Published in final edited form as:

Mol Microbiol. 2016 November ; 102(3): 417–429. doi:10.1111/mmi.13470.

The *Staphylococcus aureus* group II biotin protein ligase BirA is an effective regulator of biotin operon transcription and requires the DNA binding domain for full enzymatic activity

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SUMMARY

Group II biotin protein ligases (BPLs) are characterized by the presence of an N-terminal DNA binding domain that functions in transcriptional regulation of the genes of biotin biosynthesis and transport. The *Staphylococcus aureus* Group II BPL which is called BirA has been reported to bind an imperfect inverted repeat located upstream of the biotin synthesis operon. DNA binding by other Group II BPLs requires dimerization of the protein which is triggered by synthesis of biotinoyl-AMP (biotinoyl-adenylate), the intermediate in the ligation of biotin to its cognate target proteins. However, the *S. aureus* BirA was reported to dimerize and bind DNA in the absence of biotin or biotinoyl-AMP (Soares da Costa et al. (2014) *Mol Microbiol* 91: 110-120). These *in vitro* results argued that the protein would be unable to respond to the levels of biotin or acceptor proteins and thus would lack the regulatory properties of the other characterized BirA proteins. We tested the regulatory function of the protein using an *in vivo* model system and examined its DNA binding properties *in vitro* using electrophoretic mobility shift and fluorescence anisotropy analyses. We report that the *S. aureus* BirA is an effective regulator of biotin operon transcription and that the prior data can be attributed to artifacts of mobility shift analyses. We also report that deletion of the DNA binding domain of the *S. aureus* BirA results in loss of virtually all of its ligation activity.

Graphical abstract

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INTRODUCTION

Biotin protein ligase (BPL) is an essential enzyme that catalyzes covalent attachment of biotin to biotin-dependent enzymes in a two step reaction (Fig. 1A). In the first half reaction, BPL binds both biotin and ATP to synthesize biotinoyl-AMP (Bio-AMP) and pyrophosphate. The mixed anhydride bond of Bio-AMP then undergoes nucleophilic attack by the ϵ -amino group of the conserved lysine residue of the acceptor protein resulting in covalently attached biotin and free AMP (Fig. 1A). Microbial BPLs are classified into two groups. Both Group I and Group II BPLs have catalytic cores and C-terminal domains that are well conserved between the groups. Group II BPLs (generally called BirAs) are characterized by the presence of an N-terminal winged helix-turn-helix DNA binding domain that the Group I enzymes lack. *E. coli* BirA is the best studied example of how an enzyme can also act as a transcriptional regulator of the biotin biosynthetic operon (Beckett, 2007, Cronan, 2014). Transcriptional repression of the *E. coli* biotin operon occurs when biotin acceptor proteins have been fully biotinylated thereby allowing BirA to accumulate Bio-AMP in its active site. The presence of Bio-AMP results in BirA dimerization and subsequent DNA binding that represses transcription of the biotin biosynthetic operon (Fig. 1B). Derepression of the biotin operon transcription occurs upon low biotin levels (Barker & Campbell, 1980) (Fig. 1C) or increased levels of unmodified biotin acceptor proteins (Fig. 1D) (Abdel-Hamid & Cronan, 2007, Cronan, 1988, Solbiati & Cronan, 2010). Transfer of accumulated Bio-AMP to acceptor proteins results in monomeric BirA which is unable to bind the biotin operator. The BirA of the distantly related bacterium *Bacillus subtilis* closely follows the *E. coli* transcriptional regulation model (Henke & Cronan, 2014, Bower *et al.*, 1995).

The putative *S. aureus* BirA contains an N-terminal winged helix-turn-helix DNA binding domain. However, the protein was recently reported to dimerize in the absence of either biotin or Bio-AMP with a K_d of 29 μ M (Soares da Costa *et al.*, 2014), a concentration much lower than analogous preparations of *E. coli* BirA (Streaker *et al.*, 2002). Moreover, it was reported that *S. aureus* BirA bound the operator sequence in the absence of biotin and Bio-AMP leading to a question of the biological function of the protein. The reported electrophoretic mobility shift assays (EMSAs) showed that the unliganded *S. aureus* BirA bound the operator DNA with only a 6-fold lower affinity than the protein that bound the Bio-AMP regulatory ligand (K_d of 649 ± 43 nM vs. K_d of 108 ± 6 nM) (Soares da Costa *et*

al., 2014). This high level of DNA binding activity in the absence of biotin or Bio-AMP should preclude *S. aureus* BirA from being an effective regulator of biotin operon transcription since DNA binding would occur without regard for the cellular levels of biotin and Bio-AMP. Thus, even if the cellular concentration of biotin was low and engenders a requirement for biotin biosynthesis (and/or biotin transport), operator binding would result in transcriptional repression of the biotin biosynthesis operon (and of the biotin transporter, *bioY*). Therefore, the *S. aureus* BirA would hinder rather than expedite cellular responses to biotin deficiency. To test whether or not the *S. aureus* BirA had this seemingly perverse activity, we utilized the fact that the *S. aureus* BirA DNA binding sites *bioO* and *bioY* are very similar to that of the previously studied *B. subtilis* *bioO* operator (Fig. 2A). We replaced the well-characterized *B. subtilis* *birA* gene (Bower *et al.*, 1995, Henke & Cronan, 2014) with that encoding the *S. aureus* BirA and assayed regulation by the levels of biotin and acceptor protein. Since these experiments indicated that the *S. aureus* BirA was an efficient regulator *in vivo*, we reconsidered the EMSA results and utilized fluorescence anisotropy to test DNA binding in free solution.

Finally we tested the role of the *S. aureus* BirA DNA binding domain in the enzymatic activity of the protein. We expected that, like the *B. subtilis* protein (Henke & Cronan, 2014), deletion of the N-terminal domain would not affect ligase activity. To our surprise deletion of the DNA binding domain resulted in severely decreased ligation activity as was previously observed with *E. coli* BirA (Chakravarty & Cronan, 2013, Xu & Beckett, 1996).

RESULTS

***S. aureus* BirA binds the *B. subtilis* *bioO* operator and modifies the *B. subtilis* acceptor in vitro**

For simplicity we will call the *S. aureus* BPL SaBirA where SaBirA will be used in the formal sense as the primary translation product lacking biotin and Bio-AMP. The SaBirA form containing biotin is called SaBirA(btn), that containing ATP as SaBirA(ATP) and that containing Bio-AMP as SaBirA(Bio-AMP). Note that Mg⁺⁺ is always present in the latter two cases.

To assess the possibility that the *B. subtilis* *bioO* strain constructed previously (Henke & Cronan, 2014) could be used to test *in vivo* regulation by SaBirA, we asked if SaBirA is capable of binding *B. subtilis* *bioO*. The protein was purified (Supporting information Fig. S1) and electrophoretic mobility shift assays (EMSAs) were performed (Fig. 2B). SaBirA bound the *B. subtilis* *bioO* binding site in the presence of biotin and ATP [e.g., as SaBirA(Bio-AMP)] whereas low levels of DNA binding were observed for SaBirA and SaBirA(btn) samples which suggested that some of the protein had the biotinoyl-AMP intermediate bound in the active site. SaBirA(ATP) bound the operator almost as well as SaBirA(Bio-AMP) suggesting that the untreated, as purified BirA had biotin bound in the active site. This was surprising because biotin was reported to readily dissociate from SaBirA (Soares da Costa *et al.*, 2012). We also assayed SaBirA for the ability to biotinylate AccB-86, the C-terminal biotin accepting domain of *B. subtilis* AccB (Henke & Cronan, 2014) by transfer of biotin from Bio-AMP assayed by thin layer chromatography (Fig. 2C) and by mass spectral analysis of AccB-86 biotinylation (Supporting information Fig. S2).

The ability of SaBirA to function with the *B. subtilis* components indicated that the regulatory properties of the *S. aureus* protein could be tested with this system.

In vivo SaBirA responds to biotin and acceptor protein levels

To assay the regulatory properties of SaBirA we modified the *B. subtilis* strain used previously (Henke & Cronan, 2014) by replacement of the *B. subtilis* *birA* with the *S. aureus* *birA*. The strain also contained a *bioB141* mutation that results in biotin auxotrophy plus a *bioW-lacZ* fusion which allows measurement of *bio* operon transcription by β -galactosidase assays. The new strain was grown in defined media supplemented with kanamycin, erythromycin, licomycin and various concentrations of biotin. As the biotin concentration increased *bioW-lacZ* transcription decreased indicating SaBirA mediated transcriptional repression of the biotin operon in response to biotin levels (Fig. 3A). We also tested regulation of the biotin operon by SaBirA in response to the level of the *B. subtilis* AccB-86 acceptor protein. This required modification of the SaBirA-*B. subtilis* strain by introduction of an ectopic IPTG-inducible gene encoding the AccB-86 acceptor. This strain was grown as above with various concentrations of biotin. Upon induction of AccB-86 production β -galactosidase assays showed that expression of the biotin operon became derepressed at biotin concentrations that normally give repression (Fig. 3B). These results parallel those seen in *E. coli* and *B. subtilis* upon overproduction of acceptor proteins (Abdel-Hamid & Cronan, 2007, Chakravartty & Cronan, 2012, Cronan, 1988, Henke & Cronan, 2014, Solbiati & Cronan, 2010). Therefore, the SaBirA protein is a fully functional regulatory ligase which raised the question of how to explain the conflicting *in vitro* results reported by Soares da Costa and coworkers (Soares da Costa *et al.*, 2014)

In vitro DNA binding of *S. aureus* BirA to *S. aureus* bioO

The as purified SaBirA bound the *S. aureus* *bioO* probe in EMSA analysis without additions of biotin or ATP (Fig. 4A). The as purified SaBirA plus either biotin or ATP also bound the probe. This could not be attributed to non-specific DNA binding activity because a probe of unrelated sequence showed no shifts (Fig. 4A). These data indicated that the as purified SaBirA had acquired biotin from the *E. coli* host and converted at least some to Bio-AMP. To rid the protein of these ligands we incubated the as purified SaBirA with ATP and $MgCl_2$ to convert any biotin to Bio-AMP. We then treated the protein with neutral hydroxylamine to cleave any Bio-AMP to give AMP and biotinoyl hydroxamate. Following this treatment SaBirA no longer bound the operator DNA in the presence or absence of biotin (Fig. 4B). However DNA binding activity was still observed for the treated SaBirA in the presence of ATP, a surprising result in that biotin was shown to be required for SaBirA binding of ATP- Mg^{++} (Soares da Costa *et al.*, 2012). Incubation with an excess of the AccB-86 acceptor protein failed to prevent the ATP-dependent shift (Fig. 4C). These results argued that either biotin remained bound in the SaBirA active site or that it was not required for the observed mobility shift. We tested these hypotheses by assay of the protein samples for biotin using a sensitive bioassay that can detect as little as 1 pmol of biotin or (following hydrolysis) Bio-AMP.

