Expression of Two *Escherichia coli* Acetyl-CoA Carboxylase Subunits is Autoregulated*

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The abbreviations used are: ACC, acetyl-CoA carboxylase; BCCP, biotin carboxyl carrier protein; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobase pair.
SUMMARY

Acetyl-CoA carboxylase catalyzes the first step of fatty acid biosynthesis, the synthesis of malonyl-CoA from acetyl-CoA using ATP and bicarbonate. In *Escherichia coli* and most other bacteria, ACC is composed of four subunits encoded by *accA, accB, accC, and accD*. Prior work from this laboratory showed that the *in vivo* expression of the *accBC* operon had a strikingly non-linear response to gene copy number (S.-J Li and J. E. Cronan, Jr. *J Bacteriol.* 175, 332-340 (1993) in that the presence of 50 or more copies of the *accBC* operon resulted in only a 2 to 3-fold increase in AccB and AccC. We now report that AccB functions to negatively regulate transcription of the *accBC* operon. Expression of a chimeric protein consisting of the N-terminus of *E. coli* AccB and the C-terminal bioinylation domain of *Bacillus subtilis* AccB downregulated transcription of the *E. coli accBC* operon. A truncated form of AccB consisting of the N-terminal 68 amino acids of *E. coli* AccB was sufficient to negatively regulate the *accBC* operon. In vivo bypass of acetyl-CoA carboxylase activity by expression of a malonyl-CoA synthase from *Rhizobium trifolii* allowed construction of strain deleted for the *accA and accB* genes. Unexpectedly, the ∆*accB* mutation could not be resolved from the ∆*accA* mutation. Transcription of the *accBC* operon in the ∆*accB* ∆*accA* strain continued well into stationary phase under growth conditions that normally result in greatly decreased transcription. These data support a model in which AccB acts as an autoregulator of *accBC* operon transcription.
INTRODUCTION
The coordination of growth rate and membrane biosynthesis is a crucial feature for all organisms. Although *Escherichia coli* has been a paradigm for the study of fatty acid-containing membrane lipids, several aspects of the regulation of *E. coli* fatty acid synthesis remain unclear. A number of potential control points in the pathway exist. Blockage of phospholipid biosynthesis causes an accumulation of derivatives of acyl carrier protein (ACP) acylated with fatty acids and a dramatic decrease in the rate of fatty acid biosynthesis. The inhibition of fatty acid synthesis can be reversed by cleavage of the acyl-ACPs (1) indicating that such species might inhibit fatty acid synthetic enzymes. Acyl-ACPs were subsequently been shown *in vitro* to inhibit the enoyl-ACP reductase, 3-ketoacyl-ACP synthase III, and acetyl-CoA carboxylase activities (2-6) of *E. coli*.

Acetyl-CoA carboxylase (ACC) catalyzes the first committed step in fatty acid metabolism, the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate. In contrast to eucaryotic forms, the *E. coli* acetyl-CoA carboxylase is composed of four distinct proteins; biotin carboxyl carrier protein (BCCP; AccB), biotin carboxylase (AccC), and two proteins (AccA and AccD) catalyzing the carboxyltransferase partial reaction (7). The conversion of acetyl-CoA to malonyl-CoA takes place in two steps. Biotin carboxylase catalyzes the first reaction by transferring CO2 in an ATP-dependent fashion from bicarbonate to the biotin moiety of AccB, resulting in carboxybiotinoyl-AccB. The CO2 group is subsequently transferred from the carboxybiotin moiety to acetyl-CoA by the carboxyltransferase subunits, resulting in malonyl-CoA formation (7).

ACC seems an appropriate target for regulation of the fatty acid synthetic pathway. This reaction is the first committed step in fatty acid metabolism. In *E. coli* ACC has recently been demonstrated *in vivo* to be a rate limiting step since increased levels of *E. coli* ACC increased the rate of fatty acid synthesis (8). Additionally, the only known fate in *E. coli* of malonyl-CoA, the ACC product is in the synthesis of fatty acids. Since 94% of the ATP used in membrane phospholipid synthesis is consumed occurs during fatty acid synthesis, controlling malonyl-CoA production would be energy efficient (9).

The rates of transcription of all four acetyl-CoA carboxylase genes are regulated with respect to growth rate (10). The accB and accC genes form an operon and have pattern of regulation that differs from that of the unlinked accA and accD genes (10). It is interesting that
the accB and accC are frequently adjacent genes even in bacteria only distantly related to E. coli. Of 90 sequenced microbial genomes surveyed, 75 organisms (encompassing 54 distinct species) retain the accB and accC genes in an genomic organization suggestive of cotranscription. This result suggests that coregulation of these two genes may be a widely conserved and important feature of cellular metabolism. This hypothesis has been directly demonstrated in a gram positive organism, Bacillus subtilis, in which the accBC genes were shown to be growth-rate regulated in a manner analogous to that of E. coli (11).

The accBC genes are located in an operon located at min 73.35 (3403.1 Kb) of the E. coli MG1655 chromosome, a site distant from the carboxyltransferase subunit genes, accA (min 4.50) and accD (min 52.40). A number of unusual features are found in the DNA segment immediately upstream of the accBC operon; a long, non-translated leader region of 319 base pairs, a 98 bp sequence of bent DNA within this leader region, an unknown ORF of 188 bp, as well as a series of inverted repeats upstream of the accB initiation codon (10). Deletion analyses of these features, however, failed show any regulatory roles for these features (10). We now report that transcription of the accBC operon is regulated by accumulation of AccB.
EXPERIMENTAL PROCEDURES

_Bacteria, plasmids, and culture_ conditions—The bacterial strains and plasmids used in this study are listed in Table 1. E. coli strains were grown at 37°C in minimal E salts supplemented with 0.2% glucose and vitamin-free Casamino Acids (Difco) when necessary. Optimal induction of the Plac and Ptac promoters was determined for each gene, but maximal expression was with 1mM IPTG. Genes under control of the arabinose promoter were induced with 0.2% L-arabinose. Malonate supplementation was determined to be optimal for most strains at 2 mM. Greater concentrations of malonate were inhibitory to growth of MG1655 and other K-12 strains. Growth on malonate as a sole carbon source was conducted in minimal medium E plates supplemented with Casamino Acids (0.04%) and malonate at 2 mM. Malonate liquid media were supplemented with 5% sucrose as an osmotic stabilizer.

