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# The Genes Encoding the Biotin Carboxyl Carrier Protein and Biotin Carboxylase Subunits of *Bacillus subtilis* Acetyl Coenzyme A Carboxylase, the First Enzyme of Fatty Acid Synthesis

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**The genes encoding two subunits of acetyl coenzyme A carboxylase, biotin carboxyl carrier protein, and biotin carboxylase have been cloned from *Bacillus subtilis*. DNA sequencing and RNA blot hybridization studies indicated that the *B. subtilis accB* homolog which encodes biotin carboxyl carrier protein, is part of an operon that includes *accC*, the gene encoding the biotin carboxylase subunit of acetyl coenzyme A carboxylase.**

Our knowledge of lipid metabolism in *Bacillus subtilis* is scant. Although *B. subtilis* is often considered the paradigmatic gram-positive organism, the mechanisms involved in lipid biosynthesis have been little studied, and much is argued by analogy with *Escherichia coli* (5). Recent studies indicate that fatty acids might act as signalling molecules that are important for cellular differentiation in *B. subtilis* (19), which prompted us to identify genes involved in fatty acid synthesis. Since the first enzymatic step in a metabolic pathway is often rate limiting, we sought to isolate and characterize the genes encoding subunits of the acetyl coenzyme A (acetyl-CoA) carboxylase (ACC), which is the enzyme catalyzing the first committed step of fatty acid synthesis, i.e., the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA (12, 13). In *E. coli*, carboxylation of acetyl-CoA proceeds through two distinct reactions and involves an enzyme composed of four subunits: biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC), and carboxyl transferase (CT), a tetramer composed of two nonidentical subunits (12, 13). Cloning of prokaryotic ACCs subunits in the gram-negative bacteria *E. coli* (12, 13), *Anabaena* species (8), and *Pseudomonas aeruginosa* species (2) and in mycobacteria (16) has been reported; however, no *acc* genes from *Bacillus* species or other gram-positive species have been reported. The genes studied encode protein products similar to those of the *E. coli* BCCP and BC subunits. However, these gene arrangements have been reported to differ. In *E. coli* (12) and *P. aeruginosa* (2), the BCCP and BC genes (*accB* and *accC*, respectively) form a two-gene operon, whereas in *Anabena* species, the genes encoding BCCP and BC are unlinked (8). In contrast, genes from *Mycobacterium leprae* and *Mycobacterium tuberculosis* encode biotinylated proteins that in these organisms have both BCCP and BC functions (16), which is an arrangement that is also seen in the $\alpha$ subunit of the mammalian mitochondrial propionyl-CoA carboxylase (3, 11). The genes coding for the CT $\alpha$ and $\beta$ subunits (*accA* and *accD*, respectively) have been cloned only for *E. coli* (13).

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Here, we report that a DNA fragment containing the gene encoding the BCCP subunit of *B. subtilis* complements an *E. coli* strain with a mutant BCCP subunit. Moreover, we present the nucleotide sequence of an approximately 2.5-kb fragment that includes the *B. subtilis accB* and *accC* genes, and we demonstrate that the *accB* and *accC* genes form part of an operon located 220° downstream of *spoIIIA* (in the *sin-ahrC* interval) of the *B. subtilis* chromosome.

**Cloning of *B. subtilis accB* homolog by complementation of an *E. coli* BCCP mutant.** We cloned a *B. subtilis accB* homolog by complementation of an *E. coli* strain possessing a temperature-sensitive (Ts) mutation in the BCCP gene (*accB*), using a lambda gt11 library of *B. subtilis* chromosomal DNA (Clontech). To this end, the *E. coli accB*(Ts) strain L8 (12) was lysogenized with a lambda phage possessing a wild-type $cI^+$ repressor and a 1.2-kb segment of DNA encoding the Tn*903* neomycin phosphotransferase constructed as described by Henry and Cronan (10) to give strain DM105. For transduction with the lambda library, the recipient strain DM105 was grown at 30°C in Luria-Bertani (LB) medium (15) containing 0.2% maltose. A 20-$\mu$l volume of an overnight culture of strain DM105 was infected with the lambda library at a multiplicity of infection of 1 in the presence of 20 mM $MgSO_4$. After 30 min of absorption at 30°C, the mixture was plated on LB medium at 42°C. Several phages (lambda DM1 to lambda DM24) allowed growth of strain DM107 at 42°C. To investigate whether the recombinant phages coded for the *B. subtilis* BCCP, we took advantage of the finding that BCCP is the sole biotin-containing protein of *E. coli* (4) and that it can be specifically labeled with exogenous [$^3$H]biotin (12). Thus, crude cell extracts were prepared from several temperature-resistant lysogens of strain DM105 growing in LB medium containing [$^3$H]biotin and were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described elsewhere (12). As shown in Fig. 1, lane 2, strain DM105 has a BCCP severely defective in the acceptance of [$^3$H]biotin at 42°C. However, derivatives of strain DM105 which were complemented for growth at 42°C with the recombinant lambda phages synthesized at 42°C a biotinylated protein that migrates on SDS-PAGE as a protein of 25 kDa (Fig. 1, lanes 1 and 6 to 8). This protein shows a slightly lower mobility than the 22.5-