Samples of the as purified and treated SaBirA were denatured and centrifuged. The supernatants were spotted on paper disks in sectorial minimal plates containing a redox

indicator and the *E. coli* biotin auxotroph NRD25 which has a complete deletion of the *bio* operon (Fig. 5). As expected the as purified SaBirA samples contained biotin (or the labile Bio-AMP which would be hydrolyzed to biotin in the protein denaturation step) (Fig. 5C). In contrast the treated SaBirA contained no detectable biotin or Bio-AMP even when large amounts of the protein were assayed (Fig. 5D). As a control we incubated the treated SaBirA with an equimolar concentration of biotin. Upon denaturation and bioassay the expected level of biotin was detected indicating that the protocol did not interfere with the release of biotin into the supernatant (Fig. 5B). The bioassays confirmed that incubation of SaBirA with ATP and MgCl_2 prior to neutral hydroxylamine treatment effectively removed biotin and Bio-AMP from the as purified SaBirA preparations. To differentiate between biotin and Bio-AMP being the bound species we utilized the inability of neutral hydroxylamine to react with the carboxyl group of free biotin. In the presence of ATP any biotin bound in the active site would be converted to Bio-AMP and upon treatment with neutral hydroxylamine would become biotin hydroxamate, which lacks activity in the bioassay due to its blocked carboxyl group. In contrast, in the absence of ATP biotin would be unaffected by neutral hydroxylamine and give activity in the bioassay. Bioassay of as purified SaBirA incubated plus or minus ATP and MgCl_2 followed by neutral hydroxylamine treatment and protein denaturation showed that the addition of ATP had no effect (Fig. 5 D & E). These results demonstrated that biotin was not bound in the active site and that the species bound was Bio-AMP. Hence our data agree with the reported rapid SaBirA off-rate for biotin (Soares da Costa *et al.*, 2012).

These data raised the question why the EMSA analyses detected DNA binding activity for SaBirA(ATP) when no biotin was present. One possibility was that binding was an artifact of the EMSA analyses (Cann, 1989, Cann, 1998, Fried & Crothers, 1981, Fried, 1989, Fried & Liu, 1994). To test this possibility we turned to a fluorescence anisotropy assay that allowed DNA binding to be measured in free solution. SaBirA, SaBirA(btn), SaBirA(ATP) and SaBirA(Bio-AMP) samples were prepared from the as purified protein as described in Experimental Procedures. These preparations were then assayed for DNA binding activity by EMSA using the buffer conditions given by Soares da Costa and coworkers (Soares da Costa *et al.*, 2014) (Fig. 4D). The DNA binding activities of four SaBirA protein samples were tested with a fluorescein end-labeled 33 base pair *S. aureus bioO* binding site. The changes in anisotropy were plotted against the protein concentrations to obtain binding curves and the data were analyzed using the non-linear curve fitting function of GraphPad Prism 4 to calculate K_d values (Fig. 6). The SaBirA and SaBirA(ATP) samples had very high K_d values of $5.284 \pm 0.23 \mu\text{M}$ and $7.841 \pm 1.1 \mu\text{M}$, respectively, values that differed greatly from the SaBirA K_d value of $649 \pm 43 \text{ nM}$ reported from EMSA data by Soares da Costa and coworkers (Soares da Costa *et al.*, 2014). Unexpectedly, SaBirA(btn) had an appreciably lower binding K_d of $182.8 \pm 10.97 \text{ nM}$. As expected, SaBirA(Bio-AMP) sample had the lowest K_d . A value of $83.1 \pm 4.2 \text{ nM}$ was found which is similar to that previously derived from EMSA data (Soares da Costa *et al.*, 2014). Note that in the case of *E. coli* BirA, a major component of the 2000-fold difference in operator binding of the BirA:Bio-AMP complex versus the unliganded protein is that the unliganded BirA has essentially no dimerization ability (equilibrium dimerization constant of 1–2 mM) (Streaker *et al.*, 2002). In contrast, unliganded SaBirA has a reported equilibrium dimerization constant of 29 μM

(Soares da Costa et al., 2014). If unliganded *E. coli* BirA had the dimerization ability of SaBirA, a rough calculation suggests that the difference in binding affinities of the unliganded and Bio-AMP liganded proteins would be comparable to those of SaBirA and SaBirA(Bio-AMP).

SaBirA requires N-terminal domain sequences for full ligase activity

E. coli BirA was recently shown to require specific interdomain interactions for full ligase activity (Chakravartty & Cronan, 2013). The wing of the winged HTH structure interacts with the biotin binding loop of the ligase active site and acts to organize the active site to give high affinity binding of biotin and Bio-AMP (Chakravartty & Cronan, 2013). Unlike *E. coli*, *B. subtilis* BirA does not require an intact N-terminal DNA binding domain for full ligase activity (Henke & Cronan, 2014). We previously modeled the *B. subtilis* BirA structure on the SaBirA BirA crystal structure (PDB 4DQ2) because among the biotin ligase proteins of known structure that protein had highest amino acid sequence identity to *B. subtilis* BirA (31%). From the modeling we expected that deletions of the SaBirA N-terminal domain would retain ligase activity, as did N-terminal domain deletion derivatives of the *B. subtilis* protein (Henke & Cronan, 2014). To test this expectation we constructed N-terminal deletions of three different lengths as previously done for *B. subtilis* BirA (Henke & Cronan, 2014). SaBirA deletion 2-65 eliminated the N-terminal domain whereas SaBirA deletions 2-74 and 1-81 cut into the central domain (Fig. 7).

