Regulation of *Escherichia coli* Biotin Biosynthesis: Isolation of BirA Super-Repressor Mutant Strains

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Transcription of the *Escherichia coli* biotin (*bio*) operon is directly regulated by the biotin-protein ligase, BirA, the enzyme that covalently attaches biotin to its cognate acceptor proteins. Binding of BirA to the *bio* operator requires dimerization of the protein, which is triggered by BirA-catalyzed synthesis of biotinoyl-adenylate (biotinoyl-5′-AMP), the obligatory intermediate of the ligation reaction. Although several aspects of this regulatory system are well understood, no BirA super-repressor mutant strains had been isolated. Such super-repressor BirA proteins would repress the biotin operon transcription *in vivo* at biotin concentrations well below those needed for repression by wild type BirA. We isolated mutant strains having this phenotype by a combined selection-screening approach and resolved multiple mutations to give several birA super-repressor alleles each having a single mutation all of which showed repression dominant over the wild type allele. All of these mutant strains repressed *bio* operon transcription *in vivo* at biotin concentrations that gave derepression of the wild type strain and retained sufficient ligation activity for growth when overexpressed. All of the strains except that encoding G154D BirA showed derepression of *bio* operon transcription upon overproduction of a biotin accepting protein. The G154D BirA was a lethal mutation in single copy and the purified protein was unable to transfer biotin from enzyme bound biotinoyl-adenylate either to the natural acceptor protein or to a biotin accepting peptide sequence. Consistent with the transcriptional repression data, each of the purified mutant proteins showed increased affinity for the biotin operator DNA in electromobility shift assays. Surprisingly although most of the mutations were located in the catalytic domain all those tested excepting G154D BirA had normal ligase activity. Most of the mutations that gave super-repressor phenotypes altered residues located close to the dimerization interface of BirA. However, two mutations were located at sites well removed from the interface. The properties of the super-repressor mutants strengthen and extend other data indicating that BirA function entails extensive interactions among the three domains of the protein and shows that normal ligase activity does not ensure normal DNA binding.
INTRODUCTION

In Escherichia coli the synthesis of the essential enzyme cofactor biotin, is regulated at the transcriptional level by an unusual repressor called BirA. BirA is unusual in that it is also an essential metabolic enzyme, the sole biotin protein ligase of this bacterium. Biotin protein ligases are found throughout the three domains of life and catalyze the covalent attachment of biotin to its cognate acceptor proteins that play key roles in central metabolism. The fact that BirA (a protein of 35.3 kDa) is both an enzyme and a transcriptional repressor makes biotin (bio) operon transcription sensitive not only to the intracellular concentration of biotin, but also to the levels of cognate proteins requiring biotinylation for enzymatic activity. This property results from the small molecule ligand required for bio operon repression, which is biotinoyl-adenylate (biotinoyl-5'-AMP) rather than biotin (Fig. 1A). Biotinoyl-adenylate is the product of the first half-reaction of the ligase reaction which proceeds in an ordered manner in which monomeric BirA first binds biotin then ATP (47). The adenylate remains tightly bound to the protein and triggers formation of the BirA dimers required for DNA binding (41) (Fig. 1). The dimeric BirA biotinoyl-adenylate complex represses transcription by binding to the biotin operator (bioO), a 40-bp inverted repeat that overlaps and controls both biotin operon promoters (31, 40). In the presence of unmodified acceptor protein the enzyme-bound biotinoyl-adenylate is attacked by the ε-amino group of a specific lysine residue of the acceptor protein to give the biotinylated acceptor protein (Fig. 1A).

Maximal rates of bio operon transcription (derepression) occur when the biotin supply is severely limited (e.g., biotin starvation of a bio auxotroph) (Fig. 1C) or when high levels of a biotin acceptor protein are present (Fig. 1D). Under these conditions any biotinoyl-adenylate synthesized is rapidly consumed in biotinylation of the E. coli acceptor protein (AccB) and no significant levels of the BirA:biotinoyl-adenylate complex accumulate. Therefore BirA remains largely monomeric and the bio operator is seldom occupied resulting in maximal transcription. Repression of bio operon transcription occurs when the supply of biotin is in excess of that needed to biotinylate AccB. Under these conditions AccB becomes fully biotinylated and the BirA:biotinoyl-adenylate complex accumulates and represses transcription of both promoters (Fig. 1B). The two conditions resulting in derepression act by a common mechanism in that both decrease the levels of the BirA:biotinoyl-adenylate complex available to bind the bio operator. Hence, the degree of repression of bio operon transcription can be most simply viewed as an
antagonism between retention of biotinoyl-adenylate in the BirA active site versus consumption of the biotinoyl-adenylate bound to BirA upon transfer of the biotin moiety to unmodified acceptor proteins (14, 38).

The overall model of biotin operon regulation is well supported by in vivo experiments (1, 4-6, 15, 38) in which the collection of BirA mutants isolated by Barker and Campbell (6) provided key insights into the regulatory and enzymatic functions of BirA. This and other work indicated that repressor function and ligase activity are closely intertwined. In order to further test and extend the model we have isolated and characterized the first examples of a new class of BirA mutants. These are super-repressor mutants that remain repressed under conditions that result in derepression of the wild type strain. This class of mutants may help to close a major gap in the model. Hence, we designed a selection-screening approach using chromosomal transcriptional fusion constructs driven by the divergent bio operon promoters under conditions that ensured survival of lethal birA mutations. We report the in vivo and some in vitro properties of some members of this new class of birA mutants.
MATERIALS AND METHODS

Chemicals and culture media. All bacterial strains were derivatives of *E. coli* K-12 (Table 1). The medium used in the physiological experiments was LB as the rich medium whereas the defined medium was M9 salts supplemented with 0.4% glucose (or another carbon source as stated) and 0.1% Vitamin-Free Casamino Acids (Difco). The cultures were grown at 37°C with vigorous aeration and growth was measured by the absorbance at 600 nm using a Beckman DU600 spectrophotometer unless otherwise indicated. Glycerol was used instead of glucose for growth of strains containing the pBAD33-derived plasmid, pVC17. Oligonucleotides (Table 2) were synthesized by Integrated DNA Technologies. PCR amplification was performed using *Taq* polymerase (New England Biolabs) and *Pfu* polymerase (Stratagene) according to the manufacture’s specifications. DNA constructs were sequenced either by the Roy J. Carver Biotechnology Center of the University of Illinois or by ACGT, Inc. All reagents and biochemicals were obtained from Sigma-Aldrich unless otherwise noted and New England Biolabs supplied restriction enzymes. American Radiolabeled Chemicals provided $[^\alpha-32P]ATP$ (6000 Ci mmol$^{-1}$) whereas Perkin Elmer provided $[^\gamma-32P]ATP$ (3000 Ci mmol$^{-1}$) and $d$-[8,9-$\text{H}$(N)]biotin (32.6 Ci mmol$^{-1}$). The concentrations of antibiotics used in this study were as follows (in $\mu$g mL$^{-1}$): sodium ampicillin (Amp), 100; kanamycin sulfate (Km), 50; chloramphenicol (Cml), 25, spectinomycin sulfate (Spec) 50. The 15:1 mixture of ticarcillin disodium salt and potassium clavulanate (Research Products International) was used at 25 $\mu$g mL$^{-1}$, unless otherwise stated.

Bacterial strain constructions. Strain VC150 was constructed by the Datsenko and Wanner method (18). The chromosomal copy of the *birA* gene of strain VC146 was replaced with a kanamycin resistance cassette by transformation with the PCR product obtained using primers VCA10 and VCA11 and pKD4 as template. The replacement was confirmed using primers VCA12 and VCA13 plus the internal primers K2 and KT, respectively. The insertion was then transduced with phage P1vir into strain VC125, a derivative of strain MG1655 carrying plasmid pCY255 (17) to give strain VC156. Other DNA manipulations were performed by standard procedures (36). The antibiotic resistance was removed from strain VC156 using the Flp recombinase encoded by the temperature-sensitive plasmid pSRK20. Strain VC205 was derived from strain VC156 by phage P1 transduction of $\Delta$lacZY::cat insertion of JT33 followed by selection on LB media supplemented with ampicillin and chloramphenicol. Strain VC203 was
constructed by homologous recombination catalyzed by the phage λ red system (18). The lacA gene of in the chromosomal φ(bioF::lacZYA)501 fusion of strain CY481(15) was replaced with a kanamycin resistance cassette by transformation with the PCR product obtained using primers VCA57 and VCA58. The orientation of the construct was then validated by colony PCR with primers VCA59 and VCA60 together with the internal primers C1 and C2. Strain VC212 was constructed by phage P1 transduction of the bioF::lacZY-km fusion of strain VC203 into strain VC205 with selection on MacConkey medium supplemented with chloramphenicol, ampicillin and kanamycin. The kanamycin and chloramphenicol antibiotic resistance cassettes were then removed as described above to give strain VC218. Strain VC218 was transformed with plasmid pSRK40 with selection for resistance to both spectinomycin and ampicillin to give VC230.

Strain VC230 was then transformed with the PCR product obtained using primers VCA20 and VCA21 with pKD3 as template followed by selection for chloramphenicol resistance. Homologous recombination generated a construct in which the cat gene replaced bioA. Transformants were selected on plates of MacConkey medium supplemented with 12.5 µg mL⁻¹ chloramphenicol and tested by colony PCR for insert orientation using primers VCA71 and VCA72 together with the internal primers C1 and C2 to give strain VC235. Strain VC233, a derivative of strain JT34 (19), was constructed by phage P1 transduction of the strain VC203 bioF::lacZY construct with selection on MacConkey medium supplemented with kanamycin. The antibiotic resistance cassette was then removed as above. Strain VC233 was transformed with plasmid pVC18 to give strain VC537 which was transduced with the ΔbirA::km insertion of strain VC156 to give strain VC618.

Strain VC776 was constructed by(42) transformation of VC236 with a PCR product encoding the cat::sacB cassette (generated with template CS100 (40, 41) and primers VCC44 and VCC49. The insert was confirmed using colony PCR with primers VCA12 and VCA13 and by its birA phenotype on defined medium plates containing 4 µM biotin. A P1 lysate of strain VC776 was used to transduce strain VC730 to chloramphenicol resistance. The resulting strain was then transformed with a PCR product generated with primers VCC50 and VCC51 using plasmids pVC7 and pVC25 as templates. The transformants were plated on defined medium plates supplemented with 4 µM biotin and 5% sucrose. The resulting colonies were then scored on defined medium plates supplemented with 4 µM or 4 nM biotin, to test BirA function. The insert was amplified using primers VCC52 and VCC53 and sequenced. The positive candidates
were then transduced with a P1 phage stock grown on the polA12 strain AK25 with selection for
a closely linked Tn10 insertion to give strains VC801 and VC802. The transductants were then
scored for the UV-sensitive polA12 phenotype at 42°C, the non-permissive temperature. The
UV-sensitive candidates were then tested for growth on defined media supplemented with 4 nM
biotin and 40 μg ml−1 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) at 30°C and
42°C.

Plasmid constructions. The birA gene was amplified by PCR from MG1655 genomic DNA
using primers VCA69 and VCA70 which added terminal EcoRI and NcoI sites. This product was
then directly ligated into the same sites of the cat gene of the medium copy number plasmid
pACYC184 to give pVC7. Plasmid pVC18, which encodes S. cerevisiae Bpl1, was constructed
by PCR amplification using pCY255 as the template with primers VCA84 and VCA85, which
added flanking SmaI and XbaI sites. The SmaI plus XbaI digested BPL1 gene was then inserted
into plasmid pEAK1 cut with the same enzymes. The sequence encoding AccB-87, the C-
terminal 87-residue biotinylation domain of AccB, was amplified from the genomic DNA of
MG1655 using primers VCB44 and VCB45, which added flanking sites. This product was
digested with SacI and HindIII and ligated into the same sites of pBAD33C to give pVC17 with
AccB-87 expression under the control of an arabinose-inducible promoter. Plasmid pVC19
encoding AccB-87 was constructed by PCR amplification using MG1655 genomic DNA with
primers VCB57 and VCB58, which incorporated flanking NcoI and BamHI sites. This product
was digested and inserted between the NcoI and BamHI sites of pET16B.

Mutant enrichment. Random mutagenesis of the birA gene was performed with the GeneMorph
II random Mutagenesis Kit using primers VCA69 and VCA70 (Table 2) and plasmid pVC7 as
template at concentrations of 100 ng, 50 ng, 5 ng or 1 ng in a total reaction volume of 50 μL. The
mutagenesis conditions designed to give fairly high-to-medium mutation frequencies were used
because the complex structural transitions undergone by BirA argued that multiple mutations
might be needed to increase DNA binding. The PCR products of the mutagenesis reactions were
then digested with NcoI and EcoRI and inserted into pACYC184 digested with the same
enzymes. The ligation products were then transformed into strain VC235 with selection on LB
plates for resistance to ampicillin and tetracycline. The transformants were pooled and diluted
1000-fold in the defined medium supplemented with 3 μg mL−1 chloramphenicol and 1.6 nM
biotin. The cultures were grown at 37°C with continuous aeration until the optical density
increased 2-fold. At this time the ticarillin-clavulenate mixture (Material and Methods) and sodium ampicillin were added to final concentrations of 200 µg mL\(^{-1}\) and 50 µg mL\(^{-1}\), respectively. The cultures were then shaken for an additional 2 h until lysis. The surviving cells were harvested, washed with defined medium and plated at different dilutions on defined medium plates supplemented with 12 µg mL\(^{-1}\) tetracycline, 50 µg mL\(^{-1}\) ampicillin, 1.6 nM biotin and 40 µg mL\(^{-1}\) X-gal. Following incubation a mixture of white and blue colonies was seen. Plasmids were extracted from the white colonies (the super-repressor phenotype) digested with HpaI to linearize plasmid pCY255 and transformed into strain VC233. The mutant plasmids were then isolated and sequenced to identify the mutations that resulted in the observed phenotype. Site-directed mutagenesis was carried out using the Stratagene Quick Change II protocol and the primers of Table I. The template plasmids were digested with DpnI prior to transformation of strain DH5α.

\textbf{β-Galactosidase assays}. 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. β-Galactosidase activity was determined as described by Miller (26) following disruption of the cells by sodium dodecyl sulfate-chloroform treatment.

\textbf{In vivo complementation}. Strain VC618 was transformed with the plasmids pVC20, pVC21, pVC22, pVC23, pVC24 or pVC25 and transformants selected for on LB plates supplemented with ampicillin and tetracycline at 30°C. The temperature-sensitive plasmid, pVC18, was cured from these strains by growth at 42°C on defined media supplemented with tetracycline. The loss of the temperature-sensitive plasmid was confirmed by ampicillin sensitivity. These strains were then used to carry out growth curve analyses in which cultures were grown overnight in defined medium supplemented with 1.6 nM biotin and then diluted 1:1000 into fresh medium having the same composition supplemented with different concentrations of biotin. Bacterial growth was then monitored using a Bioscreen C instrument with a brown filter and the Norden Lab Studio Bioscreen C software. The cultures were grown with strong shaking and the absorbance at OD\(600\) with recording every 15 min.

\textbf{BirA purification}. Expression plasmids encoding a C-terminal hexahistidine tagged version of one of the BirA proteins was amplified from genomic DNA using primers VCB16 and VCB17 which added terminal NcoI and BamHI sites as well as the purification tag. The digested products were ligated into the same sites of plasmid pET19b to give plasmid pVC9. Plasmid
VC9 was then digested with NcoI, treated with mung bean nuclease to generate blunt ends and ligated to give plasmid pVC10. Plasmid pVC10 was used as the template for site-directed mutagenesis to generate single amino acid substitutions. The resulting plasmids were introduced into *E. coli* strain BL21 λDE3. The BirA proteins were expressed in LB medium containing 100 µg mL⁻¹ ampicillin with induction at an optical density of 0.8 at 600 nm by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM. The protein fraction was subjected to Ni²⁺ affinity chromatography (13) followed by dialysis against 10 mM sodium phosphate buffer (pH 7.5) containing 5% glycerol and 0.1 mM dithiothreitol (DTT) and anion exchange chromatography using a Vivapure IEX Q Maxi H spin column. The column was successively washed with the above buffer except that the phosphate concentrations were increased to 50 mM and then 100 mM. BirA was eluted with the 100 mM buffer containing 100 mM NaCl. The elute was dialyzed against storage buffer (10 mM sodium phosphate (pH 7.5), 200 mM KCl, 10% glycerol and 0.1 mM DTT) overnight, concentrated using Millipore concentrators, flash frozen and stored at -80°C. For the *in vitro* biotinylation assays the proteins were dialyzed against 50 mM Tris-HCl (pH 7.5), 200 mM KCl, 5% glycerol and 0.1 mM DTT prior to use.

**Purification of AccB-87.** Plasmid pVC19 was transformed into strain BL21 λDE3. The strain was grown in LB medium supplemented with ticarillin-clavulenate to an optical density of 0.5 at 600 nm at which point 0.1 mM IPTG was added followed by growth for an additional 4 h. The truncated AccB (AccB-87) was purified as previously described (11). The fractions containing the pure protein as judged by SDS gel electrophoresis followed by Coomassie Blue staining were dialyzed overnight in 50 mM Tris-HCl (pH 7.5), 200 mM KCl, 5% glycerol and 0.1 mM DTT. The protein was concentrated and stored at -80°C as above. The AccB-87 preparations were validated by mass spectrometry (11).

**Biotinoyl-adenylate synthesis.** Protein concentrations were determined using the extinction coefficients calculated from the ExPASY Tools website. The assays contained 100 mM sodium phosphate buffer (pH 7.5), 5 mM (tris (2-carboxyethyl) phosphine), 0.05 mM MgCl₂, 0.01 µM biotin, 0.1 µM [α⁻³²P]ATP and 1 µM BirA protein. The reactions were incubated at 28°C for 30 min, at which time the reaction was split. To one tube AccB-87 was added and incubated for an additional 15 min at 28°C whereas the other half reaction was left untreated. One µl of each reaction was subjected to cellulose thin layer chromatography on plates containing a fluorescent indicator that were developed in isobutyric acid:NH₄OH:water (66:1:33) (34). The thin layer
10 chromatograms were dried for 10 h and exposed to phosphorimaging plate and visualized using a Fujifilm FLA-3000 Phosphor Imager and Fujifilm Image Gauge software (version 3.4 for Macintosh).

Electromobility shift assay (EMSA) of DNA binding affinity. A 112-bp DNA substrate containing the bioO operator was PCR amplified with Taq polymerase from MG1655 genomic DNA using primers VCB36 and VCB37. The PCR product was subjected to electrophoresis on a 5% polyacrylamide gel and extracted from the gel using the following protocol. The DNA fragment was eluted from the polyacrylamide in diffusion buffer (0.5 mM ammonium acetate, 10 mM MgSO₄, 1 mM EDTA, 0.1% SDS [pH 8]) at 55°C for 30 min. After removing excess polyacrylamide by filtration on BioRad Poly-Prep chromatography column, the DNA was further purified using a Qiagen Spin column. The DNA concentration was determined using spectrophotometry at 260 nm. Ten pmol of the gel extracted DNA was 5’-labeled using 50 pmol of [γ-³²P]ATP and T4 polynucleotide kinase. Gel shift assays were performed in buffer C consisting of 50 mM sodium phosphate buffer (pH 7.5) containing 50 mM NaCl and 10% glycerol. Each 10 µl reaction contained buffer C, ³²P-bioO at a final concentration of approximately 40 nM, 1 mM ATP, 1 mM MgCl₂, 1 µM biotin, and various dilutions of BirA. After incubation for 30 min at room temperature the samples were loaded onto a pre-run 5% polyacrylamide gel. The gels were visualized using a phosphoimager as described above.

In vitro biotinylation assays. Isolation and purification of the apo truncated biotin carboxyl carrier protein (apo AccB-87) was carried out using expression from pVC19 in strain BL21 (λDE3) as described above. The biotin ligase activity was measured using the assay previously described (10, 11). Briefly, the reaction mixture contained 50 mM Tris-HCl (pH 8.0), 3 mM ATP, 5.5 mM MgCl₂, 5 µM biotin, 25 pmol of [³H]biotin (specific activity 32.6 Ci mmol⁻¹), 0.1 mM dithiothreitol, 0.1 mg ml⁻¹ bovine serum albumin and various concentrations of apo protein in a final volume of 100 µL. The reaction was initiated by the addition of purified BirA to a final concentration of 16 nM, except for mutant G154D where 100 nM was needed to observe detectable biotinylation activity. In order to obtain high levels of radioactive label for accurate readings we increased the specific activity 5-fold over that used previously (10, 11) and each vial was counted to obtain <5% error. The kinetic constants Kₘ and kₗₐₜ were determined using double reciprocal plots and those for the wild type enzyme were very similar to those previously reported (10, 33).
RESULTS

Isolation of BirA super-repressor mutant strains. The current model of BirA regulation (Fig. 1) argues that several classes of BirA super-repressor mutants are possible, several of which would lack enzymatic activity and thus be lethal mutations (3). This is because the sole *E. coli* biotinylated protein AccB, is a subunit of acetyl-CoA carboxylase that catalyzes the first step of the fatty acid synthetic pathway. Early blocks in the fatty acid synthetic pathway cannot be bypassed by fatty acid supplementation, acetyl-CoA carboxylase is essential (3). We therefore isolated birA super-repressor mutants in *E. coli* cells that carried a plasmid (pCY255) encoding Bpl1, the *Saccharomyces cerevisiae* biotin protein ligase. Previous work showed that expression of Bpl1 complemented a conditional birA mutation (17). To avoid possible complications of having two BirA species present in a cell we deleted the chromosomal birA gene. To avoid lethality we constructed the deletion in the presence of a plasmid-borne wild type copy of birA and transduced the mutation into a strain carrying the yeast Bpl1 plasmid (pCY255). The successful transduction indicated that Bpl1 provided full biotin ligase function. The ΔbirA construct was confirmed to have the expected genomic arrangement by Southern blot hybridization (data not shown). To provide an assay for bio operon transcription a chromosomal bioF::lacZY promoter fusion was introduced into the ΔbirA strain (6). Expression of Bpl1 in the ΔbirA strain had no effect on bio operon regulation over a wide range of biotin concentrations (see below). Homologous recombination catalyzed by the phage λred system was then used to replace the coding sequence of the chromosomal bioA gene of the bioF::lacZY strain with a chloramphenicol acetyltransferase (cat) coding sequence. This gave VC235, the bioA::cat bioF::lacZYΔbirA/pCY255 strain used in the mutant selection (Fig. 2A). The cat gene was used because chloramphenicol is bacteriostatic to *E. coli* given low concentrations and short incubation times (2). This property allowed a positive selection for birA super-repressor mutants (Fig. 2).

The hypothesis was that at low concentrations of biotin strain VC235 expressing wild type BirA would be resistant to chloramphenicol due to derepression of the bioA promoter and therefore would continue to grow in the presence of the antibiotic (Fig. 2B). In contrast, cells expressing BirA super-repressor proteins would be sensitive to chloramphenicol and growth inhibited (Fig. 2B). Upon addition of antibiotics that kill only growing cells (cell lytic β-lactams) those cells expressing wild type BirA would be killed whereas cells expressing BirA super-repressors would fail to grow due to
chloramphenicol action and thereby survive. The selection protocol was tested using a culture that
contained a mixture of two differentially marked strains, one of which carried a chromosomal cat
cassette insertion. Chloramphenicol was added to the growing culture and after two cell doublings β-
lactams (a mixture of ticarcillin and ampicillin) was added together with the β-lactamase inhibitor,
clavulanate (Materials and Methods). Following lysis of the culture the survivors were plated and
scored. The selection resulted in a ~2x10^5-fold enrichment of the chloramphenicol-sensitive strain (data
not shown).

To isolate strains encoding BirA super-repressors the birA gene was subjected to PCR
mutagenesis (Materials and Methods). The PCR products were ligated to the pVC7 backbone and the
plasmids were transformed into the ΔbirA strain VC235 which carried the Bpl1 plasmid plus the
bioA::cat bioF::lacZY construct. The resulting transformants were pooled and grown in defined
medium containing 1.6 nM biotin and 3 μg mL^-1 chloramphenicol followed by treatment with the β-
lactam plus β-lactamase inhibitor cocktail to enrich for chloramphenicol-sensitive cells. The survivors
were plated on defined medium plates supplemented with X-gal and 1.6 nM biotin to assay repression
of bioBFCD transcription. Cells that expressed BirA super-repressors gave white colonies due to
repression whereas cells expressing wild type BirA gave blue colonies due to derepression at the low
biotin concentration. It should be noted that overexpression of wild type BirA by medium copy number
plasmids such as pVC7 results in a degree of super-repression due to greater bioO occupancy (5, 15).
We found this effect was about 10-fold which agrees well with the prior estimate of 12-fold using the
same vector plasmid (5). However, the dynamic range of BirA regulation is sufficiently large (several
100-fold) that medium copy number plasmids could be used to facilitate the genetic manipulations.

The plasmids recovered from white colonies were transformed into strains VC218 and
VC233. Transformants of the ΔbirA ΔlacZY bioF::lacZY/pCY255 strain, VC218, were used to
assay β-galactosidase levels in cultures grown with 1.6 nM biotin (Fig. 3). We sequenced the
birA genes of candidate strains that showed 5 to 10-fold reductions in β-galactosidase activity
relative to the strain carrying the BirA wild type plasmid pVC7. Five of the eight mutant genes
sequenced had multiple base substitutions that resulted in several amino acid substitutions
whereas the remaining three mutants (5A, 8A and 13B) each contained a single amino acid
substitution (Fig. 3A). The multiple mutant genes (1A, 6A, 12A,16B and 3C) were deconvoluted
by site-directed mutagenesis into single and double mutants (Fig. 3B and 3C) in order to identify
the amino acid residue substitution(s) responsible for super-repressor phenotype (Fig. 3D). In
most cases only a single mutation was required for the super-repressor phenotype (an exception is shown in Fig. 3C) and thus we limited our analyses to those proteins (Fig. 3D).

**In vivo characterization of the birA mutants.** A majority of the super-repressor BirA mutants were expected to be defective in carrying out the essential ligation of biotin to AccB due to several possible scenarios such as ligand independent dimerization, dimerization with biotin in place of biotinoyl-adenylate (biotin is known to be a weak dimerization ligand *in vitro*, ref. 7) or an inability to transfer the biotin moiety of biotinoyl-adenylate to the acceptor protein. However, this was not the case. Only one such mutant, G154D BirA, was isolated. To test the *in vivo* ligation abilities of the mutant BirA proteins, strain VC618 (*Δ*birA::kan bioF::lacZY::pVC18) was transformed with each of the mutant BirA plasmids at 30°C. These strains were then cured of the temperature-sensitive yeast Bpl1 plasmid, pVC18, by growth at 42°C. Each of the mutant proteins with the exception of mutant G154D BirA, allowed robust growth of the *Δ*birA strain comparable to that given by the plasmid encoding wild type BirA even at the minimal biotin concentration (1.6 nM) (data not shown). The G154D BirA strain also grew, but more slowly; an additional day was required for visible colonies to appear. To quantify these observations growth curve analyses of the strains were done in medium supplemented with biotin at different concentrations. Only the strain expressing G154D BirA showed a growth defect at low biotin concentrations (1.6 and 4 nM) (Fig. 5A and B). Super-repressor mutant alleles generally show genetic dominance over the wild type allele and thus we tested the plasmid-borne mutant alleles for this property. All of the mutant alleles were dominant over the chromosomal wild type allele in that they gave levels of *bio* operon expression 40 to 200–fold lower than that of the plasmid borne wild type allele when grown with 1.6 nM or 4 nM biotin (data not shown).

The rate of *bio* operon transcription responds to the concentrations of both biotin and the unmodified forms of biotin accepting proteins and thus is derepressed upon high-level expression of a biotin accepting protein even at high biotin concentrations (15). The biotin accepting protein can be AccB, heterologous proteins from other organisms or even a small peptide (16, 38). We therefore asked if the super-repression phenotypes of the mutant proteins could be relieved by increased biotin acceptor protein concentrations. The most physiologically relevant biotin acceptor protein is AccB. However, overexpression of AccB is toxic to *E. coli* unless AccC, the biotin carboxylase subunit of acetyl CoA carboxylase encoded by the downstream gene is simultaneously overexpressed (1, 22) probably because AccB and AccC form a
stoichiometric protein complex (12). The effects of AccB-AccC overexpression from plasmid pCY705 were quantitated by β-galactosidase assays of cultures grown with different biotin concentrations. As a negative control pCY705 was replaced with pCY730, which encodes a K122R AccB that can neither be biotinylated nor act as a regulatory ligand (1).

Biotin operon regulation shows the greatest response over biotin concentrations from 4 to 40 nM (1, 5, 15, 38). The strains expressing the I187T, K267M, Y178C, P143T and M310L BirA proteins all responded to over-expression of AccB (plus AccC) in a manner similar to that of the strain expressing wild type BirA (Fig. 6A-F) except that the derepression levels of the mutant BirA strains were 2 to 3-fold lower than the wild type levels (Fig. 6J-K). In contrast the strain expressing the G154D mutant BirA showed no derepression of bio operon transcription upon AccB-AccC overexpression (Fig. 6G). We hypothesized that if the observed lack of derepression was due to the inability of the G154D BirA to interact with AccB, then the mutant protein might remain able to biotinylate the peptide-85 sequence, a 15-residue biotin accepting peptide isolated by Schatz (37). The peptide-85 sequence is as effective a biotin acceptor as AccB (8), despite sharing very little sequence similarity with the AccB biotinylation site (38). Expression of the peptide-85 sequence as a fusion to maltose binding protein (38) failed to relieve BirA G154D repression of bio operon transcription (Fig. 6H), although it relieved repression by wild type BirA (Fig. 6I). These observations strongly suggest that BirA G154D is defective in the transfer of the biotin moiety from biotinoyl-adenylate to the acceptor.

Strains that expressed G154D BirA from a plasmid showed a super-repressor phenotype and grew slowly at low biotin concentrations. Since these attributes could depend to some degree on plasmid copy number and the use of a foreign promoter, we replaced the wild type chromosomal birA with the mutant allele encoding BirA G154D by use of a counter-selection strategy (42). Given the lower chromosomal expression level it seemed likely that introduction of the mutant allele into the chromosome would be lethal and thus the recipient strain carried the yeast Bpl1 plasmid to provide ligase activity. Following construction and validation of the allele replacement we attempted to transduce the G154D mutation into a wild type strain. Transduction proceeded readily when the recipient strain contained a plasmid that encoded ligase activity whereas transductions into a strain that carried an empty vector repeatedly failed, indicating that the G154D birA allele was a lethal mutation.
To provide more direct evidence of lethality we introduced a temperature-sensitive mutation in DNA polymerase I into strains carrying the Bpl1 plasmid and chromosomal copies of genes encoding either the wild type or G154D mutant BirA (strains VC779 and VC780). In the absence of DNA polymerase I activity the replication origin of the Bpl1 plasmid is inactive (23, 27). At the permissive temperature of 30°C, the \textit{polA(Ts)} mutation was readily transduced into both the G154D \textit{birA} strain VC779 and the wild type \textit{birA} strain VC780. When shifted to the non-permissive temperature of 42°C to drive out the Bpl1 plasmid strain VC801 (\textit{birA}^{WT}) grew well whereas strain VC802 (\textit{birA}^{G154D}) failed to grow, thereby demonstrating the lethality of the G154D \textit{birA} allele in its native chromosomal location (Fig. 7B).

The effect of chromosomal expression of the G154D protein on the \textit{bio} operon regulation was determined by β-galactosidase assays of cultures grown with a range of biotin concentrations (the strain carried the Bpl1-encoding plasmid for viability). No detectable derepression of the \textit{bio} operon was seen at 1.6 nM biotin indicating that the presence of the Bpl1-encoding plasmid did not effect BirA regulation (Fig. 7A). A similar absence of regulation by Bpl1 resulted when the chromosomal mutation was the \textit{birA1} allele (6) (data not shown).

\textbf{Binding affinities of the BirA mutant proteins for \textit{bioO}.} To further characterize the mutant BirA proteins we purified C-terminal hexahistidine-tagged derivatives of six mutant proteins together with wild type BirA and AccB-87, the biotin accepting domain of AccB (Fig. 8). The most straightforward explanation for the super-repressor phenotypes of the mutant BirA proteins is that they would bind the \textit{bio} operator more tightly than the wild type protein. To test if this was the case we carried out electromobility shift assays (EMSAs) using a 112-bp biotin operator duplex DNA and the purified BirAs. EMSA analyses were performed on wild type BirA and four mutant proteins, G154D, I187T, M310L and K267M, which covered the range of \textit{in vivo} phenotypes observed.

The mobility shift experiments were done over a range of BirA concentrations (500 nM-31.25 nM). All of the mutant proteins showed increased ability to bind the \textit{bio} operator in that significant operator binding was seen at concentrations (31.25 nM and 61.5 nM) at which wild type BirA showed barely detectable binding (Fig. 9 A-D). Moreover, at 250 nM BirA the mutant proteins bound the probe almost quantitatively whereas a significant fraction of the probe incubated with wild type BirA remained unbound. Three of the mutant BirAs, M310L, K267M
and G154D, showed increased operator binding across the BirA concentration gradient whereas BirA I187T had only slightly enhanced binding (Fig. 9A-D). Due to the variation seen in binding by the wild type BirA the wild type protein was assayed in parallel with each of the mutant proteins. The observed variable operator binding of wild type BirA precluded quantitative analysis of these data.

**Ligation abilities of the mutant BirA proteins.** The purified BirAs were first qualitatively tested for the ability to synthesize the BirA reaction intermediate, biotinoyl-adenylate, in the presence of biotin and [α-32P]-labeled ATP. Since all of the mutant proteins allowed growth of the ΔbirA strain, they were expected to synthesize the biotinoyl-adenylate intermediate *in vitro*. Indeed, each of the mutant proteins synthesized biotinoyl-adenylate when [α25P]-ATP and biotin were added as substrates (Fig. 10). We also tested transfer of the biotinoyl-adenylate biotin moiety to AccB-87, the carboxyl terminal half of AccB, which accepts biotin as well as the full-length protein (29). In this assay addition of AccB-87 to the completed biotinoyl-adenylate synthesis reaction should result in disappearance of biotinoyl-adenylate and the appearance of AMP. Each of the mutant proteins except BirA G154D gave this result (Fig. 10A and B). G154D BirA was almost completely defective in biotin transfer *in vitro* (Fig. 10A). With the exception of BirA mutant G154D, no detectable differences were observed between the mutant and wild type BirAs in time course assays (data not shown).

**Kinetics of AccB-87 Biotinylation.** To evaluate the abilities of the mutant BirA proteins to ligate biotin to AccB *in vitro*, the kinetic constants for AccB-87 were determined using steady state kinetics. The *in vitro* biotinylation activities of the three purified mutant BirAs I187T, M310L and G154D and of the wild type BirA were determined in assays performed at saturating levels of biotin and Mg-ATP (Materials and Methods). As shown by the kinetic constants (Table 3) BirA proteins I187T and M310L proteins did not differ significantly from those of wild type BirA in their Michaelis constants for the biotin acceptor substrate. However, as expected from the indirect experiments of Fig. 9A, BirA G154D was extremely defective in biotinylation of AccB-87. Biotinylation catalyzed by G154D BirA could be detected only when the enzyme and substrate concentrations of the assay were increased to 100 nM and 75 µM, respectively. Reliable kinetic constants for BirA G154D could not be calculated because saturating AccB-87...
concentrations could not be attained. However, it is clear that the substitution of aspartate for
G154 results in a ligase that has at least a 200- to 300-fold decrease in the ability of the protein to
productively interact with AccB-87.
Prior genetic selections for birA mutants resulted in isolation of strains having decreased abilities to repress bio operon expression, many of which were also defective in biotin utilization (6). All of the mutant alleles tested were recessive to the wild type allele indicating that they were loss of function mutations (6). Subsequent work on several of these mutant strains showed that the defects in biotin utilization reflected compromised ligase activity due to decreased affinities for biotin and/or biotinoyl-adenylate (13, 24). We report isolation of the first gain of function (genetically dominant) birA super-repressor alleles. BirA super-repressor strains were isolated by use of a chloramphenicol resistance cassette as a negative selection, a manipulation that may be generally applicable. The selection method was surprisingly efficient such that BirA super-repressor strains were readily isolated and showed the expected in vivo phenotype. The current model of BirA function implies that an appreciable fraction of super-repressor mutations should be lethal. For example BirA proteins that bind bioO in the absence of biotinoyl-adenylate would not respond to biotin limitation or the need for protein biotinylation. Although the selection method ensured survival of strains carrying lethal mutations, we isolated only one lethal mutation (BirA G154D). The finding that super-repressor strains having mutations in the catalytic domain retained ligase activity indicates that communication of the catalytic domain with the DNA binding domain is more complex than previously appreciated: normal ligase activity does not necessarily result in normal repression.

There is good evidence that BirA undergoes diverse interactions among the three domains during its physiological roles and our data provide further proof. A prime example in the prior literature concerns the N-terminal DNA binding domain. In all currently available crystal structures (e.g., Fig. 4A) the domain appears well removed from the catalytic and C-terminal domains and thus it would seem that deletion of this domain would leave the ligase activity intact. However, deletion of the DNA binding domain results in greatly weakened binding of biotin which compromises biotinoyl-adenylate synthesis (46). The ability to retain biotinoyl-adenylate in the active site is also impaired (46) and the N-terminal deletion mutant is unable to grow at physiological biotin concentrations (unpublished data). Another example of inter-domain communication is the restructuring of loops in the C-terminal domain observed...
upon binding of a biotinoyl-adenylate analogue to the catalytic domain (25, 45). Among our mutants the phenotypes of the I187T and
K267M super-repressor BirA probably reflect disturbances in inter-domain communications. The I187 substitution (I187T) involves
a residue implicated in packing interactions between the adenylate binding loop and an interfacial loop (45) whereas the K267M
substitution is located within the sequence that connects the catalytic and C-terminal domains (Fig. 4A). Thus residue K267, although
exposed to solvent and removed from both the dimer interface and the DNA binding domain, somehow modulates operator binding,
the property of a domain that is far distant in the extant crystal structures. The most profound mutation we isolated was the G154D
BirA, a lethal mutation in its native chromosomal context, that had the strongest super-repressor phenotype. This protein was severely
deficient in binding the AccB biotin acceptor protein and unable to transfer biotin from biotinoyl-AMP to the acceptor. Although the
biotin transfer defect alone could explain its super-repressor phenotype, the G154D BirA also showed increased affinity for the
operator DNA. The finding that a single point mutation affects both biotin transfer and DNA binding further illustrates interactions
among the domains of BirA.

Since BirA dimerization is required for bioO binding, super-repressor mutations that facilitate or stabilize dimerization were
expected. The BirA-biotinoyl-adenylate complex forms dimers at protein concentrations of 5-10 μM whereas operator binding occurs
in the range of 30 nM (7, 28, 41, 47). Therefore a large window exists within which increased operator binding by improvement of the
dimerization properties of BirA could be obtained. Indeed, the amino acid substitutions of several of the BirA super-repressors
isolated, P143T, Y178C and M310L are located at or adjacent to the dimerization interface (Fig. 4B) and thus may directly affect
dimerization. Unfortunately, it is not straightforward to measure BirA dimerization because the monomer and dimer forms are in
rapid equilibrium. The wild type BirA dimer has a half-life of minutes (39), a time scale incompatible with size exclusion
chromatography. Moreover, size exclusion chromatography would dilute and separate the two forms and thereby skew the
equilibrium. Hence, although super-repressor BirA dimers may be more stable than the wild type protein, no valid comparison with
the wild type protein could be made. Such measurements must be made in free solution, a technically demanding task that we have
chosen to defer pending crystallization trials of the mutant proteins.
In future work we will first focus on the structure of the BirA-bioO complex because this is the major gap in our understanding of bio operon regulation due to the lack of a co-crystal structure. The present crystal structures give no clues to the modes of interaction of the DNA binding domain with the catalytic and C-terminal domains although data in this paper and elsewhere indicate that these interactions are key to both functions of BirA. This situation remains despite many attempts with the wild type protein (B. W. Matthews, personal communication). Our hypothesis is that the increased binding affinities of super-repressor BirA proteins for bioO should increase the probabilities of obtaining co-crystal structures. However, it is unclear which (if any) of the mutant proteins will provide a high quality structure. Therefore, we have adopted a “bottom line” strategy in which several super-repressor mutant proteins have been subjected to crystallization trials with operator DNA and small molecule ligands. If successful, the BirA proteins that give high quality BirA-bioO co-crystal structures will be the prime candidates for further study.
ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health (NIH) Grant AI15650 from the National Institute of Allergy and Infectious Diseases (NIAID). We thank Dr. Sharik R. Khan, Dr. Ella Rotman, Dr. Elena A. Kouzminova, Dr. Andrei Kuzminov, Margaret M. Wood, Dr. Jeffrey F. Gardner, Caryn S. Wadler and Dr. Carin K. Vanderpool for their generous gift of plasmids, strains and research materials. We thank Dr. James A. Imlay for helpful discussion of this work.
REFERENCES


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Replacement of birA::cat-sacB with birA<sup>AS490</sup> using homologous recombination.

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This study
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pSRK46  pKD46, Spec^R^  S. R. Khan unpublished
pSRK20  pCP20, Cml^R^ and Spec^R^  S. R. Khan unpublished
pCY759  plasmid encoding Maltose binding protein fusion to biotin accepting peptides 85  (38)
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<td>VCB13</td>
<td>GCTCCACCAGAATGCCTGCAAGCTTG</td>
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<td>VCB14</td>
<td>GCCACTTTAATGATTGCCCTTGTAAGCAGTGAGACTT</td>
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<td>VCB15</td>
<td>ATTCACCGTGCGAATACATGCAATTTTCAGTG</td>
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<td>VCB16</td>
<td>GCGCCATGGATAGGATAACACCGTGCC</td>
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<td>VCB17</td>
<td>CGCCGATCCTTAATGATGATGATGATGATGATTTCCTGC</td>
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<tr>
<td>VCB44</td>
<td>CGAATTCGAGCTCTCTAAGGAGGAGCAATGGAGCCGC</td>
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<tr>
<td>VCB45</td>
<td>CAGCCAGCTTTTACTCGAGTGACGACAG</td>
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<tr>
<td>VCB36</td>
<td>GCGAAATCTCGGTTGTCTCATACAGC</td>
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<tr>
<td>VCB37</td>
<td>GCCAGGCTTTGTCTCAACACGTG</td>
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33
VCB57  GATATACCATGGAAAGCGCCAGCACG
VCB58  GCCGGATCTTACTCGAATGCACGACCAGCG
VCC44  GCAATTGTTATGAGGAAAGCTGCTCTATCTTCTTCT
VCC5   GGAATAATACCTGTTGTCGTTAGAAAATCC
VCC6   GGGATTTTCACGGCAACCAGGGTTTTATTTATCC
VCC49  ATTTGCCATCACGAGTGAAGTGAGCGCAGTGGAGACAATTTCATGA
VCC50  CCCTGCCATCTGCAGTGAAGTGAGCGCAGTGGAGACAATTTCATGA
VCC51  GCCGCTAGAAGAGTTCAGACTAATTTGCAGGGGAGCGAATCTCCCCTTTCTTATTTTCCT
VCC52  GGTGAAAAATTTAATGTCTCGCTTG
VCC53  CCAGTTTAATCGCAAGAAAATGTCTAATC
VCB73  GGTGAGTGCTGTTATCGATATCGAATCGGCTGAAAG
VCB74  CTCCGCCATCAGATCGAAACTCAAACTTAAAC
Table 3. Kinetic constants for biotinylation of AccB-87 by wild-type BirA and three mutant BirA proteinsa

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type</th>
<th>I187T</th>
<th>M310L</th>
<th>G154D</th>
</tr>
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<tbody>
<tr>
<td>$K_{\text{m}}$ (μM) for AccB-87</td>
<td>5.4 ± 0.76</td>
<td>12.8 ± 2.35</td>
<td>8.6 ± 1.5</td>
<td>170</td>
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<tr>
<td>$k_{\text{cat}}$ (sec⁻¹)</td>
<td>0.86 ± 0.7</td>
<td>1.37 ± 0.04</td>
<td>1.22 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>$k_{\text{cat}}/K_{\text{m}}$ (M⁻¹sec⁻¹) x 10³</td>
<td>164.3 ± 28.8</td>
<td>116.1 ± 2.4</td>
<td>140 ± 10</td>
<td>ND</td>
</tr>
</tbody>
</table>

aThe wild type and mutant derivatives of BirA were assayed by in vitro biotinylation reactions over a range of AccB-87 protein concentrations as described in Materials and Methods. Results are presented as the average and standard error of triplicate determinations. ND, not determined.
**Figure Legends**

**FIG 1** The BPL reaction and *E. coli* biotin operon regulation.  Panel A shows the biotin protein ligase (BPL) activity of BirA. Panels B, C and D show the general model of *bio* operon regulation. Green ovals denote BirA, tailed blue ovals are AccB, black dots represent biotin and black dots with red pentagons denote biotinoyl-adenylate (bio-5'-AMP). Panel B shows the transcriptionally repressed state whereas panels C and D, respectively, show derepression of *bio* operon transcription engendered by either excess unbiotinylated AccB acceptor protein or by biotin limitation. Figure modified from (38).

**FIG 2** Selective enrichment for BirA super-repressor mutant strains. (A) The *E. coli* biotin operon consists of five structural genes that are arranged in a bi-directional operon (upper line). BirA represses the transcription of these genes by binding to *bioO*, a 40-bp inverted repeat sequence (Modified from (6)). The native *bio* operon was modified (lower line) by transduction of the *bioF::lacZY* fusion of strain CY481 into strain VC212. The *bioA* gene of the resulting construct was then replaced with the *cat* gene (which encodes chloramphenicol resistance) using recombination catalyzed by the phage *λ* red system (18) to give strain VC235, the strain used in the selection-enrichment protocol. The *lacZY* genes are fused to the rightward *bio* promoter resulting in a lactose-positive phenotype when the *bio* operon transcription is derepressed and a lactose-negative phenotype when the operon is repressed (6). The strain also carried a deletion of the chromosomal lactose operon and was a biotin auxotroph due to insertion of the *lac* genes into *bioF*. When grown with low biotin concentrations super-repressor mutants would have a higher affinity for the biotin operator such that the strains would be chloramphenicol sensitive. In contrast the strains encoding wild type copy of BirA would be resistant to chloramphenicol and continue growth in the presence of the antibiotic. The selection results from addition of a mixture of β-lactams (ticarcillin and ampicillin) plus a β-lactamase inhibitor (clavulanate) which kills only growing (chloramphenicol-resistant) cells whereas the chloramphenicol sensitive cells survive. Finally, the surviving cells were plated on defined medium plates supplemented with X-gal and 1.6 nM biotin to screen for colonies having a super-repressor phenotype (white colonies rather than the wild type blue colonies).
FIG 3  Isolation and Characterization of BirA candidates exhibiting super-repressor phenotypes. Single colonies were streaked on defined medium plates supplemented with 1.6 nM biotin and X-gal for phenotypic confirmation. Liquid cultures grown in the same medium were then assayed for bio operon transcription by β-galactosidase activity (Materials and Methods). The birA inserts of the plasmids encoding the super-repressor BirAs were then sequenced. Panel A shows the transcriptional activities and mutations of the mutants studied whereas panel B illustrates the deconvolution of the double mutants 1A and 6A (which shared the I187T mutation) to identify the mutations causing the super-repressor phenotype. Panel C shows the deconvolution of the 12A triple mutant. The single mutations were generated by site-directed mutagenic repair of each mutant allele with the wild type sequence. Strains carrying the plasmids encoding single mutant BirAs were then assayed for β-galactosidase activity. Finally (panel D) the β-galactosidase activities of the plasmid borne single mutant super-repressor strains chosen for in vivo and in vitro analysis were compared with the wild type strain and with each other.

FIG 4  The three-dimensional structure of BirA. In panel A the three different domains of the monomeric protein are shown. The single amino acid substitutions that resulted in super-repressor phenotypes are highlighted. In panel B the 2.8 Å crystal structure of dimeric BirA complexed to the co-repressor analogue, biotinol-5'-AMP (colored white), is shown. Relative to the unliganded structure co-repressor analogue binding resulted in ordering of both adenylate-binding loops and the dimer interface also undergoes a favorable transition that might be necessary for BirA binding to bioO. Several of the mutations are found within the dimer interface, suggesting that they might stabilize the dimer to give the observed super-repressor phenotypes. The model was generated by using the graphics program Chimera (32) with Protein Data Bank files 1HXD and 2EWN.

FIG 5  Complementation of the E. coli birA ΔbioABFCD strain by expression of either wild type BirA (panel A) or G154D BirA (panel B) from a medium copy number plasmid. The strains were assayed for growth in defined medium liquid cultures supplemented with various concentrations of biotin as described in the Materials and Methods section. The differing biotin...
concentrations are indicated by the colored symbols to the right of panel B. Each growth curve assay was carried out in triplicate and
the average was used in this plot. Growth was measured by optical density at 600 nm. Note that the Y-axis is plotted in a log scale.
Panels A and B have the same color code.

FIG 6  Regulation of bio operon expression in BirA Mutant strains upon over-expression of biotin acceptor proteins.

Expression of the bio operon in cultures grown with various concentrations of biotin assayed by use of β-galactosidase production
from the chromosomal bioF::lacZY fusion. The strains used were derivatives of strain VC618 cured of plasmid pVC17 that carried
the lacF\(^{\circ}\) plasmid pMS421 plus two compatible plasmids, one of which encoded a BirA mutant protein and the other encoded wild
type AccB plus AccC or AccB K122R-AccC or the biotin accepting peptide-85 fusion protein. The AccB-AccC and AccB K122R-
AccC plasmids were pCY705, and pCY730, respectively, whereas the plasmid pCY759 encoded the maltose-binding protein fused to
biotin accepting peptide-85. The AccB K122R protein that is unable to accept biotin served as control. The most responsive range of
biotin concentrations (X-axis) was 4 to 40 nM. Derivatives of strain VC618 carrying the wild type or the mutant BirA plasmids plus
pCY705 ( ) or pCY730 (△) were induced with 0.1 mM IPTG. The experiments were repeated three times in their entirety and the
results were essentially identical to those shown. Also included are strain VC618 derivatives that carried the peptide-85 plasmid
pCY759 and plasmids encoding either the BirA mutant G154D (H) or wild type BirA (I). Symbols (×) represent assays with cultures
that were induced with 0.1 mM IPTG whereas (○) represents assays of uninduced cultures. Note that the Y-axis is plotted on a log
scale. The data of panels J and K are from three independent experiments and demonstrate the effects of AccB overexpression on the
regulation of the bio operon by the wild type and birA mutant strains. The wild AccB-AccC expression (J) was performed at 40 nM
biotin (repression conditions) whereas the AccB K122R-AccC (K) expression was assayed at 4 nM (de-repression conditions). Open
bars indicate cultures induced with 0.1 mM IPTG whereas the solid bars indicate cultures that were not induced. Error bars denote the
standard error. The strain expressing the BirA G154D and pCY730 grew very poorly and therefore was unavailable for β-
galactosidase assay (panels G and K).
FIG 7  Transcriptional regulation of the biotin operon mediated by the chromosomal birA alleles in cultures grown with various biotin concentrations. All strains contained the chromosomal bioF::lacZY fusion and originally carried plasmid pCY255 encoding yeast Bpl1. In panel A the strains were grown in defined medium supplemented with various concentrations of biotin as shown on the X-axis. Grey bars denote wild type birA allele, red bars denote the birA null (deletion) allele and the black bars the birA allele encoding BirA G154D. The results show the average of three independent experiments and the error bars denote the standard error. In panel B the growth of strain VC780 (chromosomal wild type BirA) or strain VC779 (chromosomal BirA G154D) or their polA12 derivatives (strains VC801 and VC802, respectively). The strains were streaked on defined medium plates supplemented with 4 nM biotin and X-gal that were incubated overnight at either 30°C or 42°C. The A and B designations of strains VC801 and VC802 denote two different transductants from the polA12 strain constructions. Strains VC780 and VC801 were derepressed (blue colonies) and grew at both temperatures due to the expression of wild type BirA. Strain VC779 grew at both temperatures due to the presence of pCY255 whereas strain VC802 failed to grow at 42°C because pCY255 could no longer replicate due to loss of DNA polymerase I activity at the non-permissive temperature and strain VC779 grew at 42°C but remained repressed.

FIG 8  SDS-gel analysis of purified BirA and AccB-87 proteins. In Panel A, the BirA proteins were subjected to electrophoresis in a 12% polyacrylamide gel whereas in Panel B the gel used for AccB-87 was 15%. BirA G154D consistently migrates more slowly than the other BirA proteins, a behavior that has been observed with other single amino acid substitutions (30).

FIG 9  DNA binding properties of mutant BirA proteins assayed by EMSA. In Panels A-D the mutant and wild type BirAs were assayed for binding to a 112 bp bioO duplex DNA labeled at the 5’-ends with 32P prepared as described in Materials and Methods. The reaction products were separated via native gels electrophoresis and visualized via phosphorimagery. The mutant and wild type BirA proteins (0-500 nM) were assayed in parallel. The control reaction of all the panels are: NE, no enzyme added. From left to right the BirA concentrations assayed were: 500 nM, 250 nM, 125 nM, 61.5 nM and 31.25 nM. Each panel contains the wild type BirA plus a mutant BirA as given. Panels A, B, C and D, respectively, contained the G154D, K267M, I187T and M310L BirA proteins.
FIG 10. Abilities of the mutant BirA proteins to synthesize biotinoyl-adenylate and transfer the biotin moiety to AccB-87. In Panels A and B synthesis of the biotinoyl-adenylate in reaction mixtures containing BirA (1 µM), biotin (0.01 µM) and [α-32P]ATP (0.1 µM) is shown. Following a 30 min incubation each reaction mixture was split into halves and to one half (+ symbols) purified apo-AccB-87 (70 µM) was added followed by incubation for an additional 15 min whereas the other half (- symbols) were left untreated. One µl of each sample was then spotted onto a cellulose thin layer chromatography plate. Following plate development the reaction products (indicated by arrows), biotinoyl-adenylate (bio-5'-AMP) and AMP were visualized by autoradiography. In Panel B similar experiments were carried out except that two different concentrations of AccB-87 (7 µM or 70 µM) were used in the transfer assay. The control reactions of both panels are: NB, no biotin added and NE no enzyme added. The major spot on all lanes is ATP. The symbols (+) represent assays in which the acceptor AccB-87 was added whereas the symbol (-) denote assays in which the acceptor protein was not added.
<table>
<thead>
<tr>
<th>BirA</th>
<th>WT</th>
<th>NE</th>
<th>WT</th>
<th>K267M</th>
<th>P143T</th>
<th>M310L</th>
<th>G154D</th>
</tr>
</thead>
<tbody>
<tr>
<td>[AccB-87]</td>
<td>NB</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</table>

- Bio-5'-AMP
  - R<sub>r</sub> = 0.78

- AMP

- ATP

<table>
<thead>
<tr>
<th>BirA</th>
<th>WT</th>
<th>NE</th>
<th>WT</th>
<th>I187T</th>
<th>Y178C</th>
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<tbody>
<tr>
<td>[AccB-87]</td>
<td>NB</td>
<td>-</td>
<td>0</td>
<td>7</td>
<td>70</td>
</tr>
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</table>

- Bio-5'-AMP
  - R<sub>r</sub> = 0.77

- AMP

- ATP