

Coordinate Expression of the Acetyl Coenzyme A Carboxylase Genes, *accB* and *accC*, Is Necessary for Normal Regulation of Biotin Synthesis in *Escherichia coli*[∇]

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Transcription of the biotin (*bio*) biosynthetic operon of *Escherichia coli* is negatively regulated by the BirA protein, an atypical repressor protein in that it is also an enzyme. The BirA-catalyzed reaction involves the covalent attachment of biotin to AccB, a subunit of acetyl coenzyme (acetyl-CoA) carboxylase. The two functions of BirA allow regulation of the *bio* operon to respond to the intracellular concentrations of both biotin and unbiotinylated AccB. We report here that *bio* operon expression is down-regulated by overproduction of AccC, another acetyl-CoA carboxylase subunit known to form a complex with AccB. This down-regulation is eliminated when AccB and AccC are coordinately overexpressed, but only when the AccB partner is competent to bind AccC. Under AccC overexpression conditions AccB is underbiotinylated. These findings can be explained by a model in which excess AccC sequesters AccB in a complex that is a poor substrate for biotinylation. The observed disruption of biotin synthesis and attachment provides an excellent rationale for the observation that in the vast majority of sequenced bacterial genomes AccB and AccC are encoded in a two-gene operon.

Expression of the *Escherichia coli* biotin synthetic (*bio*) operon is controlled by a remarkably sophisticated regulatory system in which the rate of transcription of the operon responds not only to the supply of biotin but also to the supply of proteins (called biotin acceptor proteins) that become modified by covalent attachment of biotin (Fig. 1) (2–6, 10, 16, 17). The response to the supply of biotin acceptor proteins is readily rationalized by the fact that biotin has a biological function only when the vitamin is covalently attached to such proteins; the free vitamin is inactive (18). The enzymes of *E. coli* biotin synthesis are encoded (with one exception) by a cluster of genes located adjacent to the attachment site of phage λ called the biotin (*bio*) biosynthetic operon. Transcription of these *bio* genes occurs from two partially overlapping face-to-face promoters controlled by a common operator site that binds a dimer of the BirA protein (2–6, 29). It is the properties of BirA that allow the system to respond to the intracellular concentrations of both biotin and biotin acceptor proteins (5, 16). This is because BirA is the enzyme (biotin protein ligase) that catalyzes attachment of biotin to acceptor proteins, as well as the repressor of the *bio* operon (2, 16).

The biotin attachment activity of BirA proceeds through a biotinoyl-5'-AMP intermediate. Biotinoyl-5'-AMP is then attacked by the ϵ -amino group of a specific lysine of the acceptor protein to give the biotinylated acceptor protein (9) (Fig. 2A). In the absence of an appropriate acceptor protein the biotinoyl-5'-AMP intermediate remains firmly bound within the BirA active site, where it is quite stable (33). It is this liganded form of BirA that binds the *bio* operator. Therefore, increased

bio operon transcription is triggered either by inhibition of biotinoyl-5'-AMP synthesis by intracellular biotin limitation (Fig. 1B) or by increased consumption of biotinoyl-5'-AMP due to high levels of unmodified acceptor proteins (Fig. 1C). The two conditions act by a common mechanism in that both of them decrease the levels of liganded BirA available to bind the *bio* operator (Fig. 1B and C). Hence, the degree of repression of *bio* operon transcription can be viewed most simply as due to antagonism between retention of biotinoyl-5'-AMP in the BirA active site and consumption of the BirA-bound biotinoyl-5'-AMP by transfer of the biotinyl moiety to unmodified acceptor proteins (16). A structural context for this antagonism is provided by the model of Beckett and coworkers (30, 32), in which the unmodified acceptor protein binds monomeric BirA and thereby inhibits formation of BirA dimers, the species required for effective repression.

E. coli contains only a single species of biotin acceptor protein, the AccB subunit of acetyl coenzyme A (acetyl-CoA) carboxylase (ACC), which is the first enzyme of fatty acid biosynthesis (18, 19). AccB, which is also called biotin carboxyl carrier protein, forms an unstable complex with AccC, the subunit that catalyzes the biotin carboxylase partial reaction of acetyl-CoA carboxylase (Fig. 2B). The AccB-AccC complex was recently shown to consist of an AccC dimer plus four copies of AccB (12). This complex is thought to bind an $\alpha_2\beta_2$ heterotetramer of the AccA and AccD subunits to form active ACC, the enzyme required for production of malonyl-CoA, the key precursor of fatty acid synthesis (18). Since the *E. coli* ACC subunits are required in a defined stoichiometry, it seems surprising that only the *accB* and *accC* genes are cotranscribed. The chromosomal locations of the *accA* and *accD* genes are well removed from the locations of the *accBC* operon and each other (18). The rates of transcription of all four genes are controlled by the cellular growth rate (25), and transcriptional

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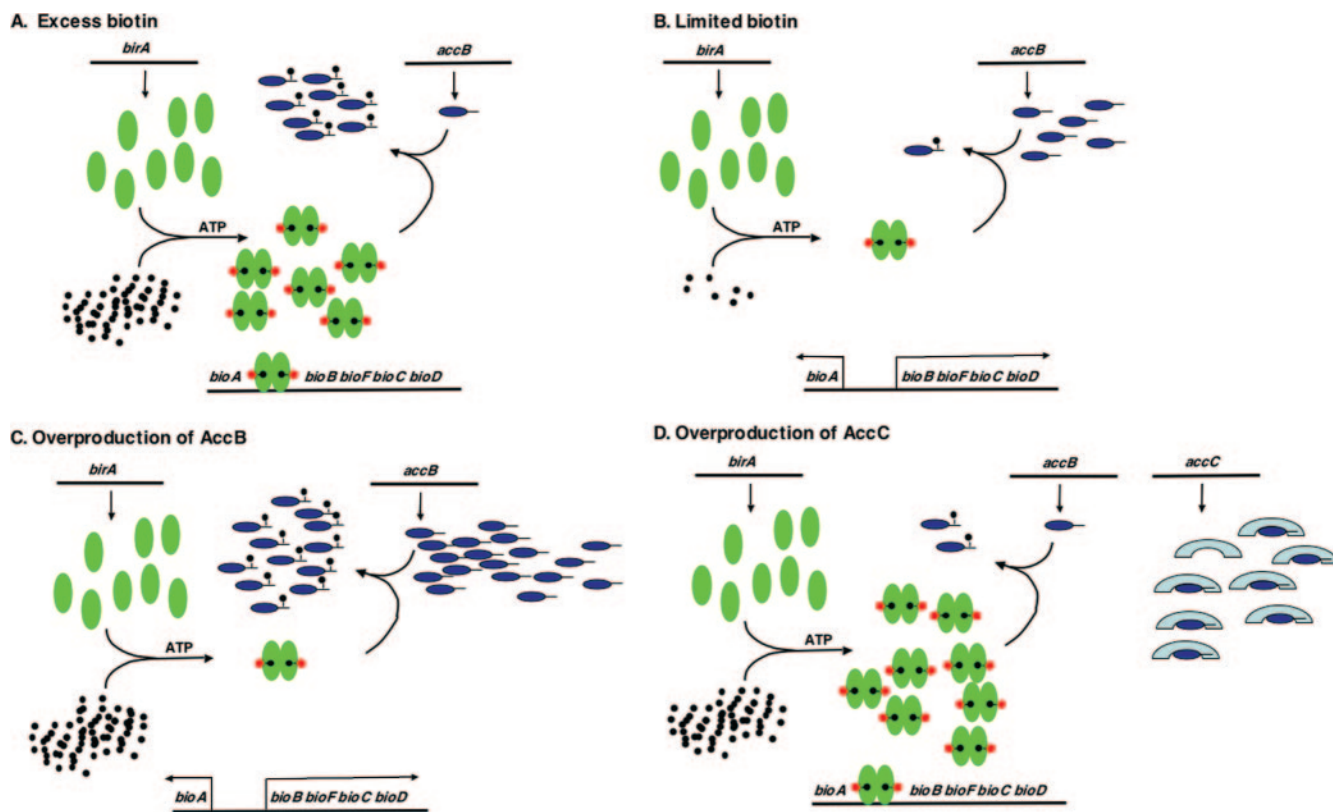


FIG. 1. Biotin regulatory system of *E. coli*. BirA is represented by green ovals, biotin is represented by black circles, the AMP moiety is represented by red pentagons, AccB is represented by dark blue ovals, and AccC is represented by light blue crescents. The arrows indicate transcription from the leftward and rightward *bio* promoters. (A to C) BirA switches from the biotin ligation function to the repressor function in response to the intracellular biotin requirement, which is indicated by the level of unbiotinylated AccB. If unbiotinylated AccB levels are high, the protein functions as a biotin ligase. Once the unbiotinylated AccB has been converted to the biotinylated form, the biotinoyl-5'-AMP is no longer consumed and remains bound to BirA. The liganded form of BirA accumulates to levels sufficiently high that the *bio* operator is fully occupied, resulting in transcriptional repression of the biotin biosynthetic genes. (D) Overproduction of AccC ties up unbiotinylated AccB in a complex that is a poor biotinylation substrate. Therefore, high levels of the liganded form of BirA accumulate, resulting in repression of *bio* operon transcription.

initiation of the *accBC* operon is autoregulated by *accB* levels by an unknown mechanism (22, 25).

The *accBC* gene arrangement first found in *E. coli* is strikingly conserved among bacteria (22). The two genes have been found to be adjacent in all sequenced proteobacterial genomes, including the smallest and largest such genomes (*Pelagibacter ubique* and the pseudomonads, respectively). The *accBC* gene arrangement is also found in gram-positive bacteria, such as the *Bacillales* and some clostridia. In other clostridia and in the *Lactobacillales* a fatty acid synthetic gene is located between the *accB* and *accC* genes. However, in these organisms all of the ACC subunits, as well as all of the fatty acid synthetic enzymes, are encoded by what appears to be a single long transcript. Therefore, even with an intervening gene, transcription of *accB* and *accC* is "hardwired," with *accB* being the upstream gene. Moreover, the *accBC* gene arrangement of *E. coli* is found in such diverse bacteria as *Thermus thermophilus*, *Chlorobium tepidum*, and *Chlamydia*.

The extremely strong conservation of the *accBC* gene arrangement coupled with the contrasting highly random genomic locations of *accA* and *accD* implies that tight regulation of the AccB:AccC ratio is essential. If this is true, an excess of either AccB or

AccC should be detrimental to bacterial physiology. It is known that overproduction of AccB in *E. coli* shuts down transcription of the *accBC* operon (22) and also results in derepression of the biotin biosynthetic operon, thereby leading to wasteful synthesis of biotin (10, 17). Would an excess of AccC also be detrimental? Karow and coworkers (23) described complex experiments which suggested that this is the case. In their studies of suppressors of null mutants with mutations in the *E. coli htrB* gene, a gene later shown to encode an acyltransferase that functions late in lipid A biosynthesis (14), these workers found that overproduction of AccC in *E. coli* resulted in significant inhibition of fatty acid synthesis, whereas overproduction of both AccB and AccC resulted in no such inhibition (23). A plausible mechanism to explain these results is that excess AccC might inhibit biotinylation of AccB, which in turn would repress the *bio* operon and inhibit biotin synthesis. This scenario posits that free AccB is the preferred substrate for BirA-catalyzed biotinylation and that excess AccC competes with BirA and ties up a portion of unmodified AccB in an AccB-AccC complex that is a poor substrate for biotinylation (Fig. 1D). We report here that AccC overproduction does indeed block derepression of biotin synthesis at low biotin concentra-

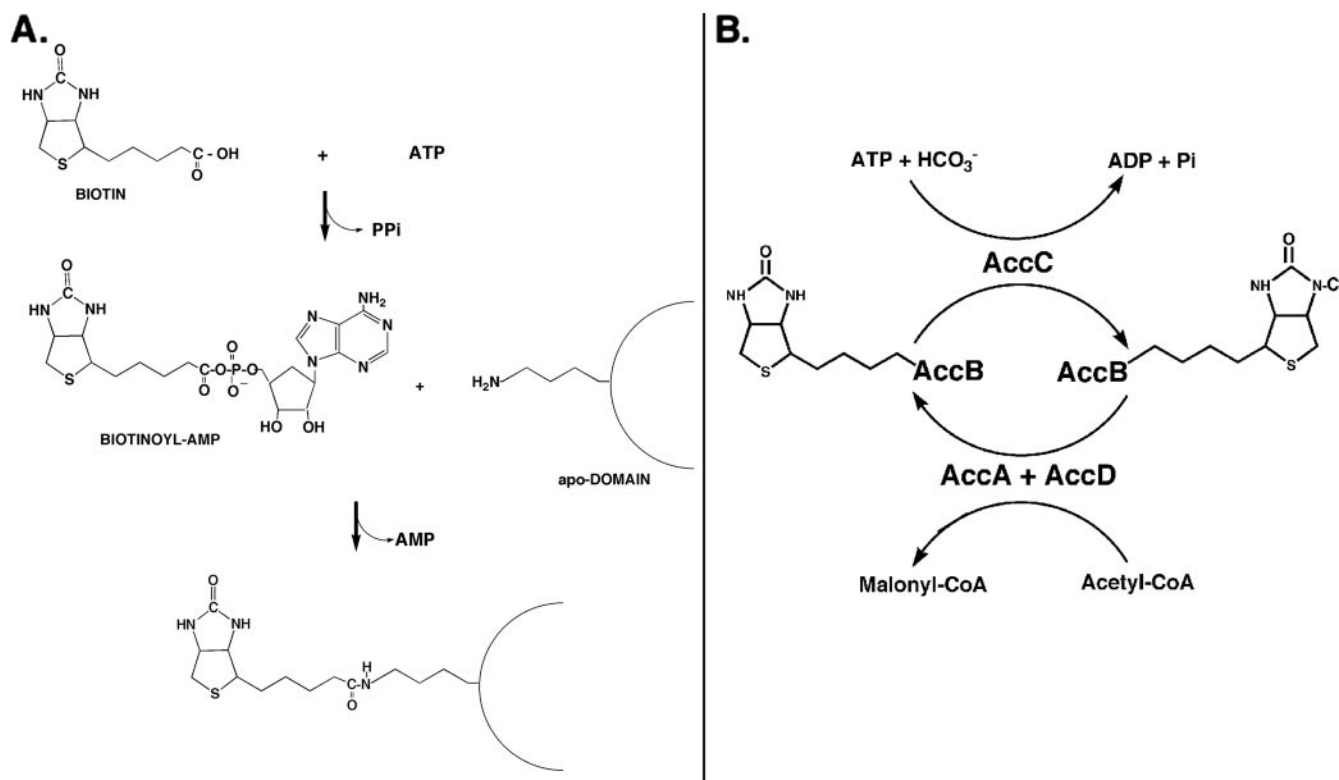


FIG. 2. BirA and acetyl-CoA carboxylase reactions. (A) BirA reaction, which is the general reaction of biotin protein ligases (9). (B) Acetyl-CoA carboxylase reaction. The functional enzyme is thought to have the composition 2AccA:4AccB:2AccC:2AccD, with the AccB-AccC and AccA-AccD complexes sufficiently stable for isolation in vitro (18).

tions and also inhibits biotinylation of AccB. The demonstrated need for precise stoichiometry during production of AccB and AccC provides an excellent rationale for the fact that in the great majority of the extant bacterial genomes the *accB* and *accC* genes are adjacent so that they can be cotranscribed to ensure stringent regulation of the ratio of the two proteins.

MATERIALS AND METHODS

Bacterial strains and media. All strains were derivatives of *E. coli* K-12. Strain CY1740, a derivative of strain CY486 (17) carrying the *lacI*^q plasmid pMS421, was used in this work. Strain CY1740 carries a chromosomal ϕ (*bioFC-lacZ*)501 fusion (4). The medium used in the physiological experiments was medium E supplemented with 0.1% vitamin-free Casamino Acids, 0.4% glucose, and 1 μ g/ml thiamine. Cultures were grown at 37°C with vigorous aeration. Glycerol was used instead of glucose for growth of strains containing the P_{BAD} *birA* plasmid, pCY216 (11). The cultures were grown overnight and then diluted 1:100 into fresh medium having the same composition and grown to the early to mid-log phase before assays were performed. Expression of *Ptac*-controlled *accB* and *accC* genes was induced with 100 μ M isopropyl- β -D-thiogalactopyranoside (IPTG), whereas *birA* expression was induced with 0.2% arabinose.