Plasmid pESJ89 is an ampicillin-resistant pBR322 derivative carrying the _accB_ gene under control of the lac promoter. Plasmid pESJ114 is an ampicillin-resistant plasmid that uses the pBR322 origin of replication and contains a truncated _accB_ gene under control of the Plac promoter. A kanamycin resistance cassette flanked by terminator regions was inserted 204 bp downstream of the _accB_ ATG resulting in expression of the first 68 amino acids of AccB and the construct was confirmed by DNA sequencing. This amino-terminal portion of AccB lacks the biotinylation domain and failed to complement an _accB_ mutation. Plasmid pESJ114 was maintained in a lacIq background to eliminate toxicity problems. Plasmid pCY216 is a chloramphenicol resistant pACYC origin plasmid carrying the BirA coding region under control of an arabinose promoter (12). Plasmid pCY335 is a kanamycin-resistant pBR322-origin plasmid carrying the coding region for the carboxy-terminal 87 amino acids of AccB under control of the arabinose promoter. The carboxy-terminal fragment encoded contains all the structural elements required for efficient biotinylation, but is unable to complement an _accB_ mutant or function _in vivo_ in the acetyl-CoA carboxylase reaction (12). Plasmid pMATop-2 is pBluescript SK containing the _matA, matB_, and _matC_ genes under their native promoter (14). Plasmid pESJ297 is a pMATop-2 derivative in which the ampicillin gene was replaced with a gentamycin-resistance cassette from p34S-GM (13). The _B. subtilis accB_ gene was cloned from strain JH642 using the polymerase chain reaction into pCR2.1 (Invitrogen) resulting in pESJ207. Plasmid pESJ207 was restriction digested with KpnI and NotI and the _accB_ fragment was ligated
into the same sites of pPROLar.A122 to give pESJ209. Plasmid pESJ225 encodes a chimeric AccB consisting of the N-terminal 44 amino acids of *E. coli* AccB fused to the C-terminal 109 residues of *B. subtilis* BCCP in vector pPROLar.A122 (Clontech). Plasmid pESJ315 encodes a chimeric AccB consisting of the N-terminal 47 amino acids of *B. subtilis* AccB and the C-terminal 109 amino acids of *E. coli* AccB in pPROLar.A122. The ability of pESJ225 and pESJ315 to provide active AccB was demonstrated by complementation of the *accB*\textsuperscript{ts} mutation of strain L8 at 42°C.

Strains for analysis of biotin operon activity include CY481, a derivative of BM4092 containing a wild type *birA* gene (14) and a *bioFZ:*lacZ fusion plus SA291, which contains a *bio* operon deletion extending from *galK* to *moaA* (15). Strain ESJ298 is SA291 containing pMATop-2. Biotin supplementation at 41 nM was used to repress *bio* operon expression, and minimal biotin supplementation was conducted at 4 nM. *E. coli* strain L8 contains an *accB*\textsuperscript{ts} allele in which growth at 40°C to 42°C causes protein instability (16).

pDEW201 is a *Photorhabdus luminescens* promoterless probe vector containing the *luxCDABE* genes necessary for light production in vivo in the absence of exogenously supplied substrates for luminescence (17). Light emission from luciferase fusions was monitored with a Lumat LB9501 luminometer (Berthold Instruments) and is reported in the form of Relative Light Units.

**Protein biotinylation assay**—Cultures were grown overnight in the presence of excess biotin (>40nM) to completely repress the biotin biosynthetic operon. The cells were then washed three times in biotin-free media and resuspended in media containing d-[8-9-\textsuperscript{3}H] biotin and allowed to grow. Metricel 0.45 μm filter disks (Millipore) were applied to a vacuum manifold, soaked in 0.1 mM biotin to reduce nonspecific biotin binding, and allowed to dry. The filters were then washed in 50% trichloroacetic acid and allowed to dry. Samples (0.1 ml) of the cultures was then pipetted onto the filter disk and followed immediately by 1 ml of 5% trichloroacetic acid solution. The filters were then washed in 1 ml of absolute ethanol to remove free biotin, dried, and the resulting radioactivity counted in a Beckman scintillation counter.

**Northern blotting**—Northern blotting procedures were modified from those of Kornblum et al. (18). Between 5 and 10 μg of total RNA in a volume of approximately 5 μl was added to a
mixture of 2 µl of 5X sodium 3-(N-morpholino)propanesulfonic acid (MOPS) buffer which consisted of 0.1 M MOPS, 40 mM sodium acetate, 5 mM EDTA pH 7.0), 3.5 µl of 37% (w/v) formaldehyde, and 10 µl of deionized ultrapure formamide (Sigma). This mixture was incubated at 65°C for 15 min and placed on ice. RNA loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol FF) (2 µl) was then added.

The RNA species were separated using 1.2% agarose gels prepared with MOPS buffer plus 1.8% formaldehyde, and electrophoresed in 10% MOPS, 2% formaldehyde at 5V/cm for approximately 3 h. Duplicate gels were run and stained with ethidium bromide to confirm the presence of sharp bands for the major ribosomal species. Transfer of RNA to positively charged nylon membranes (Roche) was conducted using a BIOS transfer apparatus with 20 X SSC as the transfer solution. Following overnight transfer, gels were stained with ethidium bromide to visually confirm efficient transfer of the ribosomal RNAs. RNA was fixed to the nylon membrane using a Fisher model FBUVXL-1000 UV-crosslinker for approximately three minutes. Northern blots were incubated in prehybridization solution consisting of 7% sodium dodecyl sulfate, 50% deionized formamide, 5 x SSC, 2% non-fat dry milk, 50 mM sodium phosphate, pH 7.0, and 0.1% N-lauroylsarcosine at 50°C for at least two hours and generally overnight in a volume of 75 ml. The probes (15 to 25 ng/ml) were boiled for 10 minutes, placed on ice for 5 min, and added to 75 ml of prehybridization solution. Hybridization was generally conducted at 60°C for at least 24 h.

