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The Atypical Occurrence of Two Biotin Protein Ligases in Francisella novicida Is Due to Distinct Roles in Virulence and Biotin Metabolism

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ABSTRACT

The physiological function of biotin requires biotin protein ligase activity in order to attach the coenzyme to its cognate proteins, which are enzymes involved in central metabolism. The model intracellular pathogen Francisella novicida is unusual in that it encodes two putative biotin protein ligases rather than the usual single enzyme. Francisella novicida BirA has a ligase domain as well as an N-terminal DNA-binding regulatory domain, similar to the prototypical BirA protein in E. coli. However, the second ligase, which we name BplA, lacks the N-terminal DNA binding motif. It has been unclear why a bacterium would encode these two disparate biotin protein ligases, since Francisella novicida contains only a single biotinylated protein. In vivo complementation and enzyme assays demonstrated that BirA and BplA are both functional biotin protein ligases, but BplA is a much more efficient enzyme. BirA, but not BplA, regulated transcription of the biotin synthetic operon. Expression of bplA (but not birA) increased significantly during Francisella novicida infection of macrophages. BplA (but not BirA) was required for bacterial replication within macrophages as well as in mice. These data demonstrate that Francisella novicida has evolved two distinct enzymes with specific roles; BplA possesses the major ligase activity, whereas BirA acts to regulate and thereby likely prevent wasteful synthesis of biotin. During infection BplA seems primarily employed to maximize the efficiency of biotin utilization without limiting the expression of biotin biosynthetic genes, representing a novel adaptation strategy that may also be used by other intracellular pathogens.

IMPORTANCE

Our findings show that Francisella novicida has evolved two functional biotin protein ligases, BplA and BirA. BplA is a much more efficient enzyme than BirA, and its expression is significantly induced upon infection of macrophages. Only BplA is required for Francisella novicida pathogenicity, whereas BirA prevents wasteful biotin synthesis. These data demonstrate that the atypical occurrence of two biotin protein ligases in Francisella novicida is linked to distinct roles in virulence and biotin metabolism.

Biotin protein ligases are highly conserved and metabolically essential enzymes that catalyze attachment of the biotin coenzyme (vitamin H) to key protein subunits (or domains) of enzymes of central metabolism (1, 2) (Fig. 1A). Bacterial biotin protein ligases fall into two groups. Group I ligases act solely as biotin attachment enzymes, whereas group II ligases (generally called BirA proteins) are bifunctional proteins that regulate biotin synthesis in addition to catalyzing biotin attachment. The two enzyme groups are readily distinguished by the presence of an N-terminal winged helix-turn-helix domain that facilitates the binding of BirA proteins to the operator sequences of biotin synthetic operons and subsequent repression of transcription and biotin synthesis (1, 3). The group II ligases lack this DNA binding domain and show structural diversity. For example, Bacillus subtilis BirA can be converted to a fully functional group I ligase by deletion of the winged helix-turn-helix domain (3), whereas similar E. coli BirA N-terminal deletions result in ligases of severely compromised activity (4, 5). DNA binding by BirA proteins requires biotinoyl-AMP (biotinoyl-adenylate), the product of the first ligase half reaction (Fig. 1A). The fact that these proteins make their own regulatory ligand allows biotin synthesis to be regulated by both the intracellular biotin concentration and the levels of proteins that are the substrates for biotin attachment (Fig. 1B to D) (2, 6). The two modes of transcriptional derepression act by a common mechanism in that both decrease the levels of the BirA–biotinoyl-AMP complex required to bind the bio operator (7). Biotinylated enzymes are rare, as mammals have only four such proteins and Escherichia coli has only a single biotinylated protein. Typically, an organism (e.g., a mammal, plant, or bacterium) encodes a single biotin protein ligase that modifies each of the biotin-requiring enzymes. Francisella novicida is a Gram-negative bacterium and model
intracellular pathogen that is a rare cause of human disease (8), often used as a surrogate for the category A select agent Francisella tularensis. Our recent studies showed that F. novicida virulence requires biotin synthesis (9). The biotin operons of F. novicida and E. coli have the same gene arrangement, although in both bacteria, the enzymes (BioJ and BioH, respectively) that catalyze the last step in synthesis of the biotin pimelate moiety are encoded outside the operons (see Fig. S1 in the supplemental material) (9).