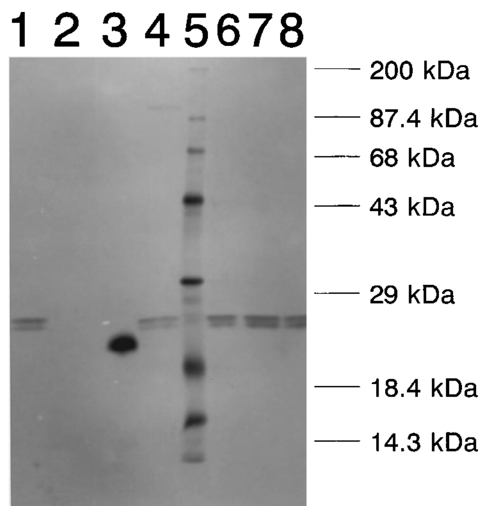


FIG. 1. Expression in *E. coli* of the gene encoding the BCCP subunit of ACC from *B. subtilis*. The strains were grown for several generations at 42°C in the presence of [$^3$H]biotin as described in the text. The cells were collected and lysed in an SDS-containing buffer, separated by SDS-15% PAGE, and fluorographed. Lanes: 1 and 6 to 8, extracts of independent $accB^+$ lysogens of strain L8 selected as described in the text; 5, $^{14}$C-labeled protein standards; 4, *B. subtilis* 168; 3, wild-type *E. coli* LE392; 2, *accB* mutant L8.

kDa BCCP subunit of wild-type *E. coli* (Fig. 1, lane 3) but has the same mobility on SDS-PAGE that a biotin-containing protein synthesized by wild-type *B. subtilis* has (Fig. 1, lane 4). These experiments strongly indicate that the recombinant lambda phages contain the gene coding for the *B. subtilis* BCCP and that this protein may be a few residues longer than the *E. coli* BCCP. However, the unusual electrophoretic properties of BCCP (12) make an accurate prediction of the polypeptide length difficult.

**Purification and N-terminal sequence determination of BCCP from *B. subtilis*.** To gain information about the *B. subtilis* 25-kDa biotinylated protein, we purified this protein from strain 168 essentially as described by Li and Cronan (12). Samples of this preparation were separated by SDS-PAGE and electroblotted to a polyvinylidene difluoride membrane. The protein band that migrated at a putative 25-kDa molecular mass was visualized with Ponceau S on the membrane and was used directly for amino acid sequence analysis. The N-terminal amino acid sequencing was performed by automatic Edman degradation in a gas phase system (Applied Biosystems). The sequence obtained (MLNIKEIHELIKAIDESTID) exactly matched 20 residues predicted from a partial sequence with homology to the *E. coli accB* gene determined by Guèrot and Stragier (9) when they sequenced the neighboring *spoIIIA* gene from *B. subtilis* (Fig. 2). These results indicate that the 25-kDa biotinylated protein is the BCCP from *B. subtilis* and that the recombinant lambda phages carry a copy of the BCCP gene that directs the synthesis of a polypeptide able to interact functionally with the other subunits of the *E. coli* ACC as well as the *E. coli* biotin apoprotein ligase that biotinylates BCCP.

**Determination and analysis of the nucleotide sequences of *accB* and flanking regions.** Synthetic oligonucleotides were used to sequence the *accB* in lambda DM11 and to determine flanking sequences on lambda DM10 and lambda DM14. The *B. subtilis* DNA insert present in lambda DM11 was sequenced with lambda DNA as a template by automated sequencing with *Taq* polymerase and fluorescent-dye-labeled terminators (17). To identify overlapping clones bearing the remaining portion of the *accB* operon contained in lambda DM11, phages that complemented the *accB*(Ts) mutation were screened by PCR amplification (7). The 3′ end of the *B. subtilis* DNA insert contained in lambda DM11 was used to design an upstream primer, and the downstream primer was the lambda gt11 reverse sequencing primer (7). The PCR products were evaluated by gel electrophoresis, and lambda DM10 and lambda DM14 were found to contain inserts larger than that of lambda DM11. The DNA insert present in lambda DM14 was cloned into the *Eco*RV site of pBluescript KS$^+$, and the resultant plasmid was named pDB1. The DNA insert cloned into pDB1 as well as the gel electrophoresis-purified PCR amplification product of lambda DM10 was sequenced by the dideoxy chain termination method with the T7 sequencing kit from Pharmacia and α-$^{35}$S-dATP. Analysis of the approximately 2.5-kb sequence revealed two potential open reading frames (ORFs), which are indicated in Fig. 2: a 477-nucleotide ORF extending from nucleotides 345 to 822 and a 1,344-nucleotide ORF spanning nucleotides 837 to 2181 (Fig. 2). Both of these ORFs are preceded by probable ribosome-binding sites. The sequence of the 477-nucleotide ORF was identical to the partial sequence determined by Guèrot and Stragier (9) downstream of the *spoIIIA* gene (Fig. 2), and it is predicted to encode a 159-amino-acid product with a molecular mass of 17.2 kDa. A multiple amino acid sequence alignment of the 477-nucleotide ORF gene product with those of the BCCP subunits of *Anabaena* species (GenBank no. L14863), *P. aeruginosa* (GenBank no. P37799), and *E. coli* (GenBank no. M80458) shows a high degree of sequence similarity throughout their lengths and identifies this ORF as the structural gene for BCCP, which is hereafter designated *accB*. Two conserved features of particular interest are the tetrapeptide EAMK (amino acids 119 to 122, shown in Fig. 2), which includes the lysine residue serving as the biotinylation site, and an alanine-proline-rich region which extends from amino acids 47 to 75 in *B. subtilis* (Fig. 2), from amino acids 61 to 76 in *E. coli* (16), and from amino acids 47 to 76 in *P. aeruginosa* (2). It has been suggested that the alanine- and 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 subunit (12). The 1,332-nucleotide ORF is predicted to encode a 444-amino-acid protein with a molecular mass of 48.9 kDa. The translated product of the 1,332-nucleotide ORF shows extensive homology with other members of the biotin-dependent carboxylase family; thus, we believe that this ORF encodes BC. The most highly scoring matches were to *Anabaena* species (GenBank no. L14863), *E. coli* (GenBank no. M80458), and *P. aeruginosa* (GenBank no. P37798) biotin carboxylases; to rat (GenBank no. P14882) and human (GenBank no. X14608) propionyl carboxylases; and to the biotinylated protein of *M. leprae* (GenBank U00012). The deduced product of *accC* also shows similarity to structural motifs found in other prokaryotic BC subunits, such as a glycine-rich sequence (amino acids 162 to 168 shown in Fig. 2) that has been proposed to constitute a region for interaction with ATP (12) and a cysteine residue, C-230, located downstream of the putative ATP binding site (Fig. 2), that might be important for biotin carboxylation (12).

Hybridization of *Eco*RI-digested *B. subtilis* 168 DNA with specific probes for either *accB* or *accC* genes revealed only one hybridization signal in each case, indicating the presence of one copy of each gene (data not shown).

**Transcriptional analysis of *accB* and *accC*.** Our finding that *accB* and *accC* are separated by only 15 nucleotides (Fig. 2) suggests that these two genes are cotranscribed. To test the possibility that *accB* and *accC* are cotranscribed and to determine whether these genes are part of a larger polycistronic