Complementation assays using the *E. coli birA1* strain BM4092 (Barker & Campbell, 1980) were used to test the ligase activity of the three SaBirA N-terminal deletion proteins. All three N-terminally deleted *S. aureus* BirAs failed to restore ligase activity to the *E. coli* mutant strain at low biotin concentrations (Fig. 8A). However at higher biotin concentrations the 2-65 construct allowed markedly better growth than the empty vector indicating that the protein was expressed and stable. To determine if, like *E. coli* BirA, the wing of the HTH structure was important for active site organization, we constructed a 48-63 wing deletion and tested the activity of this protein by complementation of the *E. coli birA* mutant strain. The wing deletion protein failed to complement ligase activity at low biotin concentrations, although at higher biotin concentrations expression of the protein largely restored ligase activity (Fig. 8B). Similar results were seen with the *E. coli* BirA wing deletion derivative (Chakravartty & Cronan, 2013). As expected from the differing operator sequences, the blue colonies formed by the strain with wild type SaBirA indicated that the protein was unable to regulate *E. coli bio* operon transcription.

DISCUSSION

The BirA proteins of *E. coli* and *B. subtilis* provide a simple, yet sophisticated, regulatory system that monitors both intracellular biotin supply and the levels of enzyme proteins that require biotinylation for activity (Abdel-Hamid & Cronan, 2007, Beckett, 2007, Cronan, 2014, Henke & Cronan, 2014). This is accomplished by use of the intermediate in the ligase reaction, Bio-AMP, as the regulatory ligand. Only when the active site is occupied by Bio-AMP do the proteins efficiently dimerize and acquire the ability to bind their cognate operator sequences. Soares da Costa and coworkers (Soares da Costa *et al.*, 2014) reported

the surprising result that SaBirA dimerizes and binds its operator in the absence of substrates/ligands with an affinity only 6-fold lower than the affinity of the Bio-AMP complex. This is in contrast to the *E. coli* BirA where the operator binding affinity of the unliganded protein is 2000-fold lower than the Bio-AMP complex as measured by nuclease foot-printing (Streaker *et al.*, 2002). Such sensitive methods have yet to be applied to *B. subtilis* BirA, but the available data argue that the Bio-AMP complex has an affinity for its operators at least 50-fold greater than the unliganded protein. Physiologically the ability of unliganded SaBirA to bind its operator would result in decreased *bio* operon transcription and transport even when biotin was limiting. Moreover, the cell would be unable to respond to unmodified acceptor protein levels and central metabolism would be compromised. Indeed, the unliganded protein could behave much as a dominant negative mutant protein by blocking binding of SaBirA(Bio-AMP) to the operator.

Our *in vivo* data demonstrate that this perverse scenario does not occur. SaBirA is a competent regulatory protein that responds to both biotin supply and the levels of unmodified acceptor protein (Fig. 3). These results raised the question of how the results of Costa and coworkers (Soares da Costa *et al.*, 2014) could be rationalized. The short answer is that EMSA analysis of interaction of SaBirA with its operator is rife with artifacts. For example we found that ATP-Mg⁺⁺ elicits operator binding in the absence of biotin (Fig. 4) although surface plasmon resonance measurements indicate that the protein cannot bind ATP-Mg⁺⁺ under these conditions (Soares da Costa *et al.*, 2012). The reported binding of unliganded SaBirA to its operator (Soares da Costa *et al.*, 2012) also seems an artifact of their EMSA conditions since we could detect no binding in our EMSA analyses (Fig. 4) and only very weak binding by fluorescence anisotropy (Fig. 6).

Although EMSA analyses are often considered straightforward, it is well documented that EMSA conditions can stabilize interactions that are weak in solution (Fried, 1989, Fried & Liu, 1994). Three overlapping mechanisms have been put forth to rationalize such results. These are interactions with the gel matrix that stabilize protein-DNA affinities, the cage effect and sequestration. The latter two mechanisms have received experimental verification. The cage effect is the inability of dissociated molecules to diffuse sufficiently far away from one another that reassociation is prevented (Cann, 1989, Cann, 1998). This effect has been demonstrated for LacI repressor-operator interactions (Fried & Crothers, 1981, Fried, 1989). Sequestration is the decreased solution volume accessible to the protein and DNA which increases the effective concentrations of the components thereby promoting interaction. Sequestration has been demonstrated for interaction of the *E. coli* cyclic AMP receptor protein with its binding site on the Lac promoter (Fried & Liu, 1994). The opposite effects have also been reported. The *E. coli* tryptophan (TrpR) repressor binds its operator at pH 8.3 as shown by nuclease foot-printing although no gel shift was seen at this pH (Carey, 1988).

The binding measurements we obtained in free solution accurately reflect the mechanism of the regulatory system *in vivo*. Indeed, the behavior of the regulatory system *in vivo* must be the litmus used to judge the relevance of *in vitro* measurements. The binding curves obtained by fluorescence anisotropy indicated that SaBirA and SaBirA(ATP) bind DNA much more weakly than SaBirA(Bio-AMP). However, SaBirA(btn) binding was comparable to that of SaBirA(Bio-AMP) showing only a modest decrease in DNA binding affinity. This

interesting result suggested that biotin alone may be sufficient for dimerization and DNA binding and play a role in the regulatory mechanism. However, the finding that high level production of an acceptor protein derepresses transcription at high biotin concentrations argues against this suggestion because SaBirA(Bio-AMP) is required for protein modification. We view the SaBirA(btn) dimerization as facilitating Bio-AMP synthesis and dimerization by raising the local concentration of biotin-bound monomer and structuring the ATP binding site. Efficient operator binding in the presence of biotin has not been observed for *E. coli* BirA. Relative to the Bio-AMP complex the biotin complex binds the operator 100-fold (Prakash & Eisenberg, 1979) or 146-fold (Streaker *et al.*, 2002) less tightly. Much of this difference is due to the weak dimerization elicited by biotin binding (Streaker *et al.*, 2002). In EMSA analyses neither *B. subtilis* BirA (Henke & Cronan, 2014) nor SaBirA (Fig. 4) showed significant operator binding in the presence of biotin. A possible explanation is that due to its charge biotin may electrophorese away from the protein.

Contrary to our expectations deletion of SaBirA N-terminal domain sequences gave results that paralleled those of the analogous *E. coli* BirA deletions (Xu & Beckett, 1996, Chakravartty & Cronan, 2013) rather than those of the more highly sequence and taxonomically related *B. subtilis* BirA (Henke & Cronan, 2014). Therefore, SaBirA like *E. coli* BirA requires interdomain communication for full ligase activity. It would be interesting to compare the regulatory efficiencies of the *S. aureus* and *B. subtilis* BirAs under conditions in which the proteins had the same level of expression and could bind its cognate operator.

EXPERIMENTAL PROCEDURES

Strains, plasmids, chemicals and culture media

All *B. subtilis* strains were derivatives of strain 168 whereas the *E. coli* strains were derivatives of strains B and K-12 (Supporting information Table S1). The plasmids used and constructed are given in (Supporting information Table S2). The rich medium for growth of *E. coli* and *B. subtilis* was LB broth. The defined medium for *E. coli* was M9 salts supplemented with 0.5% glucose or 0.5% glycerol and 0.01% vitamin-free Casamino Acids (Difco) whereas the defined medium for *B. subtilis* was Spizizen salts supplemented with 0.5% glycerol and 0.05% vitamin-free Casamino Acids (Difco) plus 0.01% each of tryptophan, tyrosine, isoleucine and phenylalanine. Antibiotics were used at the following concentrations (in µg/ml): kanamycin sulfate, 50; chloramphenicol, 25; erythromycin sulfate, 1; lincomycin, 12.5 and spectinomycin, 100. Oligonucleotides were purchased from Integrated DNA Technologies. PCR amplification was performed using Phusion high fidelity DNA polymerase (New England BioLabs) according to manufacturer protocols. DNA constructs were sequenced by ACGT, Inc. Reagents and chemicals were obtained from Sigma-Aldrich and Fisher, unless otherwise noted. New England BioLabs supplied restriction enzymes and T4 DNA ligase. Life Technologies provided SYBER Green I Nucleic Acid Gel stain and the 6% DNA Retardation Novex TBE Gels.

Plasmid constructions

The *S. aureus* *birA* gene was amplified by PCR from *S. aureus* strain Newman genomic DNA with primers SKH076 and SKH077 (Supporting information Table S3) that contained

PciI and XhoI sites. The product was digested with PciI and XhoI and ligated into the NcoI and XhoI sites of pET28b resulting in plasmid pSKH025 that encoded the protein with a C-terminal hexahistidine tag.

The 500 bp immediately downstream of *B. subtilis* *birA* was PCR amplified from *B. subtilis* strain 168 genomic DNA using primers SKH102 and SKH103 (Supporting information Table S3) that, respectively, contained SalI and XhoI sites. The product was digested with SalI and XhoI and ligated into plasmid pDG780 digested with the same enzymes to give pDG780-panB. The last 500 base pairs of *B. subtilis* *cca* was PCR amplified from *B. subtilis* strain 168 genomic DNA with primers SKH104 and SKH105 (Supporting information Table S3) that contained a BamHI site and an overlapping *S. aureus* *birA* sequence, respectively. *S. aureus* *birA* was PCR amplified from *S. aureus* strain Newman genomic DNA with primers SKH106 that contained the *B. subtilis* *cca* overlap and SKH107 that contained an EcoRI site. The last 500 base pairs of *B. subtilis* *cca* and *S. aureus* *birA* were assembled by overlap PCR using primers SKH104 and SKH107 (Supporting information Table S3). The product was digested with BamHI and EcoRI and ligated into pDG780-panB digested with the same enzymes to give pSKH031.

The plasmids encoding the SaBirA N-terminal domain deletions were constructed as follows. *S. aureus* wild type *birA*, 2-65 *birA*, 2-74 *birA*, and 2-81 *birA* constructs were amplified by PCR from *S. aureus* strain Newman genomic DNA using forward primers SKH076, SKH078, SKH079, SKH080, respectively, plus reverse primer SKH081. The primers contained PciI and SalI sites, respectively. The products were digested with PciI and SalI and ligated into the NcoI and SalI sites of pBAD322Cm, to give pSKH026, pSKH027, pSKH028, and pSKH029 respectively. *S. aureus* 48-63 *birA* was amplified by PCR from *S. aureus* strain Newman genomic DNA with forward primer SKH076 containing a PciI site and reverse primer SKH097 which contained an overlapping sequence and forward primer SKH098 which contained the complement of the SKH097 overlap and reverse primer SKH080 containing a SalI site. The two fragments were fused by overlap extension PCR using primers SKH076 and SKH080 (Supporting information Table S3). The product was digested with PciI and SalI and ligated into the NcoI and SalI sites of pBAD322Cm, to give pSKH030.

***Bacillus subtilis* strain constructions**

B. subtilis competent cell preparation and transformation were carried out as described by Dubnau and Davidoff-Abelson (Dubnau & Davidoff-Abelson, 1971). Strains SKH001 and SKH002 were transformed with either linearized pSKH026 to replace *B. subtilis* *birA* with *S. aureus* plus a kanamycin cassette to give strains SKH003 and SKH004, respectively. The integration event was verified by PCR and sequencing of the PCR product with primers SKH134 and SKH135.

Purification of *S. aureus* BirA

E. coli BL21 Star (DE3) was transformed with pSKH025. The strain was grown at 37°C in LB medium supplemented with kanamycin to an OD₆₀₀ of 0.8 and expression was induced by addition of IPTG to 1 mM. Following growth for an additional 12 h at 30°C the cells

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were recovered by centrifugation and suspended in lysis buffer. Lysis buffer was 50 mM HEPES, 250 mM NaCl, 0.1 mM tris(2-carboxyethyl)phosphine (TCEP), 10 mM imidazole and 5% glycerol (pH 7.5). The cells were lysed by passage through a French pressure cell and the lysate was centrifuged to remove unbroken cells and cellular debris and the supernatant was added to Ni NTA beads (Qiagen) and incubated for 30 min before adding to a disposable 10 ml polypropylene column. The column was washed with three column volumes of wash buffer (lysis buffer containing 60 mM imidazole). BirA was eluted in 1 ml fractions with elution buffer (lysis buffer containing 250 mM imidazole). The fractions were analyzed by SDS-PAGE (Supporting information Fig. S1) to determine purity. Pure fractions were combined and dialyzed against storage buffer (lysis buffer lacking imidazole). Aliquots were flash frozen and stored at -80°C . The SaBirA and SaBirA(ATP) samples were prepared by incubation of a 1:1 molar ratio of ATP and protein plus 1 mM MgCl_2 to convert biotin to Bio-AMP. The SaBirA(btn) sample was prepared by incubating purified protein with a 1:1 molar ratio of biotin and 1 mM MgCl_2 to ensure any ATP in the solution would be converted to Bio-AMP and could be removed by treatment with 0.2 M neutral hydroxylamine followed by extensive dialysis against storage buffer to remove hydroxylamine. SaBirA was also prepared by incubating the as purified protein with a 5-fold molar excess of purified *B. subtilis* AccB-86 plus a 2-fold molar excess of ATP and 1 mM MgCl_2 . The protein was then purified using Qiagen Ni-NTA spin columns. Purification of *B. subtilis* AccB-86 was performed as previously described (Henke & Cronan, 2014).

Electrophoretic Mobility Shift Assay (EMSA) of DNA binding

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The *B. subtilis* BirA DNA binding site upstream of *bioO* was PCR amplified from *B. subtilis* 168 genomic DNA with primers SKH014 and SKH015. Negative control DNA (*blap*) was amplified from *B. subtilis* 168 genomic DNA with primers SKH028 and SKH029. The *S. aureus* BirA binding site upstream of *bioO* was PCR amplified from *S. aureus* strain Newman genomic DNA with primers SKH099 and SKH100. All DNA fragments were 125 bp in length. 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 OD_{260} by using a NanoDrop 2000c. The DNA binding reaction contained 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 50 mM NaCl, 10% glycerol, 40 nM DNA, the indicated concentrations of BirA, 1 mM ATP, 1 mM MgCl_2 , and 1 μM biotin or were modified to the solution conditions used by Soares da Costa and coworkers (Soares da Costa *et al.*, 2014). These were 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10% glycerol, 40 nM DNA, indicated concentrations of BirA, 5 μM ATP, 1 mM MgCl_2 , and 5 μM biotin (Fig. 4D). The binding reactions were incubated at room temperature for 30 min and then loaded into a 6% DNA retardation gel. The gel was run in 0.5X TBE buffer at 100 V for 1 hour and 25 min. The gel was stained with SYBR Green I nucleic acid gel stain and visualized using Bio-Rad Chemidoc XRS and Quantity One software.

Thin layer chromatographic biotinylation assay

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The *in vitro* biotinylation assays were performed as previously reported (Henke & Cronan, 2014). Reactions contained 50 mM Tris-HCl buffer (pH 8.0), 5.5 mM MgCl_2 , 100 mM KCl, 0.1 mM TCEP, 10 μM ATP, 25 μM biotin, 2.5 μM BirA, 0.1 μM [α - ^{32}P] ATP and with or without 50 μM AccB-86 for a total reaction mixture of 20 μl . The reaction mixtures were

incubated at room temperature for 30 min. A portion of each reaction mixture (1 μ l) was spotted on cellulose thin-layer chromatography (TLC) plates and developed in isobutyric acid-NH₄OH-water (66:1:33) (Prakash & Eisenberg, 1979). 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.

β -Galactosidase assays

SKH003 was grown overnight in defined media containing 1.6 nM biotin, kanamycin, erythromycin and lincomycin. SKH004 was grown overnight in defined media containing 1.6 nM biotin, kanamycin, erythromycin with lincomycin, and spectinomycin. Cultures were diluted to OD₅₉₅ of 0.2 in defined media containing various concentrations of biotin (4, 20, 40, 80, 400 nM) and grown to an OD₅₉₅ of 0.8 and then induced with 1 mM IPTG for an additional 2 h. β -Galactosidase activity was determined as described by Harwood and Cutting (Harwood & Cutting, 1990) following permeabilization of the cells with lysozyme.

Biotin Bioassays

E. coli strain NRD25 (Choi-Rhee & Cronan, 2005) was grown overnight in defined media supplemented with chloramphenicol and 1 nM biotin. The cells were recovered by centrifugation at 15,000 $\times g$ for 5 min and washed 4 times with 1 ml of M9 medium. The cells were suspended in 1 ml of glucose M9 medium and sub-cultured into 100 ml of glucose M9 minimal media lacking biotin and containing 5 units of avidin. These cultures were incubated at 37°C for 5 h, centrifuged at 15,000 $\times g$ for 5 min and the cell pellets washed 5 times with 1 ml of M9 medium. The cells were then suspended in 1 ml M9 and added to 150 ml of minimal media agar containing the redox indicator 2,3,5-triphenyl tetrazolium chloride (0.1%, w/v) (Lin *et al.*, 2010). Six ml of the agar mixture was added to sectorized petri dishes. A sterile 6 mm paper disc was placed on the top of the agar and spotted with 10 μ l of biotin standards or the supernatants of denatured protein samples. The protein samples were denatured by heating to 99°C for 20 minutes and the supernatants were obtained by centrifugation at 15,000 $\times g$ for 5 min. The supernatants were collected and used to spot the paper discs. The pmol values given were calculated using the protein concentrations prior to denaturation. The plates were incubated at 30°C overnight. Growth of strain NRD25 was visualized as a deposit of red formazan.

Assay of DNA binding by fluorescence anisotropy

The sense strand of the 33 base pair *S. aureus* BirA binding site upstream of *bioO* was synthesized by IDT to include a 5'-fluorescein-label. The labeled sense strand (SKH162) and unlabeled antisense strand (SKH163) oligonucleotides were suspended in annealing buffer (10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂ and 1 mM DTT) and annealed by mixing 1:1 molar ratio and heating to 95°C for 5 minutes followed by slow cooling. The annealed DNA was purified by PAGE to remove single stranded DNA. The DNA concentration was determined at OD₂₆₀ by using a NanoDrop 2000c. DNA binding reactions contained 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10% glycerol, 2 nM DNA, and various concentrations of BirA (10 nM to 40 μ M). The SaBirA(btn) samples contained 200 μ M biotin, SaBirA(ATP) samples contained 400 μ M ATP and 1 mM MgCl₂ and the BirA(Bio-AMP) samples contained 200 μ M biotin, 200 μ M ATP and 1 mM MgCl₂. Buffer containing

the fluorescein-labeled DNA was added to a black 96 well microplate (Molecular Devices). The indicated amount of a BirA sample was added to the wells in a final reaction volume of 20 μ l. Reactions were incubated at room temperature for 30 minutes. Anisotropy values were measured using the fluorescence polarization function of the Analyst HT Plate Reader (Molecular Devices) at the High-throughput Screening Facility, School of Chemical Sciences at the University of Illinois. GraphPad Prism 4 software was used for analysis of DNA binding.

Complementation tests

Complementation of the *E. coli birA1* strain BM4092 (Barker & Campbell, 1980) transformed with plasmids pSKH26, pSKH27, pSKH28, pSKH29 or pSKH30 was tested as described previously (Henke & Cronan, 2014). The strains were grown at 37°C on glycerol-M9 minimal plates (Miller, 1972) containing chloramphenicol and various concentrations of biotin (4 nM, 40 nM, 400 nM) (Barker & Campbell, 1980). Strain BM4092 carrying pSKH30 was also grown with 25 μ M 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (X-gal).

DNA sequence alignments

Sequence alignments were obtained using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) and the output was processed by ESPript 3.0 (<http://esprict.ibcp.fr/ESPript/cgi-bin/ESPript.cgi>) to generate the final figure (Gouet *et al.*, 2003).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health Grant AI15650 from the National Institute of Allergy and Infectious Diseases. We thank Dr. Thomas Kehl-Fie for providing the *S. aureus* genomic DNA. We thank Dr. Chen Zhang, Director of the High-Throughput Screening Facility at The University of Illinois, for help with troubleshooting and operating the Analyst HT plate reader for the fluorescence anisotropy experiments.

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Abbreviated Summary

A prior report argued that the BirA biotin protein ligase of *Staphylococcus aureus* would be a defective sensor of synthesis and attachment of the vitamin, biotin. We report that this protein is a fully functional sensor of biotin metabolism and that the prior report was due to artifacts of the *in vitro* assays utilized.

Figure 1. The biotin protein ligase reaction and the current model of *E. coli* BirA regulation
 (A) BirA binds both biotin and ATP to synthesize biotinoyl-AMP (Bio-AMP) with release of pyrophosphate. The mixed anhydride bond of Bio-AMP then undergoes nucleophilic attack by the ϵ -amino group of the conserved lysine residue of the acceptor protein resulting in covalently attached biotin and free AMP. (B) Repression by BirA. Sufficient biotin is present that essentially all acceptor protein (AccB) is biotinylated. (C) Derepression by limited biotin results in low levels of Bio-AMP such that BirA is unable to dimerize and exert repression. (D) Derepression by an excess of AccB acceptor protein consumes the Bio-AMP resulting in low levels that preclude BirA dimerization and operator binding. Green ovals denote BirA, tailed blue ovals are AccB, black dots represent biotin, and black dots connected to small red pentagons denote biotinoyl-adenylate (Bio-AMP).

Figure 2. *S. aureus* BirA binds *B. subtilis* *bioO* and modifies *B. subtilis* AccB-86
(A) Sequence alignments of *B. subtilis* *bioO*, *S. aureus* *bioO* and *S. aureus* *bioY* DNA binding sites. The SaBirA DNA binding sites are almost identical to the *B. subtilis* *bioO* binding site. Only a single base of each *B. subtilis* *bioO* half-site differs from those of both *S. aureus* operators. Conserved nucleotides are in white text and highlighted in red. Half site binding regions are indicated with black arrows. (B). EMSA showing *S. aureus* BirA binding to *B. subtilis* *bioO*. Note the small shifts seen for SaBirA and SaBirA(btn) samples versus the large shift seen for the SaBirA(ATP) sample. (C) In vitro biotinylation thin layer chromatographic assay of biotin transfer from *S. aureus* BirA to the *B. subtilis* acceptor protein AccB-86. Transfer is detected by the production of AMP (Fig. 1A) plus consumption of ATP.

Figure 3. *S. aureus* BirA responds to biotin and apo acceptor protein levels

(A) β -Galactosidase assays of the effects of biotin concentration on *bioO*-dependent transcription. The biotin requiring strain was grown in defined medium supplemented with the indicated concentrations of biotin. Error bars denote SEM. (B) β -Galactosidase assays of the effect of *B. subtilis* AccB-86 expression on *bioO*-dependent transcription. The strain was grown in defined medium supplemented with the indicated concentrations of biotin in the presence or absence of IPTG. Note the increase of some biotin concentrations in panel B. Error bars denote SEM.

Figure 4. Electrophoretic Mobility Shift Assay of SaBirA BirA DNA binding to *S. aureus bioO* (A) SaBirA binding to *S. aureus bioO* (lanes 1-5) or to a nonspecific DNA sequence (lanes 6-10). (B) SaBirA was incubated with ATP and then treated with neutral hydroxylamine to remove any Bio-AMP intermediate. (C) SaBirA was incubated with ATP and *B. subtilis* AccB-86 to remove any biotin or Bio-AMP from the active site. (D) SaBirA was incubated with either biotin (lanes 2-5) or ATP (lanes 7-10) and then treated with neutral hydroxylamine to remove any intermediate formed. Note the binding conditions were modified to those used by Soares da Costa and coworkers (Soares da Costa *et al.*, 2014) (the composition of the gels they utilized was not reported).

Figure 5. Treatment with hydroxylamine effectively removes Bio-AMP from SaBirA
 (A) Biotin standards. (B) Recovery of biotin from a mixture of biotin and hydroxylamine-treated SaBirA. (C) The as purified SaBirA contains either biotin or Bio-AMP. (D and E). After incubation without (D) or with ATP plus $MgCl_2$ (E) the samples were treated with neutral hydroxylamine followed by hydroxylamine removal. The protein samples were then denatured and the supernatants assayed for biotin. In the absence of ATP any bound biotin present would retain its free carboxyl group and be active in the bioassay whereas the biotin moiety of Bio-AMP (either formed from bound biotin in the presence of ATP or bound to the as purified protein) would be inactive in the bioassay due to its blocked carboxyl group. The lack of bioassay activity in the sample lacking ATP indicates the absence of bound biotin. Hence, the bound biotin must be in the form of Bio-AMP. Excepting the biotin standards plate the pmol values given correspond to the amount of protein denatured.

Figure 6. Binding isotherms of SaBirA binding to *S. aureus* *bioO* obtained by fluorescence anisotropy

(A) SaBirA(Bio-AMP) gave a dissociation constant of 83.1 ± 4.2 nM. (B) SaBirA(btn) gave a dissociation constant of 182.8 ± 10.97 nM. (C) SaBirA gave a dissociation constant of 5.284 ± 0.23 μ M. (D) SaBirA(ATP) gave a dissociation constant of 7.841 ± 1.1 μ M. Note the differing abscissa protein concentrations.

Figure 7. *S. aureus* BirA N-terminal deletions

The wing that was deleted (48-63) is shown in cyan. The end point of N-terminal deletion 2-65 is shown in yellow. The end point of N-terminal deletion 2-74 is shown in red whereas that of N-terminal deletion 2-81 is shown in orange. The image was created from PDB file (4DQ2) using UCSF Chimera (Pettersen *et al.*, 2004)

Figure 8. Abilities of expressed *S. aureus* BirA and its N-terminal deletion derivatives to support growth of *E. coli birA1* strain BM4092

(A) Strains were grown on glycerol M9 minimal media with biotin (4 nM, 40 nM or 400 nM). (B) Strains were grown on glycerol M9 minimal media with biotin (4 nM, 40 nM or 400 nM) and X-gal. Note that the arabinose promoter was left uninduced in order to give a low level of expression consistent with the low expression of BirA proteins. The blue color indicates derepressed transcription of the *bioF-lacZ* fusion indicating that *S. aureus* wild type BirA cannot complement *E. coli* BirA regulatory function.