F. novicida differs markedly from E. coli and other bacteria in that it encodes two putative biotin protein ligases. These are FTN_0811, a group II candidate ligase of 320 residues encoded next to the last gene of the biotin operon (albeit divergently transcribed) and FTN_0568, a gene located far from the biotin operon that encodes a group I biotin protein ligase of 260 residues (Fig. 2). The longer protein, which we call BirA, has an N-terminal domain that is readily modeled as a winged helix-turn-helix regulatory domain, whereas the shorter protein, which we name BplA (biotin protein ligase A), lacks this domain (Fig. 2). These observations raised the questions of whether both genes encode active ligase enzymes and, if so, what the physiological rationale for the presence of the unique pair of biotin protein ligases in F. novicida is. We report that both genes encode functional biotin protein ligases, although BplA is a much more robust enzyme than BirA. BplA is required for pathogenesis, whereas BirA appears to function to prevent wasteful biotin synthesis.

RESULTS
Both bplA and birA encode active biotin protein ligases. F. novicida BplA and BirA were first assayed for ligase activity by the ability to permit growth of E. coli strain BM4062 at low biotin concentrations. The high biotin requirement of strain BM4062 is due to a point mutation (birA85) that results in a temperature-sensitive growth phenotype, decreased affinity for biotin, and deficient regulation of biotin operon transcription (10, 11).

FIG 1 Biotin protein ligase reaction and regulation of E. coli biotin operon transcription. (A) Biotin protein ligase activity of BirA and BplA. (B to D) General model of bio operon regulation by BirA. Pink ovals, BirA; tailed blue ovals, AccB; green dots, biotin; green dots with red pentagons, bio-5′-AMP (biotinoyl-5′-adenylate). Panel B shows the transcriptionally repressed state, whereas panels C and D separately show the two modes of derepression of bio operon transcription engendered by either biotin limitation or excess unbiotinylated AccB acceptor protein. Both derepression modes act by decreasing the level of biotinoyl-AMP, the ligand required for operator binding by BirA.
strain also contains a bioF::lacZ fusion, which results in biotin auxotrophy and provides a visual assay of bio operon transcription (10).

The F. novicida genes were expressed from an arabinose-inducible (p araBAD) promoter in the presence or absence of arabinose. Both proteins allowed growth of strain BM4062 at non-permissive temperatures and at low biotin concentrations. However, at low biotin concentrations, growth of the strain expressing F. novicida BirA required arabinose induction, whereas the strain expressing F. novicida BplA grew well under conditions (glucose in place of arabinose) that fully repress basal transcription from the p araBAD promoter (Fig. 3) (12). These data argued either that BplA was a much more active ligase than BirA or that BplA was more readily translated in E. coli. To address these possibilities, we constructed and expressed hexahistidine-tagged versions of the two proteins, and upon denaturing gel electrophoresis of crude extracts, we found that BirA and BplA were expressed at very similar levels (data not shown). Thus, the lack of complementation of the E. coli birA strain observed at low biotin concentrations seemed unlikely to be due to poor expression of F. novicida BirA and hence strongly suggested that BplA was a much more robust ligase than BirA.

As expected from its lack of a DNA binding domain, BplA expression had no effect on regulation of the E. coli biotin operon (assayed by X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside] hydrolysis) (Fig. 3). However, an unexpected finding was that expression of F. novicida BirA weakly repressed transcription of the E. coli bio operon (Fig. 3). This was most clearly seen upon arabinose induction of F. novicida BirA expression in the presence of 40 nM biotin (Fig. 3A) but was also seen in the absence of induction (the tiny white colonies formed in the presence of 40 nM biotin) (Fig. 3B).