RNA preparation—Culture samples were placed in microcentrifuge tubes, centrifuged at 16,000 x g for 30 sec, and the liquid was removed by aspiration. The cultures were then immediately flash-frozen in a dry ice-methanol bath. Alternatively, samples were added to 2 volumes of a tetradecyltrimethylammonium bromide RNA stabilization reagent (RNAProtect Bacterial Reagent, Qiagen), incubated for 15 min, centrifuged, and the pellet flash-frozen in dry ice-methanol. The samples were then briefly thawed and the cells lysed by 1 mg/ml lysozyme in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) for 10 min. The RNA was then purified using nucleic acid binding columns according to the manufacturer's recommendation (Qiagen RNAeasy spin columns), and the RNA quantitated in duplicate by spectrophotometry. RNase-free DNase added to remove traces of DNA. Typical yields from 2 ml of actively growing E.
coli cultures varied from 50 µg to 100 µg of total RNA. When very early phases of growth were assayed, 5 to 10 ml cultures were sampled. The RNA samples were prepared and quantitated immediately before use in northern blotting to ensure uniform loading.

**Northern probe construction and signal detection** — In general, for PCR amplifying genomic sequences to be used as probes in nucleic acid analysis *E. coli* strain MG1655 was used as a template. Bacterial colonies were taken from agar plates and used directly in standard PCR reactions with no further purification. To detect the accBC transcript, primers AccCmRNA left and AccCmRNA right (Table 1) were used to PCR amplify a 663 bp fragment internal to the accC gene, approximately 250 bp downstream of the accC initiation codon. This fragment was subsequently labeled by incorporation of DIG-labeled nucleotides using a random priming method in conjunction with DNA polymerase I Klenow fragment and quantitated before use in northern blots (Roche).

A probe specific to the accB coding region was generated by restriction digestion of plasmid pESJ89 with *Hind*III and *Kpn*I to generate a 264 bp fragment carrying a portion internal to the accB gene. Following purification the fragment was labeled using the replacement synthesis reaction in conjunction with Klenow fragment. To detect mRNA in the accBC promoter region, a segment of DNA that began in the accBC promoter region and extended to 10 bp upstream of the accB ATG was amplified using primers bccpupstleft and bccpupstright (Table 1). Identification of amplified probe sequences were confirmed using restriction digests based on published sequences of the *E. coli* chromosome.

Nucleic acid probes labeled with digoxigenin were detected using anti-digoxigenin Fab fragment antibodies conjugated to alkaline phosphatase (anti-DIG-AP; Roche). Following hybridization with the DIG-labeled probe and post-hybridization washes, anti-DIG-AP antibodies were incubated with either CDP or CDP-Star according to the manufacturers recommendations (Roche) and the resulting light-producing reaction monitored using X-ray film autoradiography.
Construction of genomic replacements and deletions — Genomic replacements were conducted using the methods of Yu et al. (19) and Datsenko and Wanner (20). The use of the defective λ prophage as a source of λred recombinase (19) had a much higher efficiency than the plasmid encoded recombinase functions, such as pKD46 and pKD20 (20). The method of generating genomic insertions was essentially that described (20), with the exception of inducing recombinase expression in pKD46 for 12-24 hours and growth of the transformants at room temperature, which increased the number of recombinant clones considerably. To remove the chromosomal FRT-flanked antibiotic resistance cassettes plasmid pCP20 was used according to the method of Datsenko and Wanner (20). The primers used for the accA kanamycin cassette insertion are accA::kan left accA::kan right. The primers used to delete accB were accBinsertionleft and accbinsertionright.

To replace the endogenous accBC promoter with the lacZYA promoter, a plasmid was constructed consisting of the lacZYA promoter from strain MG1655 inserted downstream of the kanamycin resistance cassette of pKD13 (19). This construct was used as a template for PCR using primers Plac::accbcRevand Plac::accbcRev. The resulting PCR product was recombined into the chromosome using the method of Yu et al. (19). This resulted in the Plac promoter initiating transcription at the same position (297 bp upstream of the accB initiation codon) as the wild type accBC promoter. After PCR confirmation, western blotting demonstrated an IPTG dependent increase in AccB production in this strain (data not shown).
RESULTS

Overproduction of AccB results in decreased accBC operon transcription — Our first indication that AccB might play a role in transcription of the accBC operon was that growth was severely inhibited upon expression of accB from the lac promoter of a multicopy plasmid pLS24 (21). However, if accB and accC were cotranscribed from the same vector growth proceeded normally. It therefore seemed possible that overexpression of AccB might down-regulate expression of the chromosomal accBC operon thereby resulting in an AccC deficiency. Our earliest studies used a plasmid carrying a transcriptional fusion construct consisting of the promoter region upstream of accBC fused to the luxABCDE genes of plasmid pDEW201 (17). Overproduction of AccB from a second plasmid resulted in decreased expression of luciferase (Fig. 1), a result consistent with a regulatory role for AccB.

In subsequent experiments northern blotting was used to determine the levels of the chromosomally encoded accBC transcript in the presence or absence of AccB overproduction. This allowed regulation to be examined in a more physiological context and avoided the complications introduced by use of a multicopy reporter. The chromosomal accBC transcript was monitored independently of the plasmid-encoded accB transcript by use of a probe with homology only to the accC gene portion of the message. Previous analyses of accBC transcription showed no evidence of any RNA processing events that could lead to differential transcription of the accBC transcript (21). Therefore, probing for accC mRNA provides a valid representation of transcription of the entire operon.

Several cell doublings were required before growth inhibition upon accB overexpression from plasmid pESJ89 was observed. Therefore, upon induction of increased accB expression in early- to mid-exponential cultures, growth continued unabated with only a slight reduction in final cell densities. To separate overall growth effects from any specific consequences of AccB overproduction on accBC operon transcription, cultures were grown to early log phase in glucose containing medium (glucose was included to aid repression of the lac promoter). The cells were then collected, washed, and resuspended in medium containing either glucose or IPTG. Upon resumption of growth the levels of chromosomal accBC transcripts were analyzed by northern blotting of RNA samples taken over the course of the experiment (Fig. 2). In cultures where
accB expression was repressed by addition of glucose, accBC operon transcription remained constant throughout the time course (although transcription began to diminish slightly after two hours as growth slowed due to the onset of stationary phase). In contrast induction of increased accB expression had a dramatic and rapid effect on accBC transcription (Fig. 2A).

Densitometric quantitation of accBC transcript levels showed that elevated levels of AccB resulted in a 50% decrease in transcript levels within 30 min after induction, falling to 10% of uninduced levels by 40 min after induction. The inhibition of accBC transcription was specific to AccB overexpression in that overexpression of the accA, accD, and accC gene products had no effect on accBC operon transcript levels (Fig. 2B). Incorporation of d-[8-9-3H] biotin showed that AccB expression from plasmid pESJ89 resulted in a four-fold increase in the rate of AccB production relative to uninduced cultures. Therefore, only a modest overexpression of AccB was needed to trigger the decrease in transcription.

AccB regulation is exerted at the level of transcription initiation — The unusual features of the DNA segment between the accBC promoter and the accB initiation codon suggested that the negative regulation seen upon accB over-expression might not be straightforward transcriptional control. For example, AccB could directly or indirectly influence the structure of either the DNA or the RNA downstream of the site of transcriptional initiation and thereby decrease transcript levels. Although we could not exclude these possibilities we doubted that accBC mRNA stability would be altered since prior work (10) showed no difference in message half-life between rapidly growing and slow growing cells indicating that the variations in accBC mRNA levels were due to the rates of transcription. Comparison of the accBC operon coding regions of Salmonella enterica serovar Typhimurium, a close relative of E. coli, provided a test for the involvement of the leader region in regulation. Although the AccB proteins are identical, the leader regions of the S. enterica and E. coli accBC operons differ significantly particularly in the region between the promoter and a point about 40 bp upstream of the accB initiation codon. If this region was involved in autoregulation the regulation of the S. enterica accBC operon should differ from that of E. coli. Analysis in E. coli of a β-galactosidase transcriptional fusion consisting of the S enterica accBC leader region showed that the S. enterica accBC operon was regulated identically to that of E. coli (data not shown) and thus a regulatory role seemed
precluded for much of the leader sequence. We therefore focused our attention on the promoter region.

To test if the effects of AccB over-expression was exerted on the *E. coli* accBC promoter or on the mRNA we precisely replaced the chromosomal accBC promoter with the *E. coli* lactose operon promoter. The construct was made such that the 5’-nucleotide of the lac promoter transcript would be identical to the accBC promoter transcript. Therefore, any regulatory mechanism that acted following the initiation of transcription should regulate accBC expression from the lac promoter and AccB overproduction should shut down transcription. If no regulation was observed we could then conclude that the accBC promoter was the site of regulation. In the Plac-accBC strain (strain ESJ307) transcription of accBC including the leader region was induced upon IPTG addition, although complete repression of Plac was not possible with this construct. (Upon IPTG induction the rate of AccB synthesis from Plac was increased approximately 2-fold and 4.5 to 5-fold relative to the uninduced culture and the parental strain culture having the normal accBC promoter, respectively.)

We analyzed the effects of AccB overproduction on the Plac-accBC construct of strain ESJ307. The levels of accBC mRNA were assayed by northern blotting using an internal fragment of the accB gene as probe. The transcripts from the chromosome and plasmid were of markedly different lengths and thus use of this probe allowed detection of both the chromosomal accBC transcript and the plasmid-encoded accB transcript. In contrast to accBC expression from the native promoter, overexpression of AccB had no effect on the levels of accBC mRNA initiated from the Plac promoter of strain ESJ307 (Fig. 3). Moreover, induction of the pESJ89 accB gene (the same plasmid used to show negative regulation) failed to inhibit accBC transcription (data not shown). These results demonstrated that AccB overproduction regulated transcription by direct or indirect interaction with the native promoter. These results are consistent with previous studies by Li and Cronan (10) on plasmids in which deletions within the DNA segment between the promoter and the accB gene had no effect on transcript levels.

*The biotinyl domain of AccB and the BirA repressor /ligase protein are not involved in accBC regulation* —AccB is a biotinylated protein and thus production of malonyl-CoA by ACC is
dependent on the synthesis of biotin and the attachment of biotin to apo-AccB. *E. coli* BirA is a bifunctional protein that acts as both the repressor of biotin biosynthesis and the biotin apo-protein ligase that catalyzes attachment of biotin to AccB (22,23). The fact that BirA acts both as a negative transcriptional regulator and as an enzyme interacting directly with AccB made it a good candidate for a role in accBC regulation.

Overproduction of BirA results in super-repression of the biotin biosynthetic operon as well as increased biotinylation of highly expressed biotinylation domains (14,23). However, BirA overexpression had no effect on accBC transcript levels (Figure 4A). We also tested the effects of an absence of functional BirA by use of a temperature sensitive G115S BirA allele (25). The accBC-lux fusion plasmid mentioned above was used to examine the levels of accBC operon expression in the birA1 strain BM4092. Measurement of light production in the birA1 background compared to an isogenic wild type strain showed no significant differences in accBC-lux transcriptional activity (data not shown). The possible role of biotinylation in accBC regulation was tested by producing high intracellular levels of BCCP-87, an AccB C-terminal protein fragment that contains the biotinoyl domain. BCCP-87 is known to interact with BirA as efficiently as does the full length AccB protein (24). We observed no differences in accBC transcript levels between cultures expressing high levels of BCCP-87 and those in which expression of BCCP-87 was repressed (Figure 4B). We also examined the effects of overproduction of BirA.

Specifity of AccB overexpression on accBC transcription — The *B. subtilis* accBC operon was previously isolated by complementation of a temperature-sensitive *E. coli* accB mutant (26) with a plasmid library. In order to see if the *B. subtilis* AccB functioned as well as the native *E. coli* protein we precisely replaced the coding sequence of the *E. coli* chromosomal accB gene with that of *B. subtilis*. This strain grew normally, indicating that the *B. subtilis* AccB protein could fully replace the native protein in the metabolically essential ACC reaction (data not shown). Labeling of this strain with d-[8-9-3H] biotin showed that the expression levels of the *B. subtilis* protein were very similar to those of the native protein (data not shown).
To test if production of *B. subtilis* AccB regulated transcription of the *E. coli* accBC operon the *B. subtilis* accB gene was cloned from strain JH642 and placed under control of the Paralac promoter to give pESJ209. A surprising result was that the *B. subtilis* accB plasmid gave better growth of the *E. coli* accB temperature-sensitive strain than did the *E. coli* accB gene expressed from the same vector and promoter. Thus, high-level expression of *B. subtilis* accB had no deleterious effects on cell growth. To test the specificity of accBC regulation the *B. subtilis* accB gene was expressed from the Plac promoter on plasmid pESJ207 and transcription from the *E. coli* chromosomal accBC operon was measured by northern blot analysis using a probe derived from the upstream region of the accBC operon (Fig. 5). High-level expression of *B. subtilis* AccB had no effect on transcription of the *E. coli* accBC operon consistent with the lack of growth inhibition mentioned above.

The *B. subtilis* AccB was fully functional in *E. coli*, but failed to regulate expression of the *E. coli* accBC operon. This together with our finding that the biotinoyl domain of *E. coli* AccB had no role in the observed regulation led us to construct a gene encoding a chimeric AccB protein. The encoded protein contained the N-terminal 44 residues of *E. coli* AccB and the C-terminal 109 residues of *B. subtilis* AccB. The N-terminal segment was chosen based on the work of Alix (27) who reported that a λ clone encoding this fragment plus the accBC promoter was toxic to the temperature-sensitive accB strain. As a control, the same vector (pPROLar.A122) was used to express a second chimeric protein consisting of the 47 N-terminal residues of *B. subtilis* AccB and the 109 C-terminal residues of *E. coli* AccB. Low level expression of either of these chimeric AccB proteins allowed growth of an accBts strain and western blotting using labeled streptavidin demonstrated that the chimeric proteins were efficiently biotinylated (data not shown). Moreover quantitative assays of biotinylation by incorporation of [3H]-biotin showed that the two proteins were expressed at very similar levels (data not shown). Upon high-level expression of the chimeric protein containing the *E. coli* N-terminal sequence, the level of mRNA expressed from the chromosomal accBC operon decreased greatly (densitometric analysis of northern blots showed a 54% decrease in transcript levels after 30 min, falling to 4% of the uninduced levels after 60 min). In contrast no decrease in chromosomal accBC operon expression was seen upon similar high-level expression of the chimeric AccB containing the N-terminal 47 residues of *B. subtilis* (Fig. 6). Therefore, only the
44 N-terminal residues of *E. coli* AccB were required to exert negative regulation of *accBC* transcription. These data suggest that this fragment forms a stably folded protein despite approaching the lower size limit of proteins able to attain a stably folded structure.

*The N-terminus of BCCP is sufficient to give potent negative regulation* — Although the *E. coli* AccB biotinylation domain (BCCP-87) has been extensively studied, the amino-terminal portion of the protein has not been characterized. This is due to the properties of the full-length AccB. The intact AccB very readily aggregates and is susceptible to proteolysis during purification (28,29). Indeed, it has not been possible to define the solution structure of the intact AccB since many forms of different sizes are seen in ultracentrifugation experiments (24,28,29). This aggregation behavior seems a property of the N-terminal third of the protein because the biotinylation domain (plus varying extents of the central linker region) is monodisperse and very soluble. The N-terminus seems to function in the intra- and inter-molecular interactions required to assemble the intact four-subunit active form of ACC, since small deletions within the N-terminal segment prevent the association of AccB with the AccC subunit (30).

The results obtained with the chimeric protein indicated that the structural determinants for negative regulation of the *E. coli accBC* operon resided in the AccB N-terminus. In a further test a plasmid (pESJ114) was constructed that was identical to the plasmid used for the overexpression of full-length AccB except that the C-terminal domain was deleted. The truncated gene encoded a protein consisting of the N-terminal 68 residues of *E. coli* AccB and thus contains only the interaction domain plus the most of the linker region. Construction of the plasmid encoding this protein fragment was problematical. It was necessary to construct and maintain the plasmid in a *lacIq* strain background plus catabolite repression conditions in order to very strongly repress synthesis (even basal expression levels were toxic). The growth of cultures upon expression of the AccB 1-68 protein followed a pattern similar to that observed when the full length protein was overexpressed in that initiation of synthesis during lag phase or early log phase caused a rapid cessation of growth. Induction of expression of the N-terminal construct later in exponential growth had less of an effect, although the final cell density reached was less than when the full-length protein was expressed. Interestingly, when plated on solid medium cultures expressing the N-terminal AccB fragment produced many fewer spontaneous suppressors of the toxicity phenotype that did cells expressing the full-length protein. However,
attempts at mapping these suppressors were unsuccessful. Only plasmid-borne mutants that altered expression were found.

Upon induction of the synthesis of the N-terminal AccB fragment, a rapid decrease in the levels of chromosomal accBC transcript was seen (Fig. 7). In contrast without induction the accBC transcript levels followed the previously characterized profile in which transcription levels were highest during early to middle log phase and began to decrease as cultures entered stationary phase. In contrast, upon induction of synthesis of the N-terminal AccB fragment the levels of the accBC transcript fell to 8% of uninduced levels within 10 min and became undetectable after that time. The growth of the induced cultures proceeded normally for two hr indicating that shutdown of accBC expression was not the cause or result of a general defect in growth. These data together with those obtained with the chimeric protein indicate that the structural determinants for negative regulation of the accBC operon reside within the N-terminal third of AccB.

Effects of deletion of accB — Malonyl-CoA synthase from Rhizobium trifolii catalyzes the ATP-dependent formation of malonyl-CoA from malonate and CoA, and expression of the operon encoding this enzyme has been shown bypass loss of E. coli ACC activity in vivo (31). Using the linear recombination method of Yu et al. (19) in conjunction with pESJ296 and malonate supplementation, we constructed accA::kan and accA::kan accB::cml chromosomal deletion strains. We failed to construct an accB deletion in the absence of the accA::kan allele nor could we transduce the accB::cml marker into a wild type background.

Despite our failure to separate the accA and accB mutations, the ΔaccA ΔaccB strain did provide a means to test the effects of a complete lack of AccB on accBC transcription. In these experiments the accA::kan ΔaccB strain was compared to the accA::kan strain and accBC transcript levels was assayed by use of a probe to the leader region of the mRNA which remained intact in the accB deletion strain (Fig. 8). Although the growth rates of the accA::kan and accA::kan ΔaccB strains were very slow (presumably due to the inefficiency of malonate supplementation), they were essentially identical (although the accA::kan ΔaccB strain grew to a slightly greater final density). In the accA::kan ΔaccB strain high levels of accBC transcript of
were maintained longer into stationary phase than in the accA::kan strain (Fig. 8). Densitometry of the northern blots showed that the level of the accBC operon transcript was increased an average of 5-fold throughout the growth curve. Moreover the ΔaccB strain also continued to accumulate accBC leader mRNA for almost two hours after accBC transcript accumulation had ceased in the accA::kan strain. These results complement those seen upon over-expression of AccB or of the AccB N-terminal fragment, and confirm that AccB is necessary for accBC operon regulation.
DISCUSSION

Prior work of Li and Cronan (10) showed that accBC expression from the native promoter was not linear with gene copy number. Multicopy plasmids having copy numbers ranging from 20-100 all gave only a two-fold or less overexpression of the AccB and AccC proteins. The low levels of protein over-expression were attributed to transcriptional regulation because only a two-fold or less increase of accBC mRNA levels was observed. This lack of proportionality with gene dosage was suggested to be due to titration of a positive transcriptional activator present at limiting levels (10). However, the present work shows that accBC transcriptional regulation is exerted by the AccB protein itself and therefore is an example of autogenous repression. Replacement of the native promoter in its chromosomal location with a lac promoter eliminated autogenous repression. Since the mRNA species expressed from the native promoter and the lac promoter should be identical, this rules out regulation exerted on or within the mRNA such as formation of mRNA secondary structures or mRNA degradation. Therefore, accBC autogenous repression is clearly exerted upon initiation of accBC transcription at the native promoter. Studies with chimeric proteins and with an AccB N-terminal fragment indicate that only the first 44 residues of AccB are required for autoregulation. It seems probable that the ability of the AccB N-terminal fragment to abate transcription makes the cells extremely susceptible to the high rate of AccB degradation in accBts strains (16), and that this was the cause of the N-terminal fragment toxicity reported by Alix (27).

Our data suggest a straightforward model of autoregulation, in which the accumulation of BCCP causes negative feedback on accBC transcription. The most straightforward mechanism for this repression would be for the N-terminal region of AccB to directly bind the accBC promoter and thereby prevent promoter utilization. Unfortunately, in vitro tests of this hypothesis are very problematical due to the highly intractable nature of the intact AccB protein. Although the AccB biotinyl domain fragment is a protein of almost ideal properties (12,24), the full-length E. coli AccB has never been purified as a discrete protein species from either amplified or non-amplified sources. Even at micromolar concentrations the full-length protein was reported to form aggregates with molecular weights ranging from that of the monomer (17 kDa) to 200 kDa (29). Recent data obtained using modern techniques gave data sets for the
AccB solution molecular weight that yielded values of 70, 109, and 182 kDa using a single species model although agreement with the model was poor in all data sets (24). Therefore, although we have performed numerous unsuccessful gel mobility shift experiments using the accBC operator region and various AccB preparations, the intractable nature of the protein may well have prevented detection of interactions. If AccB binds the accBC promoter DNA, it must do so by a novel DNA binding motif since there is no evidence of a known motif within the N-terminal sequence. For this reason it seems likely that AccB exerts its regulatory effect through another protein or proteins (or perhaps an RNA) and we are currently seeking candidate molecules. It is noteworthy that although AccB is the sole biotinylated protein of E. coli, the accBC regulatory mechanism has no overlap with the BirA regulatory system that controls expression of the biotin biosynthetic genes.

It has long been assumed that AccB is present in the active ACC complex at levels equimolar to the three other subunits. This assumption was based on several other biotin-dependent carboxylases in which the AccB and AccC sequences reside as separate domains on a single protein with the AccB domain being the C-terminal domain. However, recent results indicate that translation of the accBC mRNA results in two molecules of AccB per molecule of AccC and that a metastable complex is formed consisting of four AccB molecules in association with an AccC dimer (30). This complex is unstable in vitro and the same instability may posit in vivo. If so, the amount of free AccB might vary in vivo depending on the ACC activity and thereby provide a feedback between enzyme activity and enzyme synthesis. It remains possible that AccB acts indirectly. For example, elevated levels of AccB might sequester AccC such that it cannot bind the AccA-AccD carboxyltransferase complex. The free AccA-AccD complex could then be the transcriptional repressor. Future studies will involve defining the precise protein and DNA sequence requirements for regulation, as well as the existence of other accessory regulatory molecules. In addition to nonlinearity with accBC gene dosage Li and Cronan (10) also observed that accBC transcription was controlled by growth rate. Although we did not directly test growth rate control in the present experiments, it seems likely that AccB accumulation also plays a role in this phenomenon. This suggestion stems from our finding that the accB deletion strain continued accBC transcription well into stationary phase (Fig. 8); growth conditions that normally result in greatly decreased transcription (10).
Although gene orders are frequently not conserved in bacteria (32), the *accBC* arrangement is found with high frequency. In their identifications of orthologous pairs of genes found adjacent in at least two evolutionarily distant organisms, Snel *et al.* (33) placed the probability of *accBC* belonging to the same operon at 98%. This conservation of the *accBC* arrangement in distantly related bacteria suggests that the genes are co-regulated. Although large-scale genomic sequencing has revealed a great number of potential *accBC* operons, the issue of co-expression has been directly tested in only two organisms, *E. coli* and *B. subtilis*. The arrangement of the *B. subtilis accBC* genes is very similar to that of *E. coli* in that the genes are separated by only 11 bp whereas 10 bp separate the *E. coli* genes. The 5’ end of the *B. subtilis* transcript was mapped by primer extension to 56 nucleotides upstream of the *accB* initiation codon (26). The pattern of *B. subtilis accBC* transcription shows growth-dependent regulation of *accBC* mRNA levels very similar to that seen in *E. coli*. Although it was suggested that the *B. subtilis accBC* genes might be regulated by the sporulation regulator, SpoIIIA, direct analyses indicate that *accBC* transcription is unaffected by sporulation programming (11), although cells with low levels of AccB sporulate poorly and are delayed in both spore germination and outgrowth (34). Therefore, despite the complication of sporulation in this gram positive organism, two distantly related bacteria seem to regulate *accBC* transcription in a similar manner. It is tempting to postulate that AccB may play in role in *B. subtilis accBC* regulation similar to that we have observed in *E. coli*. 
References