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CAGCTGGAAACGCTGATTAAAACACAAGGTTACGAGGATGCGCTTGTTAATGCTGAAGGA   60
GATAAAATCAATATTACAGTCAAATCAGACAAACACTCTAAATCGAAGGCGACAGCCATT  120
ATAGACCTTGTGGCAAAAGAAATCAAAACAATGAAAGATGTCGCTGTCACATTTGAACCC  180
TCTAAATAAGAATGAGGGAAAAAAGCCCGCTAAACAAGCGGGCTTTTTGCGTTGCTGTCA  240
                     IR                IR
TATTAGAGTTGAATTCAAAAGTCCGCCTCCTGTAAGATGAACATAGTATGTACTTTTAGT  300
                                              accB
AGTAACCTATAAAAAAAGAAATTTCATACATAGGAGTGCGATTGAATGTTAAATATCAAA  360
                                RBS        M  L  N  I  K
GAAATCCACGAGCTGATTAAAGCAATTGACGAGTCTACAATTGACGAATTCGTATATGAA  420
E  I  H  E  L  I  K  A  I  D  E  S  T  I  D  E  F  V  Y  E
AATGAAGGTGTATCCTTAAAACTGAAAAAACACGAAGCAGGCACGGTTCAAGTCATGCAG  480
N  E  G  V  S  L  K  L  K  K  H  E  A  G  T  V  Q  V  M  Q
CAGGCACCGGCAGCACCTGTACAAGCACAGGCTCCGCAGGCAGTTCAGCCTCAAGGCGAG  540
Q  A  P  A  A  P  V  Q  A  Q  A  P  Q  A  V  Q  P  Q  G  E
CAAGCAGCGGCACCTGCCCAAGAAGCACCAAAGCAAGATGAGAATCTGCATAAAATCACT  600
Q  A  A  A  P  A  Q  E  A  P  K  Q  D  E  N  L  H  K  I  T
TCACCAATGGTAGGAACATTTTATGCTTCTTCATCACCGGAAGCTGGCCCGTATGTAACA  690
S  P  M  V  G  T  F  Y  A  S  S  S  P  E  A  G  P  Y  V  T
GCCGGTTCAAAAGTAAATGAAAACACAGTTGTCTGCATTGTAGAAGCGATGAAGCTTTTC  720
A  G  S  K  V  N  E  N  T  V  V  C  I  V  E  A  M  K  L  F
ATCGAAATCGAAGCAGAAGTGAAAGGCGAAATCGTTGAAGTATTAGTAGAAAACGGTCAG  780
I  E  I  E  A  E  V  K  G  E  I  V  E  V  L  V  E  N  G  Q
                                                          accC
CTGGTCGAATACGGACAACCTCTATTTCTTGTAAAAGCGGAGTAAGGAGACTTAACATGA  840
L  V  E  Y  G  Q  P  L  F  L  V  K  A  E  *  RBS       M  I
TTAAAAAGCTATTGATCGCCAACAGAGGAGAAATTGCTGTCAGAATCATCAGAGCCTGCA  900
  K  K  L  L  I  A  N  R  G  E  I  A  V  R  I  I  R  A  C  R
GAGAGCTCGGAATTGAGACTGTCGCTGTTTATTCAGAAGCTGATAAAGATGCCCTTCATG  960
  E  L  G  I  E  T  V  A  V  Y  S  E  A  D  K  D  A  L  H  V
TTCAAATGGCCGATGAAGCTTTTTGTATCGGACCGAAAGCATCAAAAGACAGCTATTTAA 1020
  Q  M  A  D  E  A  F  C  I  G  P  K  A  S  K  D  S  Y  L  N
ACGTTACAAATATTGTGAGTGTTGCAAAGCTGACTGGCACGGACGCCATTCATCCGGGAT 1080
  V  T  N  I  V  S  V  A  K  L  T  G  T  D  A  I  H  P  G  Y
ACGGATTTTTAGCTGAAAATGCTGATTTCGCTGAATTATGTGAAGAAGTTAATGTCACGT 1140
G  F  L  A  E  N  A  D  F  A  E  L  C  E  E  V  N  V  T  F
TTGTCGGCCCGAGCGCTGACGCCATTTCAAAAATGGGAACAAAAGACGTTGCGCGGGAAA 1200
  V  G  P  S  A  D  A  I  S  K  M  G  T  K  D  V  A  R  E  T
CGATGAAACAGGCCGGCGTGCCAATCGTACCGGGTTCACAGGGAATTATAGAAAATGTGG 1260
  M  K  Q  A  G  V  P  I  V  P  G  S  Q  G  I  I  E  N  V  E
AAGAAGCGGTTTCGCTTGCTAATGAAATTGGGTATCCTGTAATTATAAAAGCCACCGCAG 1320
  E  A  V  S  L  A  N  E  I  G  Y  P  V  I  I  K  A  T  A  G
GCGGAGGCGGAAAAGGAATCAGGGTTGCCCGTACTGAAGAGGAACTGATTAATGGCATTA 1380
  G  G  G  K  G  I  R  V  A  R  T  E  E  E  L  I  N  G  I  K
AGATTACACAGCAGGAAGCGGCAACTGCATTTGGGAATCCAGGTGTATACATCGAAAAAT 1440
  I  T  Q  Q  E  A  A  T  A  F  G  N  P  G  V  Y  I  E  K  Y
ACATAGAAGATTTTCGCCACGTTGAGATCCAAGTGCTTGCTGATAACTACGGAAATACGA 1500
  I  E  D  F  R  H  V  E  I  Q  V  L  A  D  N  Y  G  N  T  I
TCCATTTGGGCGAACGCGACTGCTCGATCCAAAGACGCCTGCAAAAGCTTTTGGGAGAAT 1560
  H  L  G  E  R  D  C  S  I  Q  R  R  L  Q  K  L  L  G  E  S
CACCATCTCCTGCCCTTGGTTCAGAAATCAGGGAGCAAATGGGAGATGCAGCGGTAAAGG 1620
  P  S  P  A  L  G  S  E  I  R  E  Q  M  G  D  A  A  V  K  A
CTGCAAAAGCGGTTGGCTATACAGGTGCTGGAACAGTTGAATTTATCTATGACTACAATG 1680
  A  K  A  V  G  Y  T  G  A  G  T  V  E  F  I  Y  D  Y  N  E
AACAGCGCTATTACTTCATGGAAATGAACACGAGAATTCAGGTAGAGCACCCAGTCACAG 1740
  Q  R  Y  Y  F  M  E  M  N  T  R  I  Q  V  E  H  P  V  T  E
AAATGGTGACGGGCACTGACCTGATCAAGGAACAAATCAAAGTAGCATCAGGAATGGAAC 1800
  M  V  T  G  T  D  L  I  L  E  Q  I  L  V  A  S  G  M  E  L
TGAGCCTCAAGCAAGAAGATGTTGAATTTGAAGGCTGGGCCATCGAATGCCGAATCAACG 1860
  S  L  K  Q  E  D  V  E  F  E  G  W  A  I  E  C  R  I  N  A
CAGAAACCCAAGTTAAATTCATGCCGTCACCTGGCGAAATAAATGTACCTGCCTCTGGCG 1920
  E  T  Q  V  K  F  M  P  S  P  G  E  I  N  V  P  A  S  G  G
GTCTTGGTGTCCGTGTCGATTCAGCTGCGTACCCTGGCTATTCCATCCCACCGTACTATG 1980
  L  G  V  R  V  V  S  A  A  Y  P  G  Y  S  I  P  P  Y  Y  D
ACAGCATGATTGCAAAGGTAATCACATACGGAAAAACGAGGGATGAAGCGATTGCTCGCA 2040
  C  M  I  A  K  V  I  T  Y  G  K  T  R  D  E  A  I  A  R  M
TGAAGCGCGCATTGGCACAATTCGTCATCGAAGGCATTGAGACAACAATCCCTTTCCATT 2100
  K  R  A  L  A  Q  F  V  I  E  G  I  E  T  T  I  P  F  H  L
TAAAACTGCTTGAACACGAAACATTTGTGAGCGGAGAGTTTAATACGAAATTTTTAGAAA 2160
  K  L  L  E  H  E  T  F  V  S  G  E  F  N  T  K  F  L  E  T
CATATGATGTAATGGGCTCATAATTTTTACGGAGGCTGAATCTGTAACTGACAAGACAAC 2220
  Y  D  V  M  G  S  *
AGCTTGCTTAAATGGAATCAC                                        2241
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FIG. 2. Nucleotide sequence of the *accBC* operon and upstream region. The predicted amino acid sequences of the AccB and AccC proteins are written in one-letter abbreviations below the nucleotide sequences. Nucleotides 345 to 822 and 837 to 2181 constitute the *accB* and *accC* genes, respectively. Presumed ribosome binding sites (RBS) are underlined and in boldface. The conserved structural motifs found in other prokaryotic BCCP and BC subunits are indicated in boldface. The nucleotide sequence encompassing nucleotides 1 to 227 corresponding to the end of the upstream operon *spoIIIA* (11) is also included. A 12-nucleotide inverted repeat (IR) that could function to terminate *spoIIIA* transcription is underlined.

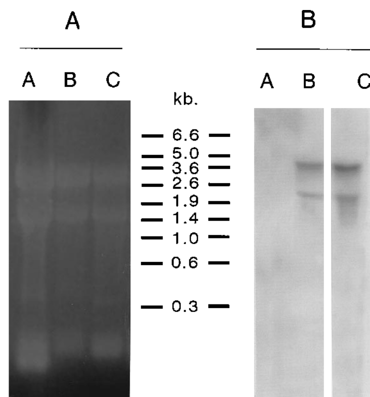


FIG. 3. Analysis of mRNA transcripts encoded by *accB* and *accC* by RNA blot hybridization. (A) Ethidium bromide-stained 1.2% agarose formaldehyde gel electrophoresis of total RNA denatured on formaldehyde-containing buffer (1). Each lane contains about 25 μg of total RNA. RNA was extracted from *B. subtilis* 168 cells grown either for 24 h (lane A) or to exponential phase (lanes B and C) in LB medium. (B) The RNAs displayed in lanes A through C from panel A were transferred to nylon membranes and hybridized with a $^{32}$P-labeled gene fragment specific for *accB* (lanes A and B) and *accC* (lane C). Molecular size markers are indicated.

message, we performed RNA blot hybridization studies. Total *B. subtilis* RNA for RNA blot analysis was prepared as previously described (14). RNA (approximately 25 μg) was electrophoretically separated in a 1.2% (wt/vol) agarose gel containing 0.66 M formaldehyde and was transferred to a nylon membrane (Hybond-N; Amersham) according to standard methods (1). Specific DNA fragments used as hybridization probes were produced by PCR amplification and were labeled with $^{32}$P by the random priming method (18). Hybridization studies performed with RNA extracted from logarithmically growing cells from strain 168 with either a probe specific for *accB* or one specific for *accC* detected two mRNA species of approximately 2.9 and 1.8 kb, respectively (Fig. 3B, lanes B and C). On the other hand, total RNA extracted from strain 168 grown in LB medium for 24 h did not show hybridization signals with the *accB* probe (Fig. 3B, lane A) (similar results were obtained with the *accC* probe [data not shown]). Since *accBC* mRNA (but not 16S and 23S rRNAs [Fig. 3A, lane A]) should have decayed after growing the *B. subtilis* cells for 24 h, this experiment eliminates the possibility that the *acc* probes could be nonspecifically hybridizing to *B. subtilis* rRNAs that migrate close to the *acc* transcripts (Fig. 3, lanes A to C). Thus, these experiments indicate that *accB* and *accC* are cotranscribed, forming part of the same operon. The two species of RNA are sufficiently long to include the BCCP- and BC-coding regions. However, with the data presented here, we cannot determine whether *accB* and *accC* are the only two genes contained in the 2.9-kb mRNA transcript. To answer this question, it will be necessary to clone and sequence the DNA region located downstream of *accC*.

**Assessment of the presence of biotinylated proteins in *B. subtilis*.** A crude cell extract was prepared from a culture of strain 168 growing logarithmically in LB medium containing [$^{3}$H]biotin, was examined by SDS-PAGE and autoradiography to assess the number of biotinylated proteins present (Fig. 4), and showed the presence of two proteins with apparent masses of 25 and 85 kDa. The 25-kDa protein is BCCP, and the difference from the predicted molecular mass (17.2 kDa on the basis of DNA sequence analysis) may be attributed to the protein's extensive alanine- and proline-rich domain. It is tempting to speculate that the 80-kDa *B. subtilis* protein is

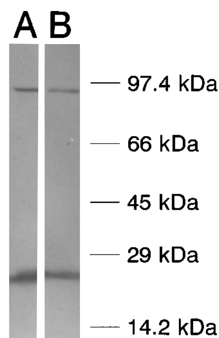


FIG. 4. Identification of biotin-containing proteins in *B. subtilis*. Exponentially growing *B. subtilis* 168 was labeled with [$^3$H]biotin as described in the text. The proteins were analyzed on an SDS-15% polyacrylamide gel, and the bands were detected by fluorography. Sizes and positions of protein molecular mass markers are indicated on the right. Lanes A and B show two independent experiments.

pyruvate carboxylase, the biotin enzyme that catalyzes the ATP-dependent carboxylation of pyruvate in *B. subtilis* (6).

**Nucleotide sequence accession number.** The nucleotide sequence of the *B. subtilis* 168 operon that contains *accB* and *accC* has been deposited under GenBank accession number U36245.

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