis suggests that *accB* and *accC* are translationally coupled. (iii) Mutant complementation studies indicate that the promoter for *accB* lies between nucleotide 572 (the *Bam*HI site of plasmid pCGN3931) (Fig. 1) and nucleotide 861 (the Met start codon of *accB*) as shown in Fig. 2. (iv) A probable terminator sequence is located immediately downstream of *accC*.

Homology with *E. coli* ACC subunits and other biotin-

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GAC GCC ATG AAG CGC GCC GGC GTC CCC ACC GTG CCG GGC TCC GAC GGC CCG CTG     1762
D   A   M   K   R   A   G   V   P   T   V   P   G   S   D   G   P   L

CCG GAA GAT GAA GAG ACC GCC CTG GCG ATC GCC CGC GAG GTC GGC TAC CCG GTG     1816
P   E   D   E   E   T   A   L   A   I   A   R   E   V   G   Y   P   V

ATC ATC AAG GCC GCC GGC GGC GGC GGT GGG CGC GGC ATG CGC GTG GTC TAC GAC     1870
I   I   K   A   A   G   G   G   G   G   R   G   M   R   V   V   Y   D

GAG TCC GAG CTG ATC AAG TCG GCC AAG CTG ACC CGC ACC GAG GCC GGC GCG GCG     1924
E   S   E   L   I   K   S   A   K   L   T   R   T   E   A   G   A   A

TTC GGC AAC CCG ATG GTC TAC CTG GAG AAG TTC CTG ACC AAC CCG CGC CAC GTG     1978
F   G   N   P   M   V   Y   L   E   K   F   L   T   N   P   R   H   V

GAA GTC CAG GTG CTT TCC GAC GGC CAG GGC AAC GCC ATC CAC CTC GGC GAC CGC     2032
E   V   Q   V   L   S   D   G   Q   G   N   A   I   H   L   G   D   R

GAC TGC TCC CTG CAG CGC CGC CAC CAG AAG GTG ATC GAA GAG GCG CCG GCC CCC     2086
D   C   S   L   Q   R   R   H   Q   K   V   I   E   E   A   P   A   P

GGC ATC GAC GAG AAG GCT CGC CAG GAA GTC TTC GCC CGC TGC GTC CAG GCC TGC     2140
G   I   D   E   K   A   R   Q   E   V   F   A   R   C   V   Q   A   C

ATC GAG ATC GGC TAC CGC GGC GCC GGC ACC TTC GAG TTC CTC TAC GAG AAC GGC     2194
I   E   I   G   Y   R   G   A   G   T   F   E   F   L   Y   E   N   G

CGC TTC TAC TTC ATC GAG ATG AAC ACT CGC GTG CAG GTG GAG CAC CCG GTA TCT     2248
R   F   Y   F   I   E   M   N   T   R   V   Q   V   E   H   P   V   S

GAG ATG GTC ACC GGT GTC GAC ATC GTC AAG GAG ATG CTG CGC ATC GCC TCC GGC     2302
E   M   V   T   G   V   D   I   V   K   E   M   L   R   I   A   S   G

GAG AAG CTC TCG ATC CGC CAG GAG GAC GTG GTC ATC CGC GGC CAT GCG CTG GAA     2356
E   K   L   S   I   R   Q   E   D   V   V   I   R   G   H   A   L   E

TGC CGG ATC AAC GCC GAA GAC CCG AAG ACC TTC ATG CCC AGC CCG GGC AAG GTC     2410
C   R   I   N   A   E   D   P   K   T   F   M   P   S   P   G   K   V

AAG CAC TTC CAC GCC CCC GGC GGC AAC GGC GTG CGC GTC GAC TCG CAC CTC TAC     2464
K   H   F   H   A   P   G   G   N   G   V   R   V   D   S   H   L   Y

AGC GGC TAC AGC GTG CCG CCG AAC TAC GAC TCG CTG GTC GGC AAG GTC ATC ACC     2518
S   G   Y   S   V   P   P   N   Y   D   S   L   V   G   K   V   I   T

TAC GGT GCC GAC CGC GAC GAG GCG CTG GCG CGG ATG CGC AAT GCC CTG GAC GAG     2572
Y   G   A   D   R   D   E   A   L   A   R   M   R   N   A   L   D   E

TTG ATC GTC GAC GGT ATC AAG ACC AAT ACC GAA CTG CAC AAG GAC CTG GTG CGC     2626
L   I   V   D   G   I   K   T   N   T   E   L   H   K   D   L   V   R

GAC GCC GCC TTC TGC AAG GGC GGG GTG AAC ATC CAT TAC CTG GAG AAG AAA CTG     2680
D   A   A   F   C   K   G   G   V   N   I   H   Y   L   E   K   K   L

GGT ATG GAC AAG CAC TGA                                                   2698
G   M   D   K   H   .
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                        TCCGTCAGTC GCTGCGCACA AGGGCTGCCT CCGGGCGGCC CTTGTCGTTT  2748
CCGCCTGCCG GCCGCCCCAA CGGTGCTATC CTCGTCGCCC CTCCACCACC GGACTCCTGC ATGGAACGCA  2818
TCCGCCGCTG GTACCGCCAA TCGCTGGCCG CCGCGACCGA GGCCGCGACC TGCTTCGCCC TGGGTTTCAG  2888
GGAAAGCCTG CAACCCGCCG CACTCTTCCG CTCCGCGAGC CTGTGCATTC TCGTCAGCGT GCTGTGCACC  2958
TGGCTGTTCG TGCATTTCTT CGAACCGATC ATCCGCCTCT GCGGCTGGGC CGCGCTGTAC ACGGCATTCA  3028
GCGTGGCCAA CTTCGCCCTG ATCCCCAGCG GCTCGCTGAT CGAGGCCGGC AGCGGTGGCC CGTACTTCGA  3098
TCCGCTGGCG GCCTTCAACG GCCTGGCGGG ACTGGCGCAA CTGGCGTTCT ATTTCGTCGG CTATGCCGCG  3168
CTGTTCTTCG TCGCCCTGTA CGCCGCCAGC ATCGTCTTCG GCATCCGCCT GGGCCTGCGC ATC        3231
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FIG. 2—*Continued.*

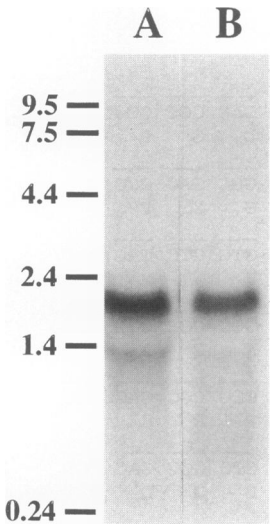

FIG. 3. Analysis of mRNA transcripts encoded by *accB* and *accC* by RNA blot hybridization. Hybridization of RNA from exponentially growing *P. aeruginosa* with a ^{32}P-labeled gene fragment specific for *accB* (A) or *accC* (B) is shown. Molecular size markers are in kilobases.

containing proteins. The BCCP subunits of the *P. aeruginosa* and *E. coli* ACCs are remarkably conserved; the two proteins are 63% identical. Two conserved features in the *P. aeruginosa* and *E. coli* BCCP sequences of particular interest are a pentapeptide, EAMKM (amino acids 119 to 123 shown on Fig. 4A), which includes the lysine residue serving as the biotinylation site, and an alanine-proline-rich region which extends from amino acids 47 to 76 in *P. aeruginosa* and from amino acids 61 to 76 in *E. coli* (Fig. 4A). It has been suggested that the alanine-proline-rich portion of BCCP serves as a mobile spacer which allows the biotinylated portion of ACC to move between active sites of the ACC subunits (21). We find it interesting that the alanine-proline-rich sequence occupies an even larger portion of the sequence in *P. aeruginosa* than it does in *E. coli* (Fig. 4A). Although alanine-proline-rich sequences are conserved in other biotin-containing proteins, they are rarely as extensive as the sequence found in the *P. aeruginosa* BCCP (30). In this respect, the biotin-containing portion (α subunit) of the *Klebsiella pneumoniae* oxalacetate decarboxylase gene is particularly interesting. The alanine-proline-rich portion of the *K. pneumoniae* oxalacetate decarboxylase extends over a 31-amino-acid stretch and includes 16 alanine residues and 9 proline residues (30).

The BC subunits of the *P. aeruginosa* and *E. coli* ACCs are also highly conserved; the two proteins are 71% identical. By homology with other ATP-requiring enzymes, Li and Cronan (21) have suggested that a glycine-rich sequence extending from residues 162 to 168 of the *E. coli* BC sequence (Fig. 4B) functions as the ATP-binding region of ACC. We note that this sequence is completely conserved in the *P. aeruginosa* sequence. Li and Cronan (21) have suggested that a cysteine residue, C-230, located downstream of the putative ATP-binding site in the *E. coli* BC, may be important for biotin carboxylation; this residue is conserved in the *P. aeruginosa* BC.

Biotinylated proteins in *P. aeruginosa*. Biotinylated proteins are rare in bacteria. *E. coli* contains only one biotinylated protein: the BCCP subunit of ACC (10). *P. citronellolis* contains two biotin-containing proteins: the 25-kDa BCCP subunit of ACC and a 65-kDa subunit of pyruvate carboxylase (10). A modification of the Western blot technique was performed to enumerate the biotin-containing proteins in *P. aeruginosa* and to determine the apparent molecular weight of the *P. aeruginosa* BCCP. Three biotin-containing proteins were detected in the soluble fraction of a *P. aeruginosa* protein extract (Fig. 5). None of the three proteins were visible on the nitrocellulose filter when the filter was preincubated with avidin, which binds specifically to biotin, or when streptavidin-alkaline phosphatase was omitted from the treatment. The sizes of these proteins are 22.1, 70.4, and 85.0 kDa. The 22.1-kDa protein is almost certainly BCCP. The difference from the predicted molecular mass (16.5 kDa on the basis of DNA sequence analysis) may be attributed to the protein's extensive alanine-proline-rich domain (21). Although it is tempting to speculate that the 70.4-kDa *P. aeruginosa* protein is pyruvate carboxylase, we are not certain that this species of *Pseudomonas* possesses this enzyme activity. The identity of the 85.0-kDa protein is unknown, but on the basis of our control experiments, it is not alkaline phosphatase.

***P. aeruginosa* homologs of the *E. coli accA* and *accD*.** Having found that the *P. aeruginosa accB* and *accC* genes are organized in a manner similar to their organization in *E. coli*, it was of interest to determine the locations of the genes encoding the *P. aeruginosa* TC. To evaluate the possibility that the *P. aeruginosa accB* and *accC* genes border the *accBC* operon, we cloned a DNA fragment containing DNA flanking both *accB* and *accC*. We constructed a pMW1216 cosmid library containing *P. aeruginosa* DNA which had been partially digested with *Hin*dIII and then screened the library for clones capable of complementing EE451, using the criteria described previously. An approximately 11.2-kb *Hin*dIII fragment from one cosmid clone was subcloned into Bluescript KS+, and the resulting plasmid, pCGN3941, was found to complement EE451. Plasmid pCGN3941 was found to contain the *P. aeruginosa accB* and a considerable length of DNA both upstream and downstream of the coding region for *accB* (Fig. 1).

Plasmid complementation experiments were performed to determine whether the gene encoding the β subunit of TC was located in the proximity of the *P. aeruginosa accB* and *accC*. Plasmids pCGN3931 and pCGN3941, which contain sequences flanking *P. aeruginosa accB* and *accC*, were introduced into *E. coli* LA1-6, which contains a temperature-sensitive defect in the β subunit of TC (23). Neither pCGN3931, which contains approximately 1.2 kb of sequence downstream of *accC* (Fig. 1), nor pCGN3941, which contains approximately 8.2 kb of sequence upstream of *accB* (Fig. 1), was able to rescue LA1-6 for growth at 42°C. A control plasmid containing the *E. coli accD*, pSJ9 (23), allowed LA1-6 to grow at 42°C. Moreover, comparison of the *E. coli* AccD protein with a 6-frame translation of the *P. aeruginosa* sequence flanking the *accBC* coding region (Fig. 2) failed to reveal significant homology.

Southern hybridization experiments were performed to identify *P. aeruginosa* DNA fragments homologous to the *E. coli accA* and *accD* genes (data not shown). No hybridization signal was observed when DNA fragments specific for the *E. coli accA* and *accD* were used to probe *Bam*HI-digested pCGN3931 or *Hin*dIII-digested pCGN3941. Hybridization of *P. aeruginosa* DNA fragments with a DNA fragment specific for the *E. coli accD* identified the following chromosomal fragments: a ~21-kb *Bam*HI fragment, a ~3.3-kb *Hin*dIII fragment, a >23-kb *Kpn*I fragment, and a ~1.3-kb *Pst*I fragment. A similar hybridization experiment using the *E. coli accA* as a probe identified the following *P. aeruginosa* DNA fragments as the potential *accA* homolog: a ~1.7-kb *Bam*HI fragment, a >23-kb *Hin*dIII fragment, a ~5.8-kb *Kpn*I frag-

A

```
                10        20        30        40        50
PA          MDIRKVKKLIELLEESGIDELEIREGEESVRISRHSKTAAQPVYAQAPAF
EC          .....I......V.....S....S..........AAPA.SF..MQ..Y.A

                60        70        80        90       100
PA          AAPVAAPAPAAAAPAAAAAESAPAAPKLNGNVVRSPMVGTFYRAASPTSA
EC          PMMQQPAQSN.....TVPSME....AEIS.HI...........TP..DAK

               110       120       130       140       150
PA          NFVEVGQSVKKGDILCIVEAMKMMNHIEAEVSGTIESILVENGQPVEFDQ
EC          A.I....K.NV..T...........Q...DK...VKA....S.......E


PA          PLFTIV
EC          ..VV.E
```

B

```
                10        20        30        40        50
PA          MLEKVLIANRGEIALRILRACKELGIKTVAVHSTADRELMHLSLADESVC
EC          ..D.IV.............................S...D.K.VL....T..

                60        70        80        90       100
PA          IGPAPATQSYLQIPAIIAAAEVTGATAIHPGYGFLAENADFAEQIERSGF
EC          .....SVK...N.....S...I...V.........S...N....V.....

               110       120       130       140       150
PA          TFVGPTAEVIRLMGDKVSAKDAMKRAGVPTVPGSDGPLPEDEETALAIAR
EC          I.I..K..T..........IA...K....C.........GD.MDKNR...K

               160       170       180       190       200
PA          EVGYPVIIKAAGGGGGRGMRVVYDESELIKSAKLTRTEAGAAFGNPMVYL
EC          RI........S...........RGDA..AQ.ISM..A..K...S.D...M

               210       220       230       240       250
PA          EKFLTNPRHVEVQVLSDGQGNAIHLGDRDCSLQRRHQKVIEEAPAPGIDE
EC          ..Y.E......I...A........Y.AE....M........V.........TP

               260       270       280       290       300
PA          KARQEVFARCVQACIEIGYRGAGTFEFLYENGRFYFIEMNTRVQVEHPVS
EC          EL.RYIGE..AK..VD............F...E.........I......T

               310       320       330       340       350
PA          EMVTGVDIVKEMLRIASGEKLSIRQEDVVIRGHALECRINAEDPKTFMPS
EC          ..I....LI..QM...A.QP...K..E.HV....V.........N..L..

               360       370       380       390       400
PA          PGKVKHFHAPGGNGVRVDSHLYSGYSVPPNYDSLVGKVITYGADRDEALA
EC          ...ITR......F...WE..I.A..T...Y...MI..L.C..EN..V.I.

               410       420       430       440
PA          RMRNALDELIVDGIKTNTELHKDLVRDAAFCKGGVNIHYLEKKLGMDKH
EC          ..K...Q...I......VD.QIRIMN.EN.QH..T..........LQEK
```

FIG. 4. Alignment of the predicted amino acid sequences of BCCP (A) and BC (B) of *P. aeruginosa* (PA) and *E. coli* (EC). Identical amino acid residues are indicated by dots.

ment, and a ~3.7-kb *Pst*I fragment. If the *P. aeruginosa accA* and *accD* homologs are similar in size to the *E. coli* genes, one would expect that the *P. aeruginosa accA* and *accD* would each require approximately 1.0 kb of coding sequence and that a relatively small DNA fragment could contain sufficient coding capacity for both genes. No significant homology was observed when the *E. coli* AccA protein sequence was compared with a 6-frame translation of the *P. aeruginosa* sequence flanking the

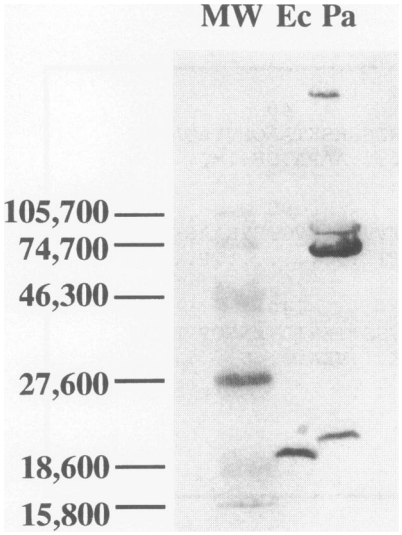

FIG. 5. Identification of biotin-containing proteins in soluble extracts of *P. aeruginosa* and *E. coli* by a modification of the Western blotting technique. Lanes: MW, molecular mass standards in daltons; Ec, *E. coli* extract; Pa, *P. aeruginosa* extract. The uppermost band in lane Pa is due to the interaction of streptavidin with *P. aeruginosa* proteins that failed to enter the gel.

accBC operon. These results suggest that genes encoding the *P. aeruginosa* AccA and AccD are unlinked, either to each other or to the *accBC* operon.

The *E. coli accA* and *accD* are located almost directly opposite each other in the chromosome, and both genes are cotranscribed with other genes essential for growth (23). Li and Cronan (23) have speculated that the *E. coli accA* and *accD* may have become separated by an inversion event. It will be interesting to learn whether the *P. aeruginosa accA* and *accD* are separated by a large distance in the chromosome and whether these genes are cotranscribed with other complex operons, as they appear to be in *E. coli*.

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ADDENDUM

After this manuscript was prepared, Gornicki and coworkers (12) reported the DNA sequences and map locations of the two genes encoding the *Anabaena* BC and BCCP subunits. The *Anabaena accB* and *accC* homologs are unlinked. Thus, there are at least two structural arrangements for the prokaryotic *accB* and *accC*: a 2-gene operon, as in *P. aeruginosa* and *E. coli*, and an unlinked set of genes, as in *Anabaena* sp. strain PCC 7120.

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