To further compare the two ligases, we purified the hexahistidine-tagged proteins to homogeneity (Fig. 4; also, see Fig. S2A and B in the supplemental material), and peptide mapping showed that each contained a significant number of the tryptic peptides predicted from the DNA sequence (see Fig. S2C and D), thereby confirming the identification of the proteins. Cross-linking with ethylene glycol bis-succinimidylsulfamate indicated that BplA was monomeric in solution (see Fig. S2E), whereas BirA formed a mixture of monomers and dimers (see Fig. S2F; dimers were also seen during purification). An attempt to detect formation of BplA-BirA mixed multimers by cross-linking gave no support for this notion (see Fig. S2G).

The purified hexahistidine-tagged proteins (Fig. 4A) were used to compare the F. novicida BplA and BirA ligases to the well-studied E. coli BirA. We used a thin-layer-chromatographic method that assays conversion of α-[32P]-labeled ATP and biotin to biotinoyl-AMP (Fig. 4B). This provides a direct assay of the first ligase partial reaction (Fig. 1A) and upon addition of acceptor protein provides an indirect assay of the second ligase partial reaction, transfer of biotin from biotinoyl-AMP to the acceptor protein (Fig. 1A). Addition of acceptor protein results in loss of the biotinoyl-AMP spot with the concomitant appearance of AMP and consumption of ATP (Fig. 4B). The increased consumption of ATP results from the fact that in the absence of acceptor, biotinoyl-AMP remains tightly bound within the ligase active site such that only one molecule is formed per molecule of ligase (i.e., biotinoyl-AMP synthesis is not catalytic). Biotin transfer to an acceptor protein allows catalysis, which results in increased conversion of ATP to AMP.

In these assays, the most striking difference between BplA and BirA was that upon addition of the acceptor protein, BplA (and also E. coli BirA) consumed most of the α-[32P]-labeled ATP,
whereas no appreciable increase in ATP consumption was seen in the *F. novicida* BirA assay (Fig. 4B). This indicates that *F. novicida* BirA catalyzes biotin attachment more slowly than the other two ligases. Moreover, residual biotinoyl-AMP was seen only in the case of *F. novicida* BirA (Fig. 4B). The *F. novicida* BirA reactions also accumulated an appreciable amount of ADP, an off-pathway product. ADP production has been previously observed only in reactions with mutant *E. coli* BirA proteins having compromised ligase activity (4). However, mass spectrometry showed that given a long incubation time, *F. novicida* BirA, like BplA and *E. coli* BirA, could catalyze the full ligase reaction (Fig. 4C to F).

**F. novicida contains only a single biotinylated protein.** One possible rationale (albeit unprecedented) for the presence of two biotin protein ligases in *F. novicida* would be that the enzymes could specifically biotinylate different acceptor proteins. The *Francisella* species genome annotations list only a single protein as containing a canonical site for biotin attachment, the AccB subunit of acetyl coenzyme A (acetyl-CoA) carboxylase. These annotations seem reliable because the gene is located immediately upstream of a gene that encodes another acetyl-CoA carboxylase subunit, AccC (biotin carboxylase), and this gene arrangement is found in many bacteria, including the well-characterized accB-accC operon of *E. coli* (13, 14). Moreover, the *Francisella* AccB proteins include a readily modeled “thumb” structure, which is essential for *E. coli* AccB function (15) and constitutes a motif allowing small biotinylated proteins to be identified as subunits of acetyl-CoA carboxylase rather than of another biotin-dependent enzyme (16). Note that in *E. coli* (and the other bacteria tested), accB is an essential gene. Moreover, its biotinylation is essential for acetyl-CoA carboxylase-catalyzed synthesis of malonyl-CoA, the
indispensable building block of fatty acid synthesis which cannot be provided by supplementation of growth media.

Notwithstanding the annotations, it remained possible that Francisella species encode a biotinylated protein that lacks a recognizable biotin attachment sequence. To test this possibility, we performed Western blots of extracts of three F. novicida strains using a streptavidin probe and detected only a single biotinylated protein in these extracts (see Fig. S3 in the supplemental material).

FIG 4 Enzymatic activities of the F. novicida BplA and BirA proteins. (A) SDS-PAGE analysis of the proteins used in these experiments. M, molecular mass standards. The SDS-PAGE results are consistent with the calculated molecular masses of the hexahistidine-tagged versions of E. coli BirA, F. novicida BirA, and F. novicida BplA, which are 36.1, 38.7, and 32.5 kDa, respectively. (B) TLC assays of biotin protein ligase activities. The synthesis of $^{32}$P-labeled biotinoyl-AMP (biotinoyl-adenylate) from $[^\alpha-32P]$ATP was assayed as described in Materials and Methods, analyzed by thin-layer chromatography on glass cellulose plates, and visualized by autoradiography. The reaction products biotinoyl-5′-AMP (bio-5′-AMP), ADP, AMP, and the ATP are indicated in the left margin. The proteins assayed are given at the top of the figure. The abilities of the F. novicida proteins to convert $[^\alpha-32P]$ATP to biotinoyl-AMP and subsequently to AMP were tested by addition of the AccB-87 acceptor protein. (C to F) Low-resolution matrix-assisted laser desorption/ionization analyses of the overall conversion of apo-AccB-87 to the biotinylated species catalyzed by the various ligases in overnight incubations as shown on the panels. The calculated masses of apo-AccB-87 and biotinylated AccB-87 are 9,333.8 and 9,560.1, respectively.
Consistent with its annotation, this protein had the characteristic and atypical SDS gel mobility first seen for E. coli AccB (13). Although according to amino acid sequencing the E. coli AccB protein is 16.7 kDa, it migrates as though it is considerably larger (ca. 20 to 22 kDa). This anomalous migration is attributed to the extended alanine/proline-rich sequences spanning residues 40 to 70 (13). Hence, F. novicida encodes two enzymes to modify a single acceptor protein. The protein extracts assayed were from F. novicida mutant strains in which the gene encoding either BplA or BirA was disrupted by insertion of a kanamycin resistance cassette or from a mutant lacking the biotin synthetic enzyme BioJ as a control (4). Biotinylated AccB was present in all three extracts, although the band in the strain lacking BplA was considerably fainter than that in the strain lacking BirA (see Fig. S3). Note that E. coli also grows well with only a fraction of its normal level of biotinylated AccB (13, 15). These data together with the enzymatic assays and E. coli complementation data demonstrate that BplA is the major F. novicida biotinylase enzyme, although BirA suffices for growth in the laboratory at some cost in growth rate (see below; also, see Fig. S5 in the supplemental material).

**F. novicida BirA binds the E. coli bio operon.** The surprising result that expression of F. novicida BirA weakly repressed transcription of the E. coli bioBFCD operon (Fig. 3) argued that the protein must bind the E. coli bioO operator (Fig. 1). Indeed, sequences related to the E. coli operator sequence are found within the Francisella bioA-bioB intragenic regions (see Fig. S4A in the supplemental material), suggesting that these two divergent bacteria might share some BirA-operator interactions. This was tested by electrophoretic mobility shift assays, which showed that F. novicida BirA bound the minimal E. coli bio operator (see Fig. S4C), although markedly less tightly than its cognate operator (see Fig. S4D). This result is in accord with the results of Fig. 3 and with reverse transcriptase PCR analyses (data not shown), which showed only a 3- to 5-fold repression of E. coli bioBFCD transcription upon high level expression of F. novicida BirA. In contrast, E. coli BirA failed to bind the F. novicida BirA (see Fig. S4F), although the BirA preparation bound its cognate operator (see Fig. S4E). The inability to bind the F. novicida operator is expected, because several of the E. coli operator bases shown to interact with E. coli BirA in previous DNA footprinting experiments are absent (17). As expected, BplA showed no binding of the F. novicida sequence (see Fig. S3B).

**F. novicida BirA represses bioF expression.** Since BirA, but not BplA, bound the cognate bioO operator in vitro, we tested the in vivo role of each protein in transcriptional regulation of biotin synthesis in F. novicida. We generated deletion mutants lacking either bplA or birA and quantified expression of the representative biotin synthesis gene bioF by quantitative real-time PCR (qRT-PCR) (Fig. 5). The levels of bioF expression were similar in the wild-type and ΔbplA strains but significantly increased in the ΔbirA strain (Fig. 5). Complementation of the ΔbirA mutation with a wild-type copy of the gene restored bioF expression to the wild-type level. These data indicate that BirA acts as a transcriptional repressor of bioF expression in F. novicida, whereas BplA lacks repressor activity.

**BplA contributes to F. novicida replication in minimal medium and in macrophages.** Given that biotin protein ligases are essential enzymes of central metabolism, we tested the physiological contributions of BplA and BirA to F. novicida growth. In a defined minimal medium (Chamberlain’s medium [CHBI]), the ΔbplA strain exhibited a modest growth defect relative to the wild-type strain (see Fig. S5A in the supplemental material). In contrast, the ΔbirA strain grew as well as the wild-type strain, as did the ΔbplA and ΔbirA complemented strains (see Fig. S5A). Moreover, addition of biotin rescued the growth defect of the ΔbplA strain (see Fig. S5B). These data indicate that BplA plays a more important role than BirA in F. novicida growth in minimal medium. This is consistent with its superior biotin ligase activity in E. coli (Fig. 3) and in vitro (Fig. 4).

We hypothesized that the contribution of BplA to F. novicida growth in minimal medium would be reflected during macrophage infection, where the bacteria must traffic through the nutrient-limited host cell phagosome. To test this premise, we infected murine bone marrow-derived macrophages with the wild-type and deletion strains and quantified levels of intracellular bacteria at 5.5 h postinfection. In contrast to wild-type bacteria, which readily replicated within these macrophages, the ΔbplA mutant strain replicated poorly and was present at roughly 10-fold-lower levels (Fig. 6A). Complementation of the ΔbplA mutation with a plasmid expressing the wild-type gene restored wild-type levels of replication. The birA mutant, however, replicated similarly to the wild-type strain (Fig. 6A), indicating that BirA is not required for F. novicida intracellular replication. These data indicate that BplA makes a much more significant contribution to F. novicida replication than does BirA. Furthermore, bplA but not birA transcripts were up-regulated during macrophage infection (Fig. 6B), further indication of a significant role for BplA.

**BplA is required for F. novicida virulence in mice.** Since BplA was required for F. novicida replication in macrophages, a process thought to be required for replication in vivo, we tested if BplA was similarly required for virulence in mice. Mice were infected subcutaneously with the wild-type strain, the ΔbplA mutant strain, or the ΔbirA mutant strain. At 48 h postinfection, the ΔbplA mutant strain was present at significantly lower levels than the wild-type strain in the skin (6-fold), spleen (27-fold), and liver (39-fold) (Fig. 7). In contrast, the ΔbirA mutant strain was not significantly attenuated compared to the wild-type strain. Taken together, these data demonstrate the strong contribution of BplA to F. novicida virulence, as well as its inability to be replaced by BirA. Hence, the two enzymes play distinct roles in F. novicida physiology.
DISCUSSION

We investigated why *F. novicida* encodes two genes encoding putative biotin protein ligases. This seemed a wasteful extravagance given its small genome (ca. 40% that of *E. coli* K-12). The low sequence conservation between the two ligases (27% identical residues) argues that neither protein evolved from the other. Indeed, virtually all of the residues conserved between the two proteins are those common to all biotin protein ligases. Both *F. novicida* proteins align almost as well with *Bacillus subtilis* BirA as they do with one another (data not shown). The location of the *birA* gene at the end of the *bioBFCD* operon (see Fig. S1 in the supplemental material) argues that *birA* would have been the first biotin ligase to appear in a *Francisella* ancestor, given that there is no regulatory or enzymatic reason that the protein must be encoded at that location. If so, then BplA would have been a later acquisition, perhaps to facilitate pathogenesis. However, the sequences encoding the two proteins give no evidence for recent acquisition; both genes have the low G+C content characteristic of *Francisella* genomes.

BplA, the main *F. novicida* biotinylation enzyme, is required for growth in nutrient-limiting environments (see Fig. S5A in the supplemental material), replication in mouse macrophages (Fig. 6A), where its expression is up-regulated (Fig. 6B), and *in vivo* virulence (Fig. 7). These data provide further evidence of the link between bacterial metabolism and *F. novicida* virulence. Given that BplA does the “heavy lifting” in AccB biotinylation, what is the physiological role of BirA? BirA plays no obvious role in pathogenesis and is a remarkably poor ligase, as is evident from its inability to support growth of the *E. coli* BirA ligase mutant strain at low biotin concentrations (Fig. 3) and its poor enzymatic activity *in vitro* (Fig. 4). Given the presence of the robust BplA ligase, the retention of BirA in *F. novicida* and several other *Francisella* species argues that its physiological role is likely to regulate biotin synthesis and thereby prevent wasteful synthesis of this coenzyme. Synthesis of a biotin molecule by the *E. coli* pathway consumes 15 ATP equivalents, and thus, unconstrained biotin synthesis could exert a significant metabolic cost in *Francisella* (which seems very likely to use the same pathway as *E. coli*). In bacteria that have only a BirA ligase (e.g., *E. coli*), *bio* operon transcription responds to both biotin limitation and increased supply of apo (unbiotinylated)-AccB acceptor protein (Fig. 1). As noted above, the two derepression modes act to decrease the levels of the BirA–biotinoyl-AMP complex required to bind the *bio* operator. However, the presence of BplA argues that in *F. novicida*, regulation of *bio* operon transcription by the supply of apo-AccB would not take place, because the very active BplA would modify AccB and thereby short circuit this mode of regulation. Hence, *F. novicida* BirA likely primarily functions to monitor the intracellular concentration of biotin and would perform this task only at high intracellular biotin concentrations, because only then could it form the key regulatory ligand, biotinoyl-AMP. The poor affinity of BirA for biotin seems to be an advantage, in that it would prevent the regulatory system from starving the BplA ligase.

FIG 6  *F. novicida* BplA is required for replication in macrophages. (A) Murine bone marrow-derived macrophages were infected with a 20:1 MOI of wild-type *F. novicida* U112 (WT) and the Δ*bplA*, Δ*birA*, *bplA* complemented (comp), and *birA* complemented (comp) strains. CFU were quantified at 5.5 h postinfection. (B) Murine BMDM macrophages were infected with wild-type *F. novicida* U112 (MOI 20:1), and expression of *bplA* and *birA* was quantified by qRT-PCR relative to expression of the housekeeping gene *uvrD* at 30 min, 1 h, and 2 h postinfection (hpi). ***, P < 0.0005.

FIG 7  *F. novicida* BplA is required for replication in mice. Mice were infected subcutaneously with 1 × 10^6 CFU of wild-type *F. novicida* U112 (WT) or the Δ*bplA* or Δ*birA* strain. At 48 h postinfection, skin samples obtained at the site of infection as well as the spleen and liver were harvested, and CFU were enumerated after plating. ***, P < 0.0005.
for biotin. The finding that one of the highly virulent *F. tularensis* strains (strain SchuS4) encodes a full-length BirA but an inactive truncated BirA indicates that BirA is sufficient to provide requisite biotin ligase activity to support growth. It also suggests that highly efficient biotin ligase activity in the absence of biotin operon repression during infection by this intracellular pathogen may optimally promote bacterial virulence. This may be a paradigm employed more broadly by diverse intracellular pathogens.

**MATERIALS AND METHODS**

**Strains and growth conditions.** All *E. coli* strains were derivatives of the wildtype K-12 strain (see Table S1 in the supplemental material) and were routinely maintained in LB medium (Luria-Bertani medium containing 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl per liter) or on LB agar plates. The defined M9 minimal medium contained 0.1% vitamin-free Casamino Acids and either 0.4% glucose or 0.2% arabinose. Antibiotics were supplemented as needed (micrograms per milliliter): sodium ampicillin, 100; tetracycline HCl, 10; and kanamycin sulfate, 50. Due to deletion of the araD gene, strain BM4062 is sensitive to arabinose due to accumulation of toxic ribulose-5-phosphate. To allow full induction of the plasmid paraBAD promoter that drives expression of the *F. novicida* ligases we repaired the ΔaraD mutation by phase P1 transduction of BM4062 with a lysate of strain CAG12095 with selection for tetracycline resistance, followed by screening for growth on arabinose as the sole carbon source.

To generate the *F. novicida* bplA and *birA* deletion mutants, PCR was used to amplify flanking DNA regions upstream and downstream of the gene of interest. A kanamycin resistance cassette was inserted between these flanking regions using Gibson assembly (New England Biolabs) and transformed into chemically competent wild-type strain U112 as previously described. The primers used to create the kanamycin-resistant deletion mutants contained Flp recombinase target (FRT) sites flanking the kanamycin resistance cassette, which allowed a clean deletion of each mutant to be made using the plasmid pFlpF, encoding the Flp recombinase, as previously described (9). The *bplA* and *birA* strains were complemented in trans by ligation of the genes into the EcoRI and BamHI sites of the broad-host-range vector pBAV1K-T5-GFP. The resulting plasmids were transformed into the clean *bplA* and *birA* deletion mutant strains, respectively.

**Ligase plasmids and DNA manipulations.** The two putative biotin protein ligase-encoding genes *bplA* ([FTN_0568](http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00591-15/-/DCSupplemental)) and *birA* ([FTN_0811](http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00591-15/-/DCSupplemental)) were amplified by standard PCR genomic DNA of *F. novicida* U112 using Phusion high-fidelity DNA polymerase (New England Biolabs). Following gel purification of the *bplA* and *birA* PCR products, they were each inserted into the medium-copy-number, arabinose-inducible expression vector pBAD322 (18) using XmaI and SphI digestions to give plasmids pBAD322-*bplA* and pBAD322-*birA*, respectively (see Table S1 in the supplemental material). Similarly, the genes were also inserted into the T7 promoter expression vector PET28(a) by use of BamHI and XhoI digestion (see Table S2 in the supplemental material), resulting in pET28-*bplA* and pET28-*birA* (see Table S1). All constructs were verified by DNA sequencing.

**Bio-5′-AMP synthesis reactions.** The assay for ligase-catalyzed in vitro protein biotinylation activity was performed as described previously (4), with some modifications. Protein concentrations were determined using the extinction coefficients calculated from the protein sequence using the ExPASY Tools website. The assays contained 50 mM Tris-HCl (pH 8), 5 mM tris-(2-carboxyethyl)phosphine, 5 mM MgCl₂, 20 μM biotin, and 5 μM ATP plus 16.5 mM [α-32P]ATP, 100 mM KCl, and 2 μM ligase. Each of the reaction mixtures was incubated at 37°C for 30 min. For each ligase protein tested, two identical tubes were incubated in parallel, and after the 30-min incubation, AccB-87 (50 μM) was added to one of each pair of tubes, while the other tube was left untreated. The tubes were incubated for an additional 15 min at 37°C. One microliter of each reaction mixture was applied to an Analtech Avicel microcrystalline cellulose thin-layer-chromatography plate, and the plates were developed in isobutyric acid-NH₂ OH-water (66:1:33 by volume) (19). The chromatograms were dried for 10 h, exposed to a phosphorimaging plate, and visualized using a Fujifilm FLA-3000 PhosphorImager and Fujifilm Image Gauge software (version 3.4 for Mac OS).

**Analyses of in vitro BirA biotin attachment activity.** Low-resolution matrix-assisted laser desorption/ionization (MALDI) was used to measure the level of ligase-catalyzed biotinylation of AccB-87 as previously reported (20). Reaction mixtures contained 100 μM AccB-87, 3 μM ligase, 100 μM biotin, 1 mM ATP, 10 mM MgCl₂, 100 mM KCl, 5 mM tris-(2-carboxyethyl)phosphine in 50 mM Tris-HCl (pH 8.0) were incubated at 37°C for 16 h, dialyzed against 25 mM ammonium acetate, lyophilized to dryness, and submitted for MALDI analyses.

**Growth of *F. novicida***. Bacteria were subcultured to an optical density of 600 nm (OD₆₀₀) of 0.03 in Chamberlain’s medium (CHB). Subcultures were read hourly using a SynergyMx BioTek plate reader (Applied Biosystems) for 24 h. Biotin (50 nM) (Merck KGaA) was added when appropriate.

**Quantitative real-time PCR.** RNA was isolated from mid-log-phase broth cultures or macrophages infected with wild-type *F. novicida* U112 (multiplicity of infection [MOI], 20:1) at various time points to TRI reagent and column purification with a Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA). Quantitative real-time PCR (qRT-PCR) was performed using the Power Sybr green RNA-to-CT one-step kit (Applied Biosystems). Relative transcript levels were calculated by normalizing C_T values to DNA helicase II (*uwvD* and *FTN_1594*) and plotted as 2^{-ΔΔC_T}.

**Macrophage infections.** Murine bone marrow-derived macrophages (BMDM) were prepared as described previously (22, 23). Briefly, bone marrow was collected from the femurs of mice. Bone marrow cells were plated in sterile petri dishes and incubated in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS)– and 15% macrophage colony-stimulating factor (M-CSF)-conditioned medium. Bone marrow cells were incubated at 37°C with 5% CO₂ and harvested after 6 days. All BMDM were incubated before and during infection in 24 well plates at 37°C with 5% CO₂. For infection, BMDM were seeded at 5 × 10⁶ cells per well and incubated overnight at 37°C with 5% CO₂. BMDM were infected at a multiplicity of infection (MOI) of 20:1. At 5.5 h postinfection, macrophages were lysed with 1% saponin. Lysates were serially diluted in phosphate-buffered saline (PBS) and plated onto TSA containing 0.1% cysteine to enumerate CFU.

**Mouse infections.** For mouse infections, female C57BL/6 mice (6 to 8 weeks old) (Jackson Laboratory, Bar Harbor, ME) were housed under specific-pathogen-free conditions at Emory University. Experimental studies were performed in accordance with the Institutional Animal Care and Use Committee guidelines. Mice were infected subcutaneously with 2 × 10⁶ CFU in 50 μl sterile PBS. After 48 h, the mice were sacrificed, and the spleens, livers, and skin at the site of infection were harvested, homogenized, and plated for CFU on Mueller-Hinton (MH) plates supplemented with 0.1% t-cysteine.

**Statistical analysis.** Macrophage replication and qRT-PCR were analyzed for significance using unpaired, two-tailed, Student’s t tests. The mouse infection data were analyzed for significance using the Mann-Whitney test.

**SUPPLEMENTAL MATERIAL**


- Text S1, DOCX file, 0.04 MB.
- Figure S1, TIF file, 0.1 MB.
- Figure S2, TIF file, 1.1 MB.
- Figure S3, TIF file, 1.7 MB.
- Figure S4, TIF file, 0.5 MB.
- Figure S5, TIF file, 0.7 MB.
- Table S1, DOCX file, 0.1 MB.
- Table S2, DOCX file, 0.1 MB.
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REFERENCES

Role of Biotin Utilization in Bacterial Virulence