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A *Francisella* Virulence Factor Catalyzes an Essential Reaction of Biotin Synthesis

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Summary

We recently identified a gene (*FTN_0818*) required for *Francisella* virulence that seemed likely involved in biotin metabolism. However, the molecular function of this virulence determinant was unclear. Here we show that this protein named BioJ is the enzyme of the biotin biosynthesis pathway that determines the chain length of the biotin valeryl side chain. Expression of *bioJ* allows growth of an *E. coli bioH* strain on biotin-free medium, indicating functional equivalence of BioJ to the paradigm pimeloyl-ACP methyl ester carboxyl-esterase, BioH. BioJ was purified to homogeneity, shown to be monomeric and capable of hydrolysis of its physiological substrate methyl pimeloyl-ACP to pimeloyl-ACP, the precursor required to begin formation of the fused heterocyclic rings of biotin. Phylogenetic analyses confirmed that distinct from BioH, BioJ represents a novel sub-clade of the α/β -hydrolase family. Structure-guided mapping combined with site-directed mutagenesis revealed that the BioJ catalytic triad consists of Ser151, Asp248 and His278, all of which are essential for activity and virulence. The biotin synthesis pathway was reconstituted *in vitro* and the physiological role of BioJ directly assayed. To the best of our knowledge, these data represent further evidence linking biotin synthesis to bacterial virulence.

Keywords

Francisella; biotin; biotin synthesis; BioJ; nutritional virulence factor

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Competing interests

The authors have declared that no competing interests exist.

Author contributions

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

Introduction

Tularemia (also called rabbit fever, <http://www.cdc.gov/tularemia>) is a serious zoonotic disease that is featured epidemiologically with sporadic/endemic cases in North America and parts of Europe and Asia (Jones *et al.*, 2012a, Celli & Zahrt, 2013). *Francisella tularensis* (*F. tularensis*), a Gram-negative intracellular pathogen, has been identified as the causative agent of this plague-like disease (Jones *et al.*, 2012a). Although numerous mammals such as squirrels and rabbits are susceptible to this bacterial pathogen, none of these acts as a reservoir. Instead, it seems likely that freshwater amoebae living therein may be the true reservoir of this bacterium (Jones *et al.*, 2012a, Celli & Zahrt, 2013). Human infection by *Francisella* is mainly acquired through the arthropod vectors (*i.e.*, tick and deer fly bites) and/or zoonotic transmission (*e.g.*, close contact with diseased animals) (Jones *et al.*, 2012a). No person-to-person transmission has been recorded thus far. The three known subspecies (biovars) of the genus *Francisella* include *F. tularensis*, *F. novicida*, and *F. philomiragia*, respectively (Weiss *et al.*, 2007, Celli & Zahrt, 2013). *F. tularensis* is the most virulent of these bacteria and that most frequently isolated from infected humans. Although *F. novicida*, and *F. philomiragia* can result in debilitating febrile illness in immunocompromised individuals, they rarely cause illness in immuno-competent persons suggesting that they are much less virulent (Weiss *et al.*, 2007). *F. novicida*, a BSL-2 pathogen serves as an excellent surrogate model to explore *Francisella* pathogenesis and host-pathogen interactions (Celli & Zahrt, 2013). The *F. novicida* genome is >98% identical to that of the BSL-3 category A pathogen, *F. tularensis* and thus shares many of the same virulence genes. Moreover, *F. novicida* can cause lethal infections in mice.

Francisella has evolved multiple strategies to successfully complete its intracellular infection life cycle (Jones *et al.*, 2012a). Before its entry into host cells, it relies on an unusual lipopolysaccharide (LPS) and a protective capsule to resist extra-cellular host defense systems such as complement (Rus *et al.*, 2005), antibodies (Crane *et al.*, 2009, Geier & Celli, 2011) and antibacterial peptides (Cederlund *et al.*, 2011). After uptake by macrophages, *Francisella* subverts innate immune recognition receptors such as Toll-like receptors (Kawai & Akira, 2010, Wilson *et al.*, 2009) to prevent inflammatory signaling (Jones *et al.*, 2012b, Sampson *et al.*, 2013) (reviewed in (Jones *et al.*, 2012a)). *Francisella* then rapidly escapes from the *Francisella*-containing phagosome into the cytosol where it can proliferate (McCaffrey *et al.*, 2010, Ray *et al.*, 2009). The *Francisella* pathogenicity island plays a critical role in bacterial pathogenesis and encodes a type VI-like secretion system to efficiently deliver virulence determinants into host cells (Nano *et al.*, 2004, Santic *et al.*, 2007, Barker *et al.*, 2009). Another important aspect is the nutritional tug-of-war between *Francisella* and its host. Bacterial acquisition of the limited nutrients such as iron (Weinberg, 1974, Sen *et al.*, 2010) and cysteine (Alkhuder *et al.*, 2009) inside host cells often determines successful infection. Very recently, the metabolite biotin (vitamin B7 or H) that normally functions as a micronutrient was suggested to be correlated with *Francisella* virulence (Napier *et al.*, 2012). However, the biochemical/metabolic mechanism used by *Francisella* to synthesize biotin and thereby facilitate its infectivity remained unknown. A distantly related scenario is seen in *Mycobacterium tuberculosis* infections where *de novo* biotin synthesis is required for the persistence required to cause chronic infections (Woong Park *et al.*, 2011).

Biotin is an important enzyme cofactor that acts as a covalently-bound prosthetic group (Beckett, 2007, Beckett, 2009). Biotin is essential in all domains of life because it plays critical roles in central metabolic processes involving carboxylation, decarboxylation and transcarboxylation (Attwood & Wallace, 2002, Knowles, 1989). Although this cofactor was discovered over 70 years ago, our understanding of its diversified biosynthetic routes still remain fragmentary (Lin *et al.*, 2010, Shapiro *et al.*, 2012). The later steps of the pathway

BioF, BioA, BioD and BioB are highly conserved and catalyze assembly of the fused heterocyclic rings of biotin (Beckett, 2007, Lin & Cronan, 2010). The earlier steps in which the precursor pimelate moiety is synthesized remained a long-standing puzzle until a recent report by Lin and coworkers (Lin *et al.*, 2010) demonstrated that *E. coli* synthesizes by use of a modified type II fatty acid synthesis pathway (FAS II) (Cronan & Lin, 2010, Lin & Cronan, 2010, Lin *et al.*, 2010). To the best of our knowledge, the BioC-BioH pathway of *E. coli* represents the first experimentally-verified paradigm for synthesis of the biotin precursor pimeloyl-acyl carrier protein (ACP) (Lin *et al.*, 2010). The BioC enzyme functions as an *O*-methyltransferase that catalyzes *O*-esterification of the free carboxyl group of malonyl-ACP (Fig. 1C) to generate malonyl-ACP methyl ester which is used as a primer for FAS II in place of the usual acetyl-thioester (Lin & Cronan, 2012). After completion of the pimelate chain, the methyl group must be removed to prevent further elongation to physiologically useless products and this “gatekeeper” function is the property of BioH (Fig. 4) (Agarwal *et al.*, 2012, Shapiro *et al.*, 2012). Unlike *E. coli* (Lin *et al.*, 2010) analyses of the extant bacterial genomes showed that *bioH* is absent in many *bioC*-containing bacteria, although *bioC* genes are widely-distributed (Rodionov *et al.*, 2002). It therefore seems likely that bacteria have evolved diverse mechanisms to make pimelate. *Francisella* (<http://www.francisella.org>) (Larsson *et al.*, 2005, Rohmer *et al.*, 2007) encodes BioC in its biotin operon but no gene encoding a *bioH* homologue is present in the operon or genome raising the possibility that a non-orthologous protein catalyzes cleavage of the methyl group of methyl pimeloyl-ACP. Given that disruption or deletion of the FTN_0818 gene of *F. novicida* and of the homologous genes of *F. tularensis* and *F. holarctica* engenders a biotin requirement for escape of the bacteria from the phagosome (Su *et al.*, 2007, Llewellyn *et al.*, 2011, Napier *et al.*, 2012), we hypothesized that the protein encoded by FTN_0818 might provide the BioH-like activity needed to complete the *Francisella* biotin synthesis pathway. We report that this is the case and provide the first biochemical mechanism for the synthesis of biotin as a *Francisella* nutritional virulence factor..

Results

***Francisella* species lack the paradigm BioH gatekeeper enzyme of biotin biosynthesis**

Given the fact that *Francisella* species have small genomes (1.9~2.1 Mb), less than half that of *E. coli* (~4.6 Mb), it is reasonable that these bacteria have evolved strategies to efficiently utilize their limited genetic information. One strategy is the organization of the metabolism-related genes into a cluster (Fig. 1A). In the paradigm organism, *E. coli*, most but not all biotin biosynthetic genes are clustered on the chromosome (Fig. 1A). The *bioH* gene is encoded at a site far removed from the *bioBFCD*A bidirectional operon as is the *birA* gene that encodes the bifunctional biotin protein ligase/biotin operon repressor (Fig. 1A). In contrast, the annotated biotin metabolic loci of all *Francisella* species are present in a similar *bioBFCD*A operon adjacent to *birA* (Fig. 1A). It seems likely that *Francisella* BirA regulates *bioBFCD*A by a mode similar to that of *E. coli* because a putative conserved BirA binding site is located in the intergenic region between *bioB* and *bioA*. Intriguingly, the *bioH* homologue that encodes the gatekeeper enzyme of the biotin synthetic pathway seemed to be missing in all the examined *Francisella* species (Fig. 1A and B), indicating functional replacement by an unknown novel enzyme. The *F. novicida* FTN_0818 and *F. philomiragia* Fphi_1796 genes are located adjacent to the respective *bioBFCD*A operons consistent with their implication in biotin metabolism, although the genes are separated from *bioA* by a small gene unique to *Francisella* that encodes a protein of unknown function (Fig. 1A). Note that the sequence of the *F. philomiragia* protein is 86% identical to that of the *Francisella tularensis* subsp. *Tularensis* protein (Fig. 2S).

BioJ, a new biotin biosynthesis gatekeeping enzyme

To test if expression of the *Fphi_1796* gene of *F. philomiragia* could provide the missing biotin biosynthesis gatekeeping enzyme we assayed complementation of an *E. coli* Δ *bioH* biotin auxotrophic strain. A derivative of arabinose inducible vector pBAD322 carrying *Fphi_1796* called p*Fphi* was transformed into the Δ *bioH* strain and the transformants were streaked on biotin-free M9 minimal media that contained either varying levels of arabinose, the inducer of the *araBAD* promoter or 0.2% glycerol (for basal expression) as sole carbon source. In the presence of 4 nM biotin the strains that carried either p*Fphi* or the empty vector pBAD322 both grew well whereas neither strain grew on plates lacking both biotin and arabinose (Fig. 2A). As expected, plasmid-borne expression of *Fphi_1796* allowed good growth of the Δ *bioH* strain on arabinose containing plates that lacked biotin but Fig. 2A) whereas the empty vector failed to support detectable growth (Fig. 2A). The growth response required induction; no growth was seen with glycerol as sole carbon source. Similar results were obtained in liquid media (Fig. 2B). Consistent with the protein encoded by the *Fphi_1796* gene acting as a BioH paralogue, its expression failed to allow growth of an *E. coli* Δ *bioH* Δ *bioC* strain (Fig. S1). Given that *Fphi_1796* and *E. coli* BioH lack sufficient sequence similarity for alignment we have designated *Fphi_1796* as *bioJ*.

Given its marked divergence from BioH we wished to characterize the enzymatic mechanism of BioJ. A hexahistidine tagged version of BioJ was expressed at high levels in *E. coli* and purified to homogeneity by Ni²⁺-chelate chromatography (Fig. 3A). Mass spectrometric analysis of the peptides of a tryptic digest of the purified protein validated the identity of the protein. Size exclusion chromatography showed that BioJ had the elution properties of a monomeric protein and its monomeric nature was confirmed by chemical cross-linking (Fig. 3A). Monomeric structure is typical of α , β hydrolases (Heikinheimo *et al.*, 1999, Jochens *et al.*, 2011) and was previously seen with BioH and BioG (Shapiro *et al.*, 2012).

We tested the ability of BioJ to convert pimeloyl-ACP methyl ester, the physiological BioH substrate, to pimeloyl-ACP using a conformationally sensitive electrophoretic mobility shift assay (Fig. 4A). Pimeloyl-ACP migrates more slowly than the substrate, pimeloyl-ACP methyl ester because the free carboxyl group destabilizes ACP structure and thereby increases the effective radius of the protein (Lin *et al.*, 2010, Shapiro *et al.*, 2012). BioJ catalyzed hydrolysis of the methyl ester bond of pimeloyl-ACP methyl ester proceeded rapidly and could proceed to completion (Fig. 4B). Mass spectrometry of the reaction mixtures showed conