Successful Conversion of the *Bacillus subtilis* BirA Group II Biotin Protein Ligase into a Group I Ligase

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Abstract

Group II biotin protein ligases (BPLs) are characterized by the presence of an N-terminal DNA binding domain that allows transcriptional regulation of biotin biosynthetic and transport genes whereas Group I BPLs lack this N-terminal domain. The *Bacillus subtilis* BPL, BirA, is classified as a Group II BPL based on sequence predictions of an N-terminal helix-turn-helix motif and mutational alteration of its regulatory properties. We report evidence that *B. subtilis* BirA is a Group II BPL that regulates transcription at three genomic sites: *bioWAFDBI*, *yuIG* and *yhfUTS*. Moreover, unlike the paradigm Group II BPL, *E. coli* BirA, the N-terminal DNA binding domain can be deleted from *Bacillus subtilis* BirA without adverse effects on its ligase function. This is the first example of successful conversion of a Group II BPL to a Group I BPL with retention of full ligase activity.

Introduction

Biotin protein ligase (BPL) is required for the covalent attachment of biotin to biotin-dependent enzymes. This attachment proceeds in a two-step reaction. First, BPL binds both biotin and ATP to synthesize biotinyl-5′-AMP (Bio-5′-AMP, also called biotinoyl-adenylate) with release of pyrophosphate [1]. The ε-amino group of the conserved lysine residue of the acceptor protein acts as a nucleophile to attack the Bio-5′-AMP mixed anhydride bond to give covalently attached biotin plus AMP (Fig. 1). Microbial BPLs are readily placed into two groups [2]. Both groups have catalytic and C-terminal domains that show strong structural conservation [3–7] whereas Group II BPLs are characterized by an N-terminal helix-turn-helix (HTH) DNA binding domain that permits transcriptional regulation of the biotin synthetic genes. *E. coli* BirA, the paradigm for regulation of biotin biosynthesis, is the best studied Group II BPL. Transcriptional repression of the *E. coli* biotin operon occurs when BirA accumulates Bio-5′-AMP because all biotin acceptor proteins have been biotinylated [8–10]. Bio-5′-AMP accumulation results in dimerization of BirA and subsequent DNA binding [6,11,12]. In all four *E. coli* BirA crystal structures [13–15] the HTH structure is spatially well removed from the other domains of the protein and thus deletion of the N-terminal DNA binding domain was expected to convert this Group II BPL into a fully functional Group I ligase. However, this was not the case: the resulting protein had severely compromised ligase activity [16]. This was also true for ligases having smaller N-terminal deletions [17].

The sequences of other Group II BPLs suggest such proteins are found in γ-Proteobacteria, Bacilli, and Clostridia [2], although only the proteins from *E. coli* [6] and (very recently) *Staphylococcus aureus* [7] have been enzymatically characterized and crystallized. One of the Group II BPLs, *B. subtilis* BirA, has only 27% amino acid sequence identity to *E. coli* BirA (Fig. 2). Despite this low sequence identity, *Bacillus subtilis* birA has been shown to complement the ligase activity of a temperature-sensitive *E. coli* birA85 strain [18]. Moreover, *B. subtilis* birA mutants show constitutive expression of a *bioW-lacZ* fusion, suggesting that BirA regulates biotin operon transcription [18]. *B. subtilis* microarray data identified two additional transcripts, *yuIG* and *yhfU*, regulated by BirA and BirA [19]. Both YuiG and YhiU have strong sequence similarity to the structurally characterized BioY biotin transporter of *Lactococcus lactis* [20] and other well characterized energy-coupling factor (ECF) biotin transporters [21]. All three transcripts have similar predicted BirA binding sites [2,19]. *B. subtilis* has two known biotinylated proteins, pyruvate carboxylase (PyC) and the biotin carboxyl carrier protein (AccB) subunit of acetyl-CoA carboxylase (http://genodb.pasteur.fr). A third *B. subtilis* protein, biotin/lipoil attachment protein (BLAP) encoded by the *yngH* gene was found to be biotinylated and lipoylated when expressed in *E. coli* [22] although subsequently this protein was found not to be lipoylated by the *B. subtilis* enzymes that modify the known cognate lipoic acid acceptor proteins [23].

A major shortcoming of our picture of the enzymatic and *in vivo* regulatory activities of Group II BPLs is that it is based on a single example, *E. coli* BirA. For this reason we decided to study the enzymatic and regulatory properties of the BirA of *B. subtilis*, a bacterium that is evolutionarily diverse from *E. coli*. Moreover the amino acid sequence of *B. subtilis* BirA differs markedly from that of the *E. coli* protein and *in vivo* *B. subtilis* BirA biotinylates multiple proteins whereas *E. coli* BirA modifies only a single protein. Here
we report that *B. subtilis* BirA is a Group II BPL that follows the *E. coli* model of regulation, but unlike the *E. coli* protein, *B. subtilis* BirA can be successfully converted into a fully active Group I BPL.

**Materials and Methods**

**Strains, chemicals and culture media**

The bacterial strains used were derivatives of *B. subtilis* 168 and *E. coli* K-12 (Table 1). The rich medium used to grow *E. coli* and *B. subtilis* was LB. The defined medium for *E. coli* was M9 salts supplemented with 0.5% glucose and 0.01% vitamin-free Casamino Acids (Difco) whereas the defined medium for *B. subtilis* was Spizizen salts [24] supplemented with 0.5% glycerol and 0.05% vitamin-free Casamino Acids (Difco) in addition to 0.01% each of tryptophan, tyrosine, isoleucine and phenylalanine. Antibiotics were used at the following concentrations (in µg ml⁻¹): sodium ampicillin, 100; kanamycin sulfate, 50; chloramphenicol, 25; tetracycline HCl, 12; erythromycin, 100; lincomycin, 12.5; streptomycin sulfate, 50 and spectinomycin sulfate, 50. The 15:1 mixture of ticarcillin disodium salt and potassium clavulanate (PyC-77) were amplified by PCR from *B. subtilis* strain 168 genomic DNA with primers SKH049 and SKH043, and SKH042 respectively, thereby adding EcoRI and SalI sites. The products were digested with EcoRI and SalI and ligated into the same sites of pET19b, with an N-terminal hexahistidine tag to give pSKH001. The plasmids used and constructed are given in Table 2. The plasmids used and constructed are given in Table 2. The *B. subtilis* birA gene was amplified by PCR from *B. subtilis* strain 168 genomic DNA with primers SKH001 and SKH002 (all primers are given in Table 3) that added NdeI and XhoI sites. The product was digested with NdeI and XhoI and ligated into the same sites of pET19b, with an N-terminal histidine tag to give pSKH001.

The C-terminal 86 residues of *B. subtilis* AccB (AccB-B6) were amplified by PCR from *B. subtilis* strain 168 genomic DNA with primers SKH005 and SKH006 that added NcoI and XhoI sites. The product was digested with NciI and XhoI and ligated into the same sites of pET28 to give pSKH003 which encoded the untagged protein. The C-terminal 77 residues of *B. subtilis* PyC (PyC-77) were amplified by PCR from *B. subtilis* strain 168 genomic DNA with primers SKH007 and SKH008 and inserted into pET-28 by the same procedures.

*B. subtilis* BirA, Δ2-64 BirA, Δ2-66 BirA, Δ2-74 BirA, and Δ1-81 BirA were amplified by PCR from *B. subtilis* strain 168 genomic DNA with forward primers SKH038, SKH036, SKH046, SKH048, SKH045, respectively, plus reverse primer SKH037. The products were digested with EcoRI and Sall sites. The products were digested with EcoRI and Sall and ligated into the same sites of pBAD332K, to give pSKH006, pSKH005, pSKH009, pSKH010 and pSKH011, respectively. The products were also ligated into the same sites of pBAD332Cm to give pSKH013, pSKH012, pSKH016, pSKH017 and pAKH015, respectively. *E. coli* BirA and Δ2-64 BirA were amplified by PCR from *E. coli* MG1655 genomic DNA with primers SKH049 and SKH043, and SKH042 and SKH043, respectively, thereby adding EcoRI and Sall sites. The products were digested with EcoRI and Sall and ligated into the same sites of pBAD332K, to give pSKH008 and pSKH007 respectively. The products were also ligated into the same sites of pBAD332Cm to give pSKH015 and pSKH014 respectively.

*B. subtilis* Δ2-64 BirA, Δ2-66 BirA, Δ2-74 BirA, and Δ1-81 BirA were amplified by PCR from *B. subtilis* strain 168 genomic DNA with forward primers SKH040, SKH047, SKH050, SKH044, respectively plus reverse primer SKH041. The primers added NdeI and XhoI sites. The products were digested with NdeI and XhoI and ligated into the same sites of pET-19b, adding an N-terminal histidine tag, to give pSKH019, pSKH020, pSKH021, and pSKH022, respectively.

A 500 base pair internal fragment of *B. subtilis* bioW was PCR amplified from *B. subtilis* strain 168 genomic DNA with primers SKH057 and SKH050 that added EcoRI and BamHI sites. The product was digested with EcoRI and BamHI and ligated into the same sites of pMUTIN4 to give pSKH023.

*B. subtilis* accB-86 was PCR amplified from strain 168 genomic DNA with primer SKH069 which added a HindIII and a strong RBS site (AAGGAGGAAAAATATG) plus primer SKH070 which contained an SphI site. The product was digested with HindIII and SphI and ligated into the same sites of pDR111 to give pSKH024.

**Bacillus subtilis** strain construction

*B. subtilis* competent cell preparation and transformation were carried out as described by Dubnau and Davidoff-Abelson [23]. To create a bioW-lacZ chromosomal fusion, pSKH023 was transformed into strain 1A330 creating strain SKH001. Single crossover integration into bioW was verified by PCR and sequencing. To construct an IPTG inducible chromosomal copy of accB-86, strain SKH001 was transformed with linearized DNA with primer SKH069 which added a HindIII and a strong RBS site (AAGGAGGAAAAATATG) plus primer SKH070 which contained an SphI site. The product was digested with HindIII and SphI and ligated into the same sites of pDR111 to give pSKH024.
Figure 2. Sequence alignments of *S. aureus* BPL, *B. subtilis* BirA and *E. coli* BirA. *B. subtilis* BirA has 31% amino acid identity to *S. aureus* BPL and 27% amino acid identity to *E. coli* BirA. Conserved residues are in white text and highlighted in red and similar residues are in red text and boxed in blue. The *S. aureus* BPL secondary structure (PDB: 4DQ2) is shown above the amino acid sequence.
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Table 1. Bacterial strains.

| Strain | Relevant Genotype or Description | Reference or Derivation |
|--------|----------------------------------|-------------------------|
| **B. subtilis** | | |
| 168 | trpC2 | Lab collection |
| 1A330 | argG932 bioB141 | BGSC |
| SKH001 | 1A330 bioW::lacZ | This study |
| SKH002 | SKH001 amyE::P spac acc8-86 | This study |
| **E. coli** | | |
| MG1655 | E. coli K-12 wild type | Lab collection |
| DH5α | Δ(lacZΔM15) lacY1 Δ(fruA-1) thr-1 thi-1 hsdR17 | Lab collection |
| BM4092 | [araD139lacB lacZ169gala44 thi88] Δ(lacZΔM15) glnA96 recA1 relA1 endA1 thi-1 hsdR17 | [33] |
| BL21 λ (DE3) | ompT, hsdSB (rB–, mB–), dcm, gal, lacIq (DE3) | Novagen |
| VC618 | Δ lacZ807, bioF::lacZYA, rpsL150streptomycin | [36] |
| VC832 | BL21 λ (DE3), pVC36 | [17] |
pSKH024 creating strain SKH002. Double crossover integration into amyE was verified by the amylase production screen of Harwood and Cutting [26]. Integration was also verified by PCR and by sequencing with primers SKH065 and SKH066, SKH067 and SKH075 and SKH073 and SKH074.

**Structural modeling and sequence alignment**

The *B. subtilis* AccB biotin attachment domain was identified by InterProScan [27] and structural modeling to *E. coli* AccB-87 crystal structure (PDB 1A6X) using Swiss-Model automated mode [28-30]. The *B. subtilis* PyC biotin attachment domain was identified by InterProScan [27] and structural modeling to the *Staphylococcus aureus* pyruvate carboxylase biotin attachment domain crystal structure (3HBL) using Swiss-Model as above. *B. subtilis* BirA N-terminal deletions were determined by modeling to *S. aureus* BirA crystal structure (4DQ2) as a template using Swiss-Model automated mode as above. The final image was created using UCSF Chimera package [31]. Sequence alignments were created using the Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and the output was processed by ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) to generate the final figure [32].

**Complementation analyses**

Two *E. coli* birA strains were tested. In the more straightforward test strain BM4092 [33] was transformed with either plasmid pSKH001 or pSKH002 followed by selection for transformants by plating on LB supplemented with ampicillin or kanamycin, respectively. These strains were grown at 37°C on M9 minimal plates [34] with 25 mM 5-bromo-4-chloro-indolyl-D-galactopyranoside (X-gal) and varying concentrations of biotin (1.6 nM, 4.1 nM, 41 nM, 4.1 mM, or 41 mM) [33]. Note that gene expression from T7 promoter based multi-copy plasmids in the absence of T7 RNA polymerase has been shown to be equivalent to that of a single copy plasmid [35]. Derivatives of strain BM4092 expressing the mutant BirAs encoded by kanamycin resistant plasmids pSKH005, pSKH006, pSKH007, pSKH008, pSKH009, pSKH010 or pSKH011 were similarly obtained and tested.

Strain VC618 which carries a deletion of the chromosomal birA gene was transformed with pSKH012, pSKH013, pSKH014, pSKH015, pAKH016, pSKH017, or pSKH018 and transfor-
### Table 3. Oligonucleotides utilized.

| Oligo-nucleotide | Description | Sequence |
|------------------|-------------|----------|
| SKH001           | BirA F Ndel | GAGTGGCTGAACATATGCGGTCAAC |
| SKH002           | BirA stop R Xhol | GCCGTGACCCCTGAGTATGCCCAATTC |
| SKH005           | accB F Ncol | CCATGGAAAAGCAAGATGAGAATCTGCATAAA |
| SKH006           | accB stop R Xhol | CTGAGGTACTTCTTTCAATTTCAAGGAG |
| SKH007           | pyc F Ncol | CCATGGAAAAGCAAGATGAGAATCTGCATAAA |
| SKH009           | pyc stop R Xhol | CTGAGGTACTTCTTTCAATTTCAAGGAG |
| SKH014           | bioO F     | GATCTTTTCTTTATGAGACAGAAC |
| SKH015           | bioO R     | CGC CTTTCATGATAACTGAGAAC |
| SKH016           | yhfU F     | ACCATCAAAAAAGGCTGTCCGATAC |
| SKH017           | yhfU R     | CCAAAATGAATCAAATATGGTTTAC |
| SKH018           | yuiG F     | ATGATGCGGACTTCTTTGTT |
| SKH019           | yuiG R     | CTCCATGTGACATACACAA |
| SKH026           | EC bioO F  | GATATGCGGACTTCTTTGTT |
| SKH027           | EC bioO R  | CTTTCATGATAACTGAGAAC |
| SKH034           | bioO half F | GAAAGGACCGTTTTGTG |
| SKH035           | bioO half R | ATCCAAAGTTAAACATATTATATATTATCTTCTTGG |
| SKH028           | Non 125 F  | GCATGACGGTGACATACAAATGAGG |
| SKH029           | Non 125 R  | AACCGTCCGATTCATATTCGATTC |
| SKH036           | BirA 64-325 EcoRI F | ACTGCGAATTCGACATACAAATGAGG |
| SKH037           | BirA Sall R | CTTTCATGATAACTGAGAAC |
| SKH038           | BirA EcoRI F | CTGACGAATTCGACATACAAATGAGG |
| SKH040           | BirA 64-325 Ndel F | CAGTCATATGAAAACCCGAAAACCTCGTAAAGG |
| SKH041           | BirA BamHI R | ACTGCGAATTCGACATACAAATGAGG |
| SKH042           | EC BirA 65-321 EcoRI F | ACTGCGAATTCGACATACAAATGAGG |
| SKH043           | EC BirA Sall R | CTTTCATGATAACTGAGAAC |
| SKH044           | BirA 82-325 Ndel F | CTTTCATGATAACTGAGAAC |
| SKH045           | BirA 82-325 EcoRI F | ACTGCGAATTCGACATACAAATGAGG |
| SKH046           | BirA 66-325 EcoRI F | ACTGCGAATTCGACATACAAATGAGG |
| SKH047           | BirA 66-325 Ndel F | CTTTCATGATAACTGAGAAC |
| SKH048           | BirA 75-325 EcoRI F | ACTGCGAATTCGACATACAAATGAGG |
| SKH049           | EC BirA EcoRI F | ACTGCGAATTCGACATACAAATGAGG |
| SKH050           | BirA 75-325 Ndel F | CTTTCATGATAACTGAGAAC |
| SKH057           | bioW 500 F  | ACTGCGAATTCGACATACAAATGAGG |
| SKH058           | bioW 500 R  | GACGCGAATTCGACATACAAATGAGG |
| SKH065           | ycgB F     | CTTACAGAAGAGGCTTAAGAAGAAATAAAAAG |
| SKH066           | lacI R     | CGCTCTGCTGTAAGAAGAAATAAAAAG |
| SKH067           | ldh R      | CTTCGCTTTTCTTTCATGACTCAGTCCAA |
| SKH069           | RBS accb-86 F | ACCTGCGAAGAGGAAGAAATAAAAATGGAAGACCAAAACCCAGATG |
| SKH070           | accb-86 R  | GACGCGAATTCGACATACAAATGAGG |
| SKH073           | pDR111 F Seq | CTCCGAGTAAATATGAGAAGG |
| SKH074           | pDR111 R Seq | GAAATATCATATATGAAATGTTAATGACAGT |
| MM1              | 5' BioW F   | CGA TCC TTT CTT CTA TTG AGA ACA AAG |
| MM2              | LacZ R     | GGT GTA GAT GCC ATC GTA AC |
| MM3              | pSPAC F     | CTA CAC AGC CCA GTC CAG ACT ATT CAG |
| MM4              | 3' BioW R   | ATG GCG TCA TCT AGT TCT TTT TGG CCG |
mants were selected on LB plates supplemented with ampicillin and chloramphenicol at 30°C. These strains were cured of the temperature sensitive plasmid VC18 which expresses the *Sarcophago cerevisiae* Bpl1 ligase by growth at 42°C on the defined medium supplemented with chloramphenicol and biotin. Loss of the temperature-sensitive plasmid was indicated by ampicillin sensitivity [36]. These strains were then grown at 37°C on M9 minimal plates [34] with 25 μM X-gal and varying concentrations of biotin as above [33].

### Protein purification

For purification of the wild type and *B. subtilis* N-terminally deleted BirA Proteins *E. coli* strain BL21 (λ DE3) was transformed with pSKH001, pSKH019, pSKH020, pSKH021, or pSKH022. The strains were grown at 37°C in LB medium supplemented with ticarcillin-clavulanate to an OD600 of 0.8 and induced by addition of IPTG to 1 mM for an additional 6 h at 37°C. Cells were centrifuged and resuspended in lysis buffer which was 50 mM Tris-HCl, 500 mM NaCl, 0.1 mM tris(2-carboxyethyl)phosphine (TCEP), 10 mM imidazole, 5% glycerol, pH 8.0. The cells were lysed by passage through a French pressure cell and the lysate was centrifuged. The supernatant was added to Ni NTA beads (Qiagen) and incubated for 30 min. The mixture was added to a disposable 10 ml polypropylene column (Pierce) and washed with three column volumes of wash buffer (50 mM Tris-HCl, 500 mM NaCl, 0.1 mM TCEP, 60 mM imidazole, 5% glycerol, pH 8.0). BirA was eluted in 1 ml fractions with elution buffer (50 mM Tris-HCl, 500 mM NaCl, 0.1 mM TCEP, 250 mM imidazole, 5% glycerol, pH 8.0). Fractions were subjected to SDS-PAGE to determine purity. Pure fractions were combined and dialyzed against storage buffer (50 mM Tris-HCl, 500 mM NaCl, 0.1 mM TCEP, 5% glycerol, pH 8.0). Aliquots were flash frozen and stored at −80°C.

The *B. subtilis* acetyl-CoA carboxylase biotin carboxyl carrier protein biotin attachment domain (AccB-86) was purified from *E. coli* strain BL21 (λ DE3) transformed with pSKH005. The strain was grown at 37°C in LB medium supplemented with canamycin to an OD600 of 0.8 and induced by addition of IPTG to 1 mM for an additional 4 h at 30°C. The cells were centrifuged and resuspended in starting buffer (20 mM Tris-HCl, 1 mM NaCl, 0.1 mM TCEP, 5% glycerol, pH 8.0). The cells were lysed by passage through a French pressure cell. The lysates were centrifuged and the supernatant was subjected to 60% isopropanol precipitation followed by anion exchange chromatography using a HiTrap Q FF column (GE Healthcare) and fast liquid chromatography (AKTA) [37,38].

The *B. subtilis* pyruvate carboxylase biotin attachment domain (PyC-77) was purified from *E. coli* strain BL21 (λ DE3) transformed with pSKH004. The strain was grown at 37°C in LB medium supplemented with kanamycin to an OD600 of 0.8 and induced by addition of IPTG to 1 mM for an additional 4 h at 30°C. The cell were harvested by centrifugation and resuspended in starting buffer (50 mM sodium acetate, 100 mM NaCl, 0.1 mM TCEP, 5% glycerol, pH 4.9). The cells were lysed by passage through a French pressure cell and centrifuged. The supernatant was subjected to cation exchange chromatography using HiTrap SP FF column (GE Healthcare) and fast liquid chromatography (AKTA). PyC-77 was eluted by an NaCl gradient. Fractions were subjected to SDS-PAGE and fractions containing PyC-77 were combined and dialyzed against 50 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer (pH 7.5) containing 500 mM NaCl and 0.1 mM TCEP. To further purify PyC-77 the protein was subjected to Superdex-75 size exclusion chromatography. Eluted fractions were analyzed by SDS-PAGE to determine purity. Fractions containing pure PyC-77 were combined, dialyzed against 50 mM HEPES buffer (pH 7.5) containing 500 mM NaCl, 0.1 mM TCEP, and 5% glycerol, flash frozen and stored at −80°C.

Plasmid pQC026C a derivative of vector pET28b encoding a terminal hexahistidine tagged BLAP was transformed into *E. coli* strain BL21 (λ DE3). The strain was grown in LB medium to an OD600 of 0.8 and induced by addition of IPTG to 1 mM for an additional 4 h. BLAP was purified by Ni²⁺ affinity chromatography as previously described [23]. The C-terminal hexahistidine tagged ΔΔ-65 BirA was purified as previously described [17].

### Electrophoretic Mobility Shift Assays (EMSA) of DNA binding

The predicted *B. subtilis* BirA binding sites upstream of the coding sequences of bioO, yhfU and yuiG were PCR amplified from *B. subtilis* 168 genomic DNA with primers SKH014 and SKH015, SKH016 and SKH017 and SKH018 and SKH019, respectively. The PCR products were sized on a 1.8% agarose gel and purified using a QIAquick PCR Purification Kit (Qiagen). DNA concentrations were determined at OD260 by using a NanoDrop 2000c. Purified BirA was incubated with the small molecule hydroxylamine at neutral pH to cleave any Bio-5'-AMP bound in the active site [39] and then dialyzed against storage buffer. The reaction contained 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 50 mM NaCl, 10% glycerol, 40 nM DNA and various concentra-

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**Figure 3. Purification of the wild type and N-terminal deletion BirA proteins and the biotin acceptor proteins.** The proteins were purified as described in Materials and Methods and subjected to SDS-electrophoresis on a 4–20% polyacrylamide gel. M: molecular weight standards (Precision Plus Protein Standard Kaleidoscope from BioRad). Lane 1: *B. subtilis* N-terminally hexahistidine-tagged BirA (38.9 kDa), Lane 2-5: *B. subtilis* N-terminally hexahistidine-tagged ΔΔ-63 BirA (31.8 kDa), ΔΔ-65 BirA (31.6 kDa), ΔΔ-74 BirA (30.4 kDa) and ΔΔ-81 BirA (28.7 kDa), respectively. Lanes 6-11 are the *B. subtilis* acceptor proteins AccB-86 (9.4 kDa), PyC-77 (8.3 kDa) and birin lipoyl attachment protein (BLAP) (8.73 kDa). Lane 10 is *E. coli* C-terminal hexahistidine-tagged BirA. Lane 11 is *E. coli* C-terminal hexahistidine tagged ΔΔ-65 BirA (29.18 kDa) and lane 11 is *E. coli* AccB-87. doi:10.1371/journal.pone.0096757.g003
trations of BirA (500 nM, 250 nM, 125 nM, 62.5 nM, 31.25 nM, 15.6 nM), 1 mM ATP, 1 mM MgCl₂, and 1 μM biotin [36]. The binding reactions were incubated at room temperature for 30 min and then loaded into a 6% DNA retardation gel (Invitrogen). The gel was run in 0.5X TBE at 100 V for 85 min. The gel was stained with SYBR Green I nucleic acid gel stain (Invitrogen) and visualized using Bio-Rad Chemidoc XRS and Quantity One software.

Figure 4. Sequence alignments of B. subtilis BirA DNA binding sites and electrophoretic mobility shift assay of DNA binding by BirA. A. B. subtilis has three predicted BirA DNA binding sites: 5′ UTR of the bioWAFDBI operon, 5′ UTR of yuiG, and 5′ UTR of the yhfUTS operon. Conserved residues are highlighted in red and similar residues are highlighted in yellow. B. C. and D. B. subtilis BirA binding to bioO, the yuiG operator and the yhfU operator, respectively. Note that only in the presence of biotin and ATP is binding observed. E. Quantitation of DNA binding by BirA (Quantity One software). The results show the average of three independent experiments, and the error bars denote standard error of the mean. F. BirA binding to non-operator DNA (a 125 bp internal fragment of the yngHB gene that encodes BLAP). G. BirA binding to bioO without hydroxylamine treatment. Bio-5′-AMP accumulates in the active site during expression in E. coli and survives purification of BirA. H. B. subtilis BirA binding to a half site of the inverted repeat of B. subtilis bioO. Note lane 2 is positive control full-length bioO. I. B. subtilis BirA binding to E. coli bioO. A collection of all putative BirA binding sites in diverse bacteria can be found in the RegPrecise database (http://regprecise.lbl.gov/RegPrecise/).

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Chemical Cross-linking of BirA

Purified BirA was dialyzed against 50 mM HEPES buffer (pH 7.5) containing 500 mM NaCl, 0.1 mM TCEP and 5% glycerol. BirA (30 μM) was incubated with or without biotin (0.1 mM) and ATP (0.1 mM) at room temperature for 30 min. Various concentrations of ethylene glycol bis[succinimidylsuccinate] (EGS) (0.125, 0.25, 0.375, 0.5 mM) were added and incubated at room temperature for 30 min. SDS-loading dye was added and samples were heated to 99°C for 5 min. Samples were loaded on 4–20% gradient SDS polyacrylamide gels (Bio-Rad) and run at 110 v for 1 h.

Mass Spectrometry

Purified acceptor proteins AccB-86, PyC-77, and BLAP (10 μM), with or without incubation with BirA (0.5 μM), ATP (3 mM), and biotin (250 μM), were dialysed against 2 mM ammonium acetate, dried under a stream of nitrogen and subjected to electrospray mass spectrometric analysis [38].

Bio-5′-AMP Synthesis Assays

The assays contained 50 mM Tris-HCl buffer (pH 8.0), 5.5 mM MgCl₂, 100 mM KCl, 0.1 mM TCEP, 10 μM ATP, 25 μM biotin, 2.5 μM BirA, 0.1 μM [α-32P]ATP and with or without 50 μM AccB-86, PyC-77, or BLAP for a total reaction mixture of 20 μl [17,36]. The reaction mixtures were incubated at room temperature for 30 min. A portion of each reaction mixture (1 μl) was spotted on cellulose thin-layer chromatography (TLC) plates and developed in isobutyric acid-NH₄OH-water (66:1:33) [40]. The thin-layer chromatograms were dried for 10 h and exposed to a phosphorimaging screen and visualized using a Fujifilm FLA-3000 Phosphor Imager and Fujifilm Image Gauge software.

Figure 5. In vitro biotinylation of the *B. subtilis* biotin acceptor proteins. **A.** Sequence alignment of the *B. subtilis* biotinylated proteins. Conserved residues are in white text and highlighted in red and similar residues are in red text and boxed in blue. The black arrow indicates the conserved lysine residue that becomes biotinylated. **B.** Mass spectrometry values for purified acceptor proteins AccB-86, PyC-77, and BLAP. **C.** Thin layer chromatographic analysis of *B. subtilis* BirA ligase reaction; synthesis of Bio-5′-AMP and transfer of biotin to AccB-86, PyC-77, and BLAP. doi:10.1371/journal.pone.0096757.g005

Figure 6. Model of *B. subtilis* BirA based on the *S. aureus* BirA crystal structure (PDB 4DQ2) [7]. The UCSF Chimera package [31] was used to create the image. Residues corresponding to the N-terminal deletion end points are given. Modeled domains are indicated. doi:10.1371/journal.pone.0096757.g006
β-Galactosidase Assays

Cultures were grown overnight in defined medium containing 1.6 nM biotin. The cultures were diluted to an OD595 of 0.2 in defined media containing various concentrations of biotin (1.6, 4, 20, 40, 80, 400 nM, 4 μM) and grown to OD595 of 0.8 and induced with 1 mM IPTG for an additional 2 h. β-Galactosidase activity was determined as described by Harwood and Cutting for Bacillus following permeabilization with lysozyme [26].

Results

B. subtilis BirA is a Group II BPL that binds three operator sites

To test the relative affinities of the predicted B. subtilis BirA binding sites we purified the protein (Fig. 3) and performed electrophoretic mobility shift assays (EMSAs) on DNA fragments containing the three sites (Fig. 4A). Full dependence of binding on ATP and biotin required treatment of the protein with neutral hydroxylamine to remove Bio-5'-AMP accumulated in the active site during expression in E. coli. With the treated BirA binding of the biotin biosynthetic operator (bioO) was observed only in the presence of both biotin and ATP (Fig. 4B). BirA also bound the yhU and ywG operators only in the presence of biotin and ATP (Fig. 4C, D). Although the three binding sites have slightly different DNA sequences (Fig. 4A), analysis of binding over a range of BirA concentrations showed that the three sites had very similar binding affinities (Fig. 4E).

B. subtilis BirA did not interact with the E. coli bioO DNA site (Fig. 4I). B. subtilis BirA interacted only very weakly with the E. coli bioO DNA site (Fig. 4I).

B. subtilis BirA biotinylates three cognate proteins and the reactions proceed via Bio-5’-AMP

Each of the acceptor proteins, AccB-86, PyC-77 and BLAP (Fig. 5A), was purified after high-level expression in E. coli (Fig. 3). In the first two cases the N-terminal halves of the proteins were deleted to avoid protein aggregation during purification (the deleted segments are responsible for interaction with other proteins of the enzyme complexes and play no role in biotinyla-
Electrospray ionization mass spectrometry results matched the theoretical masses of the apo forms of the three proteins and showed that the preparations were free of the biotinylated forms (Fig. 5B). When the apo forms of AccB-86, PyC-77 and BLAP were incubated with ATP, biotin and purified *B. subtilis* BirA and subsequently analyzed by mass spectrometry, mass values very similar to the theoretical values for biotinylated forms of all three acceptor proteins were obtained (Fig. 5B). In the presence of α-32P-labeled ATP and biotin *B. subtilis* BirA formed labeled Bio-5′-AMP. Upon addition of any of the three acceptor proteins (AccB-
The EGS concentrations are in mM.

regulated by experimentally verified the bioinformatic analyses of the genes to each of the acceptor proteins (Fig. 5C). In conclusion, we have detected and AMP accumulated which indicated transfer of biotin activity.

86, PyC-77, or BLAP) the Bio-5'-AMP intermediate was no longer detected and AMP accumulated which indicated transfer of biotin to each of the acceptor proteins (Fig. 5C). In conclusion, we have experimentally verified the bioinformatic analyses of the genes regulated by B. subtilis BirA and the proteins modified by its ligase activity.

B. subtilis BirA does not require the N-terminal DNA binding domain for normal ligase activity

The in vitro data presented above indicate that B. subtilis BirA behaves in a manner that strongly parallels that of E. coli BirA despite the low sequence similarity of the two proteins. However, E. coli BirA has recently been shown to undergo extensive inter-domain communication that is required for full ligase activity [17]. These observations confirm and extend those of Xu and Beckett [16] and demonstrate that the E. coli BirA N-terminal domain plays a role in organizing the active site of BirA [17]. Specifically, the wing of the winged HTH structure interacts with the ligase active site biotin binding loop and acts to organize the active site to give high affinity binding of biotin and Bio-5'-AMP [17].

To determine if another Group II BPL, that of B. subtilis, requires such communication of the catalytic and N-terminal domains for full ligase activity, we constructed genes encoding several N-terminal B. subtilis BirA deletion proteins. The deletion endpoints were based on structural modeling of B. subtilis BirA based on the crystal structure of S. aureus BirA (PDB 4DQ2) [7] (Fig. 6). BirA deletions Δ2-63 and Δ2-65 eliminated the predicted N-terminal domain whereas BirA deletions Δ2-74 and Δ1-81 also cut into the predicted central catalytic domain. Complementation assays using the E. coli birA1 mutant strain BM4092 and the E. coli birAΔ1-81 deletion strain VC618 were used to test the ligase activities of the B. subtilis BirA N-terminally deleted proteins. These assays showed that upon expression of the B. subtilis Δ2-63 BirA, Δ2-65 BirA and wild type proteins in strain BM4092 all three BPLs supported growth equally well on medium containing 1.6 nM biotin (the minimal level allowing growth of E. coli) (Fig. 7A). These results indicated that the ligase activities of these two deletion proteins were essentially normal. In contrast, upon expression of the E. coli BirA lacking its amino terminus (Δ2-65 BirA), growth of the transformed strain required a biotin concentration that was 1000-fold greater. The complementation activities of the other two B. subtilis deletion proteins, BirA Δ2-74 and BirA Δ1-81 were either partially (BirA Δ2-74) or totally (BirA Δ1-81) compromised (Fig. 7A). To ensure that residual activity of the host BirA1 protein did not play a role in growth restoration we also performed complementation of the birA E. coli strain VC618 and obtained a similar complementation pattern (Fig. 7B).

The ligase activities of the purified B. subtilis BirA N-terminal deletion proteins (Fig. 3) were also tested by in vitro biotinylation assays. As expected from the complementation results the Δ2-63 and Δ2-65 BirA proteins showed Bio-5'-AMP synthesis and biotin transfer activities indistinguishable from those of the wild type protein (Fig. 8). In contrast the Bio-5'-AMP synthetic abilities of the Δ2-74 and Δ1-81 BirAs were significantly reduced relative to wild type BirA. However, the biotin transfer activity of the Δ2-74 BirA was comparable to wild type levels. Biotin transfer by the Δ1-81 BirA was significantly reduced compared to the wild type protein (Fig. 9). In agreement with prior work [16,17] the E. coli Δ2-65 BirA was significantly reduced in Bio-5'-AMP synthesis and in biotin transfer (Fig. 8).

Bacillus subtilis BirA dimerizes in the presence of biotin and ATP

Dimerization is required for DNA binding of E. coli BirA and dimerization is dependent on biotin and ATP binding. To determine the oligomerization state of B. subtilis BirA, purified

Figure 9. Chemical crosslinking of the B. subtilis wild type and Δ2-65 BirA proteins. A. Wild type BirA. B. The Δ2-65 BirA. Note that efficient dimer formation requires the presence of both biotin and ATP. The EGS concentrations are in mM.

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Figure 10. β-Galactosidase assays of the effect of AccB-86 levels on bioO-dependent transcription. The bioW:lacZ spo accB-86 strain 504002 was grown in defined medium supplemented with the indicated concentrations of biotin plus or minus IPTG addition to induce synthesis of the AccB-86 acceptor protein. The results are the average of three independent experiments and the error bars denote standard error of the mean.

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BirA was incuated with EGS in the presence or absence of biotin and ATP. Chemical crosslinking indicated that BirA forms a dimer only in the presence of both biotin and ATP (Fig. 9A). Dimerization was also observed for the N-terminally truncated Δ2-65 BirA (Fig. 9B). Note that the characterized Group I BPLs can either dimerize as is the Propionibacterium freudenreichii protein [3,41] or monomeric as are the Mycobacterium tuberculosis [4], Aquifex aeolicus [5] and Propionibacterium freudenreichii subsp. shermanii [42] BPLs.

**Bacillus subtilis** BirA follows the *E. coli* BirA regulatory model

In *E. coli* regulation of the biotin biosynthetic gene transcription depends not only on the concentration of biotin but also the levels of unbiotinylated AccB [8-10]. This is an important attribute because biotin is only active in central metabolism when it is protein bound. Hence, the rate of biotin operon transcription is sensitive not only to the intracellular concentration of biotin, but also to the supply of the proteins to which the biotin must be attached. Moreover, accumulation of the unmodified protein increases the rate of biotin synthesis thus ensuring replacement of the biotin consumed in protein modification.

To determine if this regulatory facet also applies to another Group II BPL, we constructed a *B. subtilis* strain that could be used to monitor regulation of biotin operon transcription upon alteration of the levels of the AccB-86 acceptor protein. This strain contains the *bioB141* mutation rendering it a biotin auxotroph, a *bioW-lacZ* transcriptional fusion and an ectopic IPTG-inducible gene encoding AccB-86. This strain was grown in chemically defined media containing various concentrations of biotin. Upon induction of AccB-86 expression with IPTG β-galactosidase assays showed that expression of the biotin operon was derepressed at biotin concentrations that normally cause repression (Fig. 10) in a manner similar to that seen in *E. coli* [8-10].

**Discussion**

*B. subtilis* BirA complements the ligase activity of *E. coli* BirA mutant strain BM4092, but fails to complement the regulatory function of *E. coli* BirA. This latter result is not surprising since *E. coli* and *B. subtilis* BirA bind different operator sequences and have different spacing of the palindromic elements. We have shown that *B. subtilis* BirA is a Group II BPL that binds equally well to the three operators predicted by others (Fig. 4). Binding is observed only in the presence of both biotin and ATP indicating that Bio-5′-AMP is the regulatory ligand. Interestingly, EMSA showed that BirA weakly binds *E. coli* bioO (Fig. 4E), suggesting some distant relationship between the operators. Similar to *E. coli*, dimerization of *B. subtilis* BirA occurs only in the presence of biotin and ATP, a further indication that Bio-5′-AMP is the regulatory ligand (Fig. 9A). The *B. subtilis* Δ2-65 BirA also formed dimers indicating that the N-terminal domain is not required for dimerization (Fig. 9B).

Deletions of the N-terminal DNA binding domain of *E. coli* BirA result in proteins having only very weak ligase activities indicating inter-domain interactions are required for full ligase activity [16,17]. These interactions have been shown to occur between the wing of the winged HTH domain and the biotin-binding loop of the catalytic domain. Deletion of only the fourteen-residue wing has as drastic an effect as deletion of the entire N-terminal domain, but was largely restored by insertion of a foreign wing of similar structure [17]. Moreover, a mutation within the wing can restore the biotin moiety normally suggesting that the putative α-helix near residue 74 may be involved in stabilizing biotin and ATP or Bio-5′-AMP binding. Although *B. subtilis* △1-81 BirA failed to replace the *E. coli* ligase in vivo (Fig. 7), it retained weak Bio-5′-AMP synthesis and biotin transfer activities (Fig. 8) suggesting that the loop predicted near residue 81 is required for efficient binding of biotin and ATP. These data indicate that unlike *E. coli*, *B. subtilis* BirA does not require an intact N-terminal DNA binding domain for full ligase activity. Instead, the first α-helix and loop of the modeled central domain seem to be important in binding biotin and ATP.

*E. coli* BirA is a highly dynamic protein as shown both by physical [43-45] and mutational [36] analyses. In the latter case mutations in the catalytic and C-terminal domains as well as in the HTH domain can result in super-repressor phenotypes. These proteins are BirAs that repress bio operon expression even at biotin concentrations that normally fail to repress transcription. Although *E. coli* BirA has been studied for over 30 years [40,46] and four different crystal structures have been solved, the details of how the protein binds its operator site remain unknown. Despite much effort in several laboratories no diffraction grade crystals of the operator DNA with liganded *E. coli* BirA have been obtained. This could be due to competing interactions of the N-terminal domain with the catalytic domain and operator giving a mixture of molecular species that preclude crystallization. If so, the apparent lack of such interactions in *B. subtilis* BirA may allow crystallization of this protein with its operator DNA.

The BLAP protein was biotinylated by *B. subtilis* BirA in vitro suggesting that BLAP may be the biotin carboxyl carrier protein for a biotin-dependent enzyme. The genes that neighbor *yngHB*, the BLAP encoding gene, are annotated as propionyl-CoA carboxylase subunits. However, a recent report suggests that these genes may comprise a methylcrotonyl-CoA carboxylase involved in leucine degradation [47].

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**Author Contributions**

Conceived and designed the experiments: SKH JEC. Performed the experiments: SKH. Analyzed the data: SKH JEC. Contributed reagents/materials/analysis tools: SKH JEC. Wrote the paper: SKH JEC.
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