### Table 1. Bacterial Strains and Plasmids Employed

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Genotype or Description</th>
<th>Source or Reference</th>
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<tr>
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<td>Coli Genetic Stock Center</td>
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<td>JH642</td>
<td><em>Bacillus subtilis</em> phe trp</td>
<td>D. Mendoza</td>
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<tr>
<td>CY481</td>
<td>BM4092</td>
<td>(35)</td>
</tr>
<tr>
<td>SA291</td>
<td>Δ(galK-moa) bio operon deletion</td>
<td>(15)</td>
</tr>
<tr>
<td>L8</td>
<td><em>accB</em>&lt;sup&gt;ts&lt;/sup&gt;</td>
<td>(21)</td>
</tr>
<tr>
<td>BW25113</td>
<td><em>lacI</em>&lt;sup&gt;q&lt;/sup&gt; <em>rrnB</em>&lt;sup&gt;T14&lt;/sup&gt; ΔlacZWJ16 <em>hsdR</em>&lt;sup&gt;514&lt;/sup&gt;</td>
<td>(20)</td>
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<td></td>
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<td></td>
</tr>
<tr>
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<td>BW25113 ΔaccA ΔaccB/pESJ296</td>
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<td>ESJ307</td>
<td>MG1655 Plac::accBC</td>
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<td>DBY329</td>
<td>W3110 λc1857 Δ(cro-bioA)</td>
<td>(19)</td>
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<td>pESJ89</td>
<td><em>accB</em> under Plac control, pBR322 ori, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td><em>accB</em> N-terminus derivative of pESJ89 Kan&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pBR322 origin</td>
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<td>pCY216</td>
<td><em>birA</em> under Para control, pACYC184 ori</td>
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<td>pCY335</td>
<td>BCCP-87 under control of <em>ParaBAD</em>, pBR322 origin, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pMATop-2</td>
<td><em>matABC</em>, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pProLarA.122</td>
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<td>pESJ208</td>
<td><em>accB</em> in pProLarA.122</td>
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<td><em>lacI</em> gene, pSC101 ori Strep^R/Spec^R</td>
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<td>pKD46</td>
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Table 2. Oligonucleotide primers used in this work.

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<tr>
<td>AccCmRNA right</td>
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<tr>
<td>bccpupstleft</td>
<td>CGAAATCGTTATAATGAGCG</td>
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<tr>
<td>bccpupstright</td>
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<td>accA::kan left</td>
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<td>accA::kan right</td>
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<td>accBinsertionleft</td>
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<td>Plac::accbcRev</td>
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Figure legends

Fig. 1. **Light emission of a lux::accBC transcriptional fusion in the presence of AccB overexpression.** Plasmid pESJ208 produced low levels of AccB protein and induction had little effect on growth when compared to a control strain containing the parental cloning vector pProLarA.122. Overnight cultures were diluted 500-fold into fresh medium and grown at 37°C until very early log phase (OD$_{600}$ of 0.05). The cultures were then split in half and arabinose (0.2%) plus IPTG (0.1 mM) was added to one half to induce the Para/lac promoter whereas the other culture remained uninduced. Light production (closed symbols: • , no induction; ▲ , induction) was measured using a Berthold Lumat luminometer and reported in terms of Relative Light Units. Growth was at 37°C and was monitored at 600 nm (open symbols: □ , no induction; ◊ , induction).

Light production in the control strain follows the previously described growth phase dependent pattern of accBC operon transcription with transcription progressively decreasing upon entry to stationary phase (10).

Fig. 2. **Specifically decreased levels of chromosomally encoded accBC mRNA in the presence or absence of AccB overexpression.** Panel A. Strain MG1655 carrying plasmid pESJ89 was grown overnight in the presence of 0.2% glucose to repress expression from the Plac promoter. A 200 ml overnight culture was diluted one thousand-fold into the same medium and grown at 37°C until early log phase (OD$_{600}$ of 0.25). The cells were then harvested, washed with RB medium, resuspended in 100 ml of pre-warmed RB medium, and split into two cultures. One culture contained 0.2% glucose whereas the other culture contained IPTG (0.1 mM). The growth rates of the two strains over the time course examined were essentially identical. Samples were taken at the indicated time points. RNA was then extracted and quantitated as described in Experimental Procedures. Total RNA (5 µg) from the samples was loaded into each lane and separated by electrophoresis in a formaldehyde/MOPS buffer system. Chromosomal accBC transcription was detected using a DIG-labeled DNA probe specific to the accC gene.

Panel B. Transcription accBC in the presence of Acc subunit expression. Actively growing *E. coli* cultures of assayed for accBC operon mRNA levels following expression of an acc gene from plasmids pESJ100 (AccC), pCYL2 (AccD), pESJ195 (AccA), and pESJ208 (AccB).
Cultures were grown in the presence of glucose and the cells were harvested at an OD$_{600}$ of 0.3, resuspended in fresh medium containing inducer (0.2% arabinose and 0.5 mM IPTG), and sampled after 90 min of induction.

Fig. 3. Analysis of accBC transcription following replacement of the accBC promoter with the lac promoter. Strain ESJ307 was constructed such that the accBC promoter was precisely replaced by the *E. coli* lac promoter. Transcription of the accBC genes and the upstream leader region was inducible with IPTG, although complete repression of P$_{lac}$ promoter could not be attained. RNA analysis by northern blotting was conducted with strain ESJ307 carrying pCY465 using a probe consisting of a fragment internal to the accB gene. This probe allowed detection of both chromosomal accBC transcription (upper band) in addition to the plasmid accB mRNA production (lower band), which served as an internal control. The cultures were grown in RB medium containing 0.2% glucose until an OD$_{600}$ of about 0.4 was attained. The cells were then harvested by centrifugation, washed in RB medium, and resuspended in medium containing either 0.4% glucose or 0.1 mM IPTG plus 0.4% arabinose to induce AccB production from plasmid pCY465. The cultures were then sampled at the indicated time points. Further experimental details are given in Fig. 2 and Experimental Procedures except that RNA samples of 7 µg were loaded.

Fig. 4. Effects of the expression of BirA overexpression and AccB biotinylation domain expression on accBC operon transcription. Panel A. *E. coli* strain MG1655 containing plasmid pCY375 was grown in RB medium and supplemented with 0.2% glucose to an OD$_{600}$ of 0.35. The cells were harvested, washed with RB medium, resuspended in pre-warmed RB medium, and split into two cultures. One culture contained 0.2% glucose to repress transcription and the other culture contained 0.2% arabinose to induce expression of the truncated accB gene present on plasmid pCY375. The cultures were sampled at the indicated time points. Panel B. *E. coli* strain MG1655 containing plasmid pCY216 was grown in RB medium supplemented with 0.2% glucose to an OD$_{600}$ of 0.35. The cells were then harvested, washed in RB media, resuspended in pre-warmed RB medium and split into two fractions. One culture contained 0.2% glucose to repress birA transcription and the other culture contained 0.2% arabinose to induce expression of the birA gene on plasmid pCY216. The samples and analyzed as described in Fig. 2 and Experimental Procedures except that 7 µg RNA samples were loaded in panel B.
Fig. 6. **Effects of *B. subtilis* accB expression on expression of the *E. coli* accBC operon.** An overnight culture of strain MG1655 carrying plasmid pESJ209 which contained the *B. subtilis* accB gene under control of the Para/lac promoter was grown in RB medium and inoculated one thousand-fold into RB medium in the presence of glucose and grown at 37˚C to early log phase (OD$_{600}$ of 0.4). Cells were then harvested, washed in RB medium, and resuspended in 100 ml of pre-warmed RB medium and split into two cultures of 50 ml each. One culture was supplemented with 0.2% glucose (final concentration) and the other with IPTG (0.1mM final concentration) plus 0.2% arabinose. Samples were taken at the indicated time points. RNA was then extracted and quantitated as described in the Materials and Methods. Further experimental details are given in Fig. 2 and Experimental Procedures except that 7 µg RNA samples were loaded.

Fig. 6. **Effects of expression of chimeric AccBs on expression of the *E. coli* accBC operon.** Two chimeric genes were tested. A control plasmid (called Bs-Ec chimera in the figure) encoding a second chimeric protein consisting of the N-terminal 47 residues of *B. subtilis* AccB fused to the C-terminal 109 residues of *E. coli* AccB was constructed in vector, pPROLar.A122. A second gene (called Ec-Bs chimera in the figure) encoding an in-frame translational fusion between the N-terminal 44 amino acids of *E. coli* AccB and the carboxy terminal 109 residues of *B. subtilis* AccB was similarly constructed. Overnight cultures grown in RB medium supplemented with 0.2% glucose and diluted one thousand-fold into the same medium. Growth was monitored spectrophotometrically until an OD$_{600}$ of 0.4 was reached. The cells were then harvested, washed in RB medium, resuspended in 100 ml of pre-warmed RB medium and split into two cultures of 50 ml each. One culture was supplemented with 0.2% glucose and the other contained 0.1 mM IPTG. The cultures were sampled at the indicated time points and analyzed as described in Fig. 2 and Experimental Procedures.

Fig. 7. **Effects of expression of an N-terminal fragment of AccB on accBC transcription.** The accB gene was truncated by insertion of a kanamycin cassette, resulting in plasmid pESJ114, as described in Experimental Procedures. The resulting mutant protein consists of the first 68 amino acids of AccB. Strain MG1655 containing plasmid pESJ114 was grown in RB medium at supplemented with 0.2% glucose and antibiotics to an OD$_{600}$ of 0.25. The cells were then
harvested, washed in RB medium, resuspended in pre-warmed RB medium and split into two cultures. One culture contained 0.2% glucose and the other contained 0.1mM IPTG to induce expression of the truncated accB gene on plasmid pESJ114. The cultures were sampled at the indicated time points and analyzed as described in Fig. 2 and Experimental Procedures.

Fig. 8. **Northern blot analysis of accBC transcription in the presence of ΔaccA and ΔaccA accB alleles.** Strains containing either the ΔaccA or ΔaccA ΔaccB mutations were grown in the presence of 2 mM malonate overnight and diluted 50-fold into RB medium containing 0.2% glucose and 2 mM malonate. Samples of 5 to 10 ml were taken at the indicated time points of the above growth curve to which an RNA stabilization reagent was added and RNA prepared (see Experimental Procedures). Due to the absence of the entire accB gene both transcripts were detected with a probe homologous to the accBC leader region. The smaller size of the ΔaccA ΔaccB samples reflects the absence of the accB gene in the transcript. The cultures were sampled at the indicated time points and analyzed as described in Fig. 2 and Experimental Procedures except that a DIG-labeled DNA probe specific to the leader region of the accBC operon was used to assay accBC transcription.
Fig. 1
Fig. 2

A. Time (min)  

<table>
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<tr>
<th>AccBC mRNA</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
</table>

Repressed accB  

Induced accB

Fig. 3

B. ACC Subunit Overexpression  

Vector AccA AccB AccC AccD

accBC mRNA

Fig. 3
Fig. 4.

A. Repressed birA Expression  
Time (min) | 0 | 10 | 20 | 30 | 40 | 50 | 60 | 90

Induced birA Expression  
Time (min) | 0 | 10 | 20 | 30 | 40 | 50 | 90

B. Repressed Domain Expression  
Time (min) | 0 | 10 | 20 | 30 | 40 | 50 | 60 | 90

Induced Domain Expression  
Time (min) | 0 | 10 | 20 | 30 | 40 | 50 | 60 | 90

accBC transcript
Fig. 5.

\begin{tabular}{|c|c|c|c|c|c|}
\hline
Time & 0 & 15 & 30 & 45 & 90 \\
\hline
(min) & & & & & \\
\hline
\end{tabular}

\begin{tabular}{|c|c|c|c|c|}
\hline
Time & 0 & 15 & 30 & 45 & 90 \\
\hline
(min) & & & & & \\
\hline
\end{tabular}

\begin{tabular}{|c|c|c|c|c|c|}
\hline
B. subtilis & B. subtilis \\
accB Repressed & accB Induced \\
\hline
\end{tabular}

Fig. 6.

\begin{tabular}{|c|c|c|c|c|}
\hline
Bs-Ec Chimera & Ec-Bs Chimera \\
\hline
0 & 15 & 30 & 40 & 60 & 90 \\
\hline
\end{tabular}
Repressed AccB
1-68

Induced AccB
1-68

Time (min) 0 10 20 30 40 60 120

Fig. 7

\[ \Delta accA \]

Time (hours) 5 7 8 10 11 28

\[ \Delta accA \Delta accB \]

Fig. 8.