Plasmid construction. The sources of the *accB* and *accC* genes are shown in Table 1 together with descriptions of the derived plasmids. The vector used was the medium-copy-number *Ptac* promoter plasmid pKK223-3 (8), although the high-copy-number *Ptac* promoter plasmid pHSG397 (31) was used in some early experiments.

Plasmid pCY701 was constructed by digestion of plasmid pLS83 with EcoRI plus NsiI (whose sites lie within the multiple cloning site of the parental plasmid), and the 3,282-bp fragment was ligated to the medium-copy-number *tac* promoter plasmid pKK223-3 digested with EcoRI and PstI. Plasmid pCY703 was constructed by digesting plasmid pGB7 with HindIII and BssHIII and plasmid pCY465 with HindIII and MluI. The 1,836-bp fragment of pGB7 was then ligated to the 2,640-bp fragment of pCY465. The 2,098-bp fragment of pCY703

obtained by digestion with EcoRI plus BglII was then ligated to the 5,014-bp fragment of pCY701 (obtained by digestion with the same enzymes) to obtain pCY705, a *tac* promoter plasmid carrying *accB* in its normal chromosomal position upstream of *accC*. Plasmid pCY708, in which *accB* was expressed from the *tac* promoter, was obtained by the same manipulations except that the EcoRI-BglII fragment was the 1,672-bp fragment of pCY465. Plasmid pAH1, which encoded the C-terminal 87-residue biotinylation domain of AccB plus a kanamycin resistance marker, was constructed by PCR amplification using pCY708 as the template with primers incorporating flanking EcoRI and HindIII sites and then cloned into pKK223-3. The fragment containing the *accC* gene was cut from pCY705 with SstII and HindIII, gel purified, and cloned into pAH1 to generate pAH2. A high-copy-number *Ptac* plasmid carrying *accC* was constructed by insertion of the XhoI-SacI fragment of pLS83 into pHSG397 cut with the same enzymes. Plasmid pCY703, a Δ *accB* derivative of pCY703, was constructed by digestion with KpnI, followed by ligation at a low DNA concentration.

Site-directed mutagenesis of *accB* was carried out using the Stratagene Quick-Change II protocol and the primers shown in Table 1. PCR amplification was carried out using *Pfu* Turbo DNA polymerase for 16 cycles. The template plasmid DNAs were digested with DpnI, and the PCR product containing the mutated plasmid was used to transform DH5 α . Codon K122 of the *accB* gene of pCY708 was changed to glutamate and methionine codons in order to construct plasmids pAH13 and pAH14, respectively. The mutant *accB* genes were sequenced to confirm the expected mutations at the Keck Center for Comparative and Functional Genomics at the University of Illinois. Plasmid pCY730, which expressed K122R AccB, was constructed by exchanging the BsiWI-KpnI fragment of plasmid pER73 for the fragment of pCY705. E. Choi-Rhee of our laboratory constructed plasmid pER73 using the primers shown in Table 1. Plasmid pAH7 was constructed from pCY730 by replacement of the *accC* gene with the SstII-BglII kanamycin resistance cassette of pCY708. The fragment containing the *accC* gene was cut with SstII and BglII and used to replace the kanamycin resistance gene of plasmids pAH13 and pAH14 in order to construct plasmids pAH15 and pAH16, respectively. Two plasmids, pAH8 and pAH9, which encoded a derivative of the 87-residue C-terminal fragment of AccB in

TABLE 1. Plasmids and oligonucleotides used in this work

Plasmid or oligonucleotide	Properties or sequence (5'-3') ^a	Reference
Plasmids		
pKK223-3	<i>Ptac</i> promoter vector, pBR322 origin, Amp ^r	8
pCY701	pKK223-3 containing <i>accC</i> under <i>Ptac</i> control	This study
pCY705	pKK223-3 containing <i>accB</i> and <i>accC</i> under <i>Ptac</i> control	This study
pCY70	pKK223-3 containing <i>accC</i> under <i>Ptac</i> control	This study
pCY708	pKK223-3 containing <i>accB</i> under <i>Ptac</i> control	This study
pAH1	pKK223-3 containing ' <i>accB</i> ' under <i>Ptac</i> control	This study
pAH2	pKK223-3 containing ' <i>accB</i> ' and <i>accC</i> under <i>Ptac</i> control	This study
pCY730	pKK223-3 containing <i>accB</i> K122R and <i>accC</i> under <i>Ptac</i> control	This study
pAH15	pKK223-3 containing <i>accB</i> K122E and <i>accC</i> under <i>Ptac</i> control	This study
pAH16	pKK223-3 containing <i>accB</i> K122M and <i>accC</i> under <i>Ptac</i> control	This study
pAH7	pKK223-3 containing <i>accB</i> K122R under <i>Ptac</i>	This study
pAH13	pKK223-3 containing <i>accB</i> K122E under <i>Ptac</i> control	This study
pAH14	pKK223-3 containing <i>accB</i> K122M under <i>Ptac</i> control	This study
pCY216	<i>birA</i> under P _{BAD} arabinose promoter control in a p15a vector, chloramphenicol resistant	11
pMS421	<i>lacI</i> ^q in a pSC101 origin, spectinomycin resistant	21
pLS24	<i>accB</i> chromosomal segment in pGEM5	24
pLS83	<i>accC</i> chromosomal segment in pGEM7	24
pCY465	<i>accB</i> chromosomal segment in pCY462	15
pGB7	<i>accB accC</i> chromosomal segment in pGEM7	24
Oligonucleotide primers^b		
accB87-1	GGAATTCCATGGAAGCGCCAGCAGC	
accB87-2	AGAAAGCTTTTCCATGGACGCGTG	
accB-E1	GCATCGTTGAAGCCATGGAAATGATGAACCAGATCG	
accB-E2	CGATCTGGTTCATCATTTCATGGCTTCAACGATGC	
accB-M1	CGTTGAAGCCATGATGATGATGAACCAGATCG	
accB-M2	CGATCTGGTTCATCATTTCATGGCTTCAACG	
BCCPKR-FA	GTGCATCGTTGAAGCCATGAGAATGATGAACCAGATCGAAG	
BCCPKR-RE	CTTCGATCTGGTTCATCATTTCATGGCTTCAACGATGCAC	

^a The '*accB*' gene encodes the last 87 residues of AccB. Any mutational alteration of *accB* is indicated by the amino acid residue alteration.

^b Primers accB87-1 (forward) and accB87-2 (reverse) were used to clone a gene encoding the N-terminally truncated protein consisting of the carboxyl 87 residues of AccB. Primers accB-E1 (forward) and accB-E2 (reverse) were used to construct the *accB* gene encoding the K122E protein. Primers accB-M1 (forward) and accB-M2 (reverse) were used to construct the *accB* gene encoding the K122M protein. Primers BCCPKR-FA and BCCPKR-RE were used by E. Rhee of our laboratory to construct the *accB* gene encoding the K122R protein.

which the biotinylated lysine was replaced with arginine, were constructed by cloning the NdeI-BsiWI fragment of plasmid pAH1 into pAH7 and pCY730, respectively.

Assays. β-Galactosidase activity was determined as described by Miller (27) following disruption of the cells by sodium dodecyl sulfate-chloroform treatment. Protein biotinylation was measured by pipetting 100-μl samples of cultures grown on [8,9-³H]biotin (1 μCi/ml) at the required biotin concentrations onto 2.5-cm disks of Whatman 3MM filter paper previously soaked with 50 μl of 0.5 mM nonradioactive biotin (11, 17). The filter disks were washed twice with 10% trichloroacetic acid and then twice with 5% trichloroacetic acid. The radioactivity was determined by scintillation counting with an LS6500 multipurpose scintillation counter (Beckman Coulter). The counting efficiency was obtained by quantitative elution of the labeled proteins from the filter disks by boiling the disks for 15 min in 0.1 M NaOH (17).

RESULTS

Overproduction of AccC results in down-regulation of *bio* operon transcription. We first assayed expression of the biotin biosynthetic operon using the *bio-lac* fusion strain BM2661 of Barker and Campbell (4) or an essentially identical strain, strain CY486, constructed by Cronan (17). In these strains the *lacZ* and *lacY* genes are fused to the rightward *bio* promoter (the promoter of *bioBFCD*), resulting a lactose-positive phenotype when *bio* operon transcription is derepressed and a lactose-negative phenotype when the operon is repressed. These strains also carry a deletion of the chromosomal lactose operon and are biotin auxotrophs due to insertion of the lac-

tose utilization genes into *bioF*. These strains form bright red colonies when they are spread on MacConkey agar plates due to lactose utilization resulting from derepression of *bio* operon transcription by the low concentration of biotin present in the medium (15 nM according to data provided by the manufacturer). However, upon plating on MacConkey agar supplemented with additional biotin (40 nM or a higher concentration) white colonies are formed, indicating that there is repression of *bio* operon transcription by biotin (4). In preliminary tests we found that introduction of a plasmid that expressed high levels of AccC resulted in white colonies on MacConkey plates that were not supplemented with additional biotin, indicating that there was inhibition of the depression of *bio* operon transcription normally seen on this medium (Fig. 3). Three different *accC* plasmids gave this result. These plasmids were (i) a medium-copy-number *tac* promoter plasmid (see below), (ii) a very-high-copy-number *lac* promoter plasmid (similar to the plasmid used by Karow et al. [23]) (data not shown), and (iii) a low- to medium-copy number plasmid in which *accC* was expressed from a plasmid *tet* promoter (data not shown). In contrast, an *accC* plasmid derived from pCY465, a plasmid expected to express AccC at a level similar to the level expressed by the chromosomal gene (15), failed to block depression. Hence, significant overexpression of AccC was needed to observe inhibition of *bio* operon transcription. It

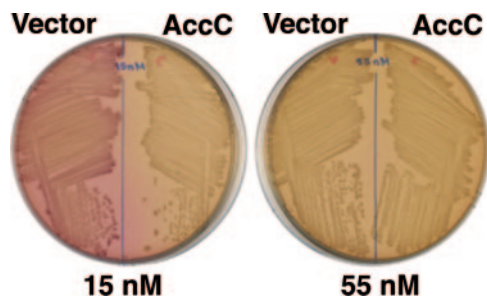


FIG. 3. Repression of *bio* operon transcription upon overproduction of AccC. The plates are MacConkey agar plates containing 100 μ M IPTG, and the biotin concentrations are indicated below the plates. The red color is due to depression of the *bio*BFC_D promoter that drives expression of the *lacZ* and *lacY* genes (4). The host was strain CY1740, and the *accC* and vector plasmids were pCY701 and pKK223-3, respectively. Essentially identical results were obtained with strains BM2661 and CY486.

should be noted that *accB*, *accC*, and *birA* are all essential for growth of *E. coli*.

The effects of AccC overproduction on *bio* operon transcription were quantitated by assaying β -galactosidase activity in strain CY1740 (Fig. 4). The presence of high levels of LacI in this strain allowed the effects of induction of the *tac* promoter-controlled *acc* genes to be tested. Derivatives of strain CY1740 carrying either the *tac* vector plasmid or plasmids that encoded AccC, AccB, or both AccB and BirA (on separate compatible plasmids) were constructed. The strain carrying the vector plasmid showed the expected decreases in *bio* operon transcription with increasing biotin concentrations first reported by Barker and Campbell (4) and subsequently confirmed in our laboratory (17). The most responsive range of biotin concentrations is about 4 to 40 nM (4, 17), as shown by the slope of the curve for the vector-containing strain (Fig. 4). As expected from previous work, addition of biotin at a concentration of 40 nM resulted in almost maximal repression of *bio* operon transcription in the vector-containing strain, whereas addition of 10 nM biotin resulted in only slight repression (Fig. 4). In contrast, the slope for the strain overproducing AccC was much steeper, and at 10 nM biotin the strain overproducing AccC showed almost full repression (Fig. 4). This down-regulation, which increased with the biotin concentration up to a concentration of about 100 μ M, was dependent on induction of AccC overproduction since in the absence of inducer the strain had an induction profile similar to that of the vector-containing control strain (Fig. 4). Derepression due to AccB overproduction was partially offset by overproduction of BirA (Fig. 4), as expected from previous work (17).

Overproduction of both AccB and AccC relieves the down-regulation observed with overproduction of AccC alone. A plausible mechanism for the down-regulation observed when AccC is overproduced is that AccC forms a complex with the AccB produced by the chromosomal *accB* gene and the complex is a poorer substrate for BirA-catalyzed biotinylation than free AccB is. Thus, high intracellular levels of BirA liganded to biotinoyl-5'-AMP accumulate, which result in increased occupancy of the *bio* operator and in greater repression of biotin synthesis (given sufficiently high intracellular biotin levels) (Fig. 1D). If this is true, overexpression of AccB together with

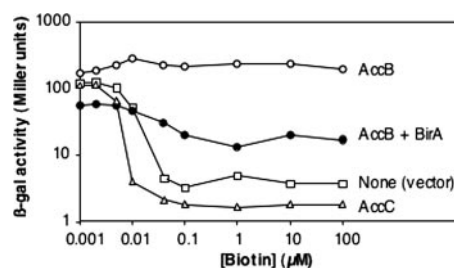


FIG. 4. Expression with various biotin concentrations of the chromosomal ϕ (*bio*FC-*lacZ*)501 fusion of derivatives of strain CY1740 carrying a plasmid encoding AccB (○) or AccC (△) or the vector pKK223-3 (□). A derivative carrying two compatible plasmids, one encoding AccB and the other encoding BirA (●), was also assayed. The *accC*, *accB*, and *birA* plasmids were pCY701, pCY708, and pCY216, respectively. Note that both axes have log scales. The experiment was repeated twice in its entirety, and in addition, the vector and AccC overproduction cultures were compared two more times. The results were essentially identical to those shown. β -gal, β -galactosidase.

overexpression of AccC should eliminate the inhibition observed when AccC is overproduced. Therefore, we constructed a series of plasmids based on the *tac* promoter vector that coexpressed both AccB and AccC or expressed only AccB using the native ribosome binding sites. Derivatives of strain CY1740 carrying these plasmids were then tested on MacConkey plates containing various concentrations of biotin. In studies done in parallel with the AccC plasmid and the empty vector we found that the strain carrying the *accBC* plasmid produced red colonies on unsupplemented MacConkey medium (containing 15 nM biotin), indicating that coordinated overproduction of AccB and AccC relieved the inhibitory effects seen when AccC alone was overproduced (data not shown). As expected from previous work (17), overproduction of AccB resulted in derepression of *bio* operon transcription (red colonies) with both 15 nM and 55 nM biotin and pink colonies with 1 μ M biotin due to consumption of the BirA-bound biotinoyl-5'-AMP and concomitant freeing of the *bio* operator (data not shown). These results were supported by β -galactosidase assays that showed that the effects of AccC overproduction were completely abolished when AccB was overproduced simultaneously with AccC (Fig. 5). Indeed, overproduction of both AccB and AccC resulted in derepression of *bio* operon transcription similar to the derepression seen when only AccB was overexpressed (Fig. 4).

AccB-AccC interaction is required for AccB overproduction to counter the down-regulation resulting from AccC overproduction. The reversal of the inhibitory effects of AccC overproduction by coupled overproduction with AccB indicated that when there was no longer an excess of AccC, AccB became biotinylated and was assembled into AccB-AccC complexes. If this is true, a mutant AccB protein that is unable to bind AccC should not reverse the down-regulation seen when AccC is overproduced. This is because excess AccC would remain free to interact with the AccB encoded by the chromosome. However, due to the regulatory circuitry, interpretation of this experiment is complicated by the derepression of *bio* operon transcription resulting from overproduction of a biotin acceptor protein. Hence, we expected to observe levels of

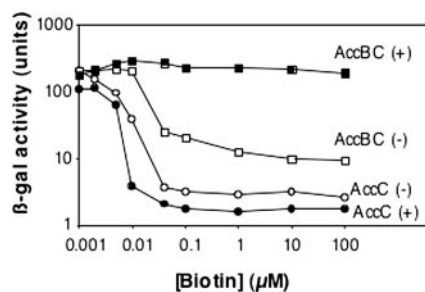


FIG. 5. Expression with various biotin concentrations of the chromosomal $\phi(bioFC-lacZ)501$ fusion of derivatives of strain CY1740 carrying a plasmid encoding AccBC (■ and □) or AccC (● and ○). The solid symbols and plus signs indicate induction by addition of 0.1 mM IPTG, whereas the open symbols and minus signs indicate uninduced cultures. The *accC* and *accBC* plasmids were pCY701 and pCY705, respectively. The experiment was repeated twice in its entirety, and in addition, the AccBC and AccC overproduction cultures were compared two more times. The results were essentially identical to those shown. β -gal, β -galactosidase.

repression intermediate between those seen when the mutant AccB alone was overexpressed and those seen when the mutant AccC alone was overexpressed, and perhaps the intermediate levels would be seen only at high biotin concentrations, when much of the AccB would have become biotinylated (and hence neutralized). The mutant AccB used was a protein comprising the carboxyl-terminal 87 residues of AccB. This protein is unable to bind AccC (1, 12). Coupled overexpression of AccC with the truncated AccB protein resulted in increased repression compared to that of a strain that overexpressed only the truncated AccB protein (Fig. 6) or a strain that overproduced both the native AccB and AccC proteins (Fig. 5). However, as expected, these different effects were seen only at high biotin concentrations; the effects at lower concentrations were masked by biotin acceptor protein overproduction. Similar results were obtained using a plasmid that encoded wild-type AccC plus a mutant AccB in which a stretch of three N-terminal residues required to form the metastable AccB-AccC complex (12) was deleted (data not shown). However, this construct showed greater repression at low biotin concentra-

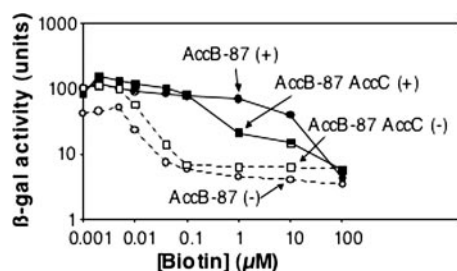


FIG. 6. Expression with various biotin concentrations of the chromosomal $\phi(bioFC-lacZ)501$ fusion of derivatives of strain CY1740 carrying plasmid pAH1 encoding the carboxyl 87 residues of AccB (AccB-87) (● and ○) or plasmid pAH2 encoding AccB-87 plus AccC (■ and □). The solid symbols and plus signs indicate induction by addition of 0.1 mM IPTG, whereas the open symbols and minus signs indicate uninduced cultures. The experiment was repeated twice, and the results were essentially identical to those shown. β -gal, β -galactosidase.

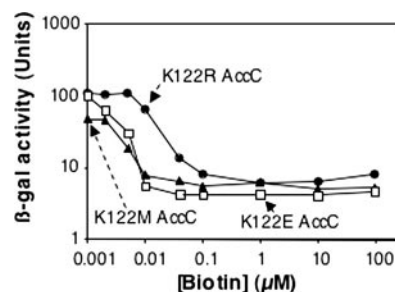


FIG. 7. Expression of the chromosomal $\phi(bioFC-lacZ)501$ fusion of derivatives of strain CY1740 carrying plasmids encoding AccC plus mutant AccB proteins. The AccB K122R protein (●), mutant AccB K122E protein (□), and mutant AccB K122M protein (▲) were encoded by plasmids pAH7, pAH13, and pAH14, respectively. The experiment was repeated twice, and the results were essentially identical to those shown. β -gal, β -galactosidase.

tions than the C-terminal 87-residue construct showed, probably because the full-length AccB protein is a poorer BirA substrate (it has a higher K_m) than the 87-residue protein (28).

In order to remove the complication of derepression of *bio* operon transcription by increased biotin acceptor protein levels, we tried to construct a mutant AccB that bound AccC but was not biotinylated. Our hypothesis was that upon coexpression with AccC this mutant AccB protein would interact with and thereby neutralize the overproduced AccC, thus relieving the repression seen when AccC was overproduced alone. The obvious approach to construct an AccB protein that could not be biotinylated was to substitute another residue for the lysine residue (K122) to which biotin is attached. However, because there is not a good mimic of lysine among the natural amino acids, we constructed *accBC* plasmids encoding three different K122 substitutions, K122R, K122M, and K122E, in the hope that one or more of the proteins would retain the ability to fold normally and efficiently bind AccC. The K122R substitution would preserve a positive charge like that of lysine and an aliphatic chain (albeit a longer chain). K122E would provide an aliphatic chain (albeit a shorter chain) plus a charge (albeit the opposite charge), whereas K122M would provide an uncharged mimic of the lysine aliphatic chain (although shorter and kinked due to the thioether bonds). Coexpression of AccB K122R with AccC almost fully relieved the down-regulation of *bio* operon transcription seen when AccC was overexpressed alone; almost full derepression was seen with 10 nM biotin (Fig. 7). In contrast, the other two K122 mutant proteins, K122M and K122E, only weakly relieved down-regulation when they were overexpressed along with AccC, suggesting that these proteins bound AccC poorly. Relief of down-regulation by AccC overexpression required interaction of the mutant AccB with AccC because AccC coexpression with a K122R derivative of a protein consisting of the carboxyl 87 residues of AccB resulted in levels of repression very similar to the level seen when AccC was overproduced alone (data not shown). Previous work showed that the K122R AccB protein cannot be biotinylated, as would be expected from the very different pK_a values of the lysine and arginine side chains (15). Consistent with this result, we found that high-level expression of all three K122 mutant AccB proteins in the absence of

coupled AccC expression did not derepress *bio* operon transcription (data not shown).

Overproduction of AccC inhibits biotinylation of AccB. The *bio* operon expression data indicate that inhibition results from interaction of excess AccC with AccB to form a complex that is a poor (compared to free AccB) BirA substrate. Therefore, a strain overproducing AccC should contain less biotinylated AccB than a wild-type strain contains. Indeed, this was the case. At biotin concentrations less than about 10 nM, AccB (encoded by the chromosomal gene) was 50 to 75% underbiotinylated when AccC was overproduced (Table 2). This deficiency in biotinylation was reversed when there was simultaneous overproduction of BirA, indicating that AccC and BirA compete for AccB, as expected from the *bio* operon expression results. Increased biotin concentrations also partially relieved underbiotinylation, presumably by increasing the activity of BirA (Table 2). Finally, expression of K122R AccB together with AccC relieved underbiotinylation of the chromosomally expressed AccB (Table 2).

DISCUSSION

The transcriptional coupling of the *E. coli accB* and *accC* genes can now be rationalized by the fact that overproduction of either AccB or AccC without the other disrupts regulation of the *bio* operon, leading to either overproduction or underproduction of biotin. Moreover, Karow et al. (23) reported that overproduction of either protein alone results in appreciable inhibition of the rate of fatty acid synthesis. This effect cannot be attributed to disturbed biotin production because these workers used a medium that contained biotin. However, in the absence of an exogenous source of biotin, the decreased biotinylation of AccB seen when AccC is overproduced has obvious physiological consequences. It should also be noted that overproduction of biotin is metabolically expensive despite the low levels of this vitamin required by *E. coli*. This is because the pathway requires expenditure of at least seven ATP equivalents per biotin molecule and six different enzymes, one of which, BioB, appears to be degraded often as a consequence of its catalytic cycle (13).

Our data suggest that free AccB is the preferred substrate for BirA-catalyzed biotinylation in vivo and that biotinylated AccB is the form normally incorporated into the AccB-AccC complex. Thus, in vivo it seems that AccC and BirA compete for unbiotinylated AccB, as shown in Table 2, and the system is set such that BirA wins the competition. The bifunctional nature of BirA precludes unambiguous interpretation of *bio* operon regulation experiments in which BirA and AccC are both overexpressed. Interpretable experiments could be done using a BirA mutant protein that was completely defective in operator binding but that retained full biotinylation activity. Unfortunately, no such mutant is known. Indeed, removal of the N-terminal DNA binding domain from BirA severely compromises the ability of the protein to bind biotin and biotinoyl-5'-AMP (34). It should be noted that our laboratory previously reported that unbiotinylated AccB complexed with AccC was a substrate for BirA-catalyzed biotinylation in vitro (12). However, the effect was modest, and due to the metastable nature of the isolated complex we cannot exclude the possibility that unbiotinylated AccB dissociated from the complex became

TABLE 2. Inhibition of AccB biotinylation by AccC overproduction and reversal of this inhibition by BirA overproduction^a

Biotin concn (nM)	Biotinylated AccB concn (pmol/10 ⁹ cells) with:			
	Vector	AccC	AccC + BirA	AccC + AccB (K122R)
1	2.5	1.1	4.1	10.5
2	3.6	1.2	3.7	9.8
5	6.1	4.1	7.2	23.1
10	13.1	4.0	16.5	18.7
40	13.8	11.6	17.4	17.1
100	17.5	12.9	18.2	21.4

^a Cultures of strain CY1740 carrying plasmids encoding AccC (pCY701), AccC plus BirA (pCY701 and pCY216), AccB K122 plus AccC (pCY730), or the vector (pKK223-3) were used for AccC plasmid construction. The strains were grown in the presence of ³H-labeled biotin at the concentrations indicated, and attachment to AccB (the sole biotinylated protein of *E. coli*) was measured as described in Materials and Methods.

biotinylated and then reformed a complex. Our interpretation that free AccB is the preferred substrate for BirA-catalyzed biotinylation in vivo is consistent with the finding that fusion proteins having the 87-residue AccB biotin domain as the downstream partner compete well with the full-length AccB protein for biotinylation in vitro (10, 24).

Further work is necessary to determine if the AccB-AccC complex formed when AccC is overproduced has the normal 2:1 stoichiometry or a different stoichiometry. However, if AccC overproduction results in aberrant complexes, these complexes may be less stable than the normal complex and thus very difficult to isolate. It would be interesting to determine if the effects of AccC overproduction seen in *E. coli* are also seen in the distantly related bacterium *Bacillus subtilis*. *B. subtilis* BirA regulates transcription of its *bio* operon (7), and the *accB* and *accC* genes are cotranscribed (26). It would also be interesting to determine if and how stoichiometric production of AccB and AccC occurs in cyanobacteria, where the *accB* and *accC* genes are unlinked (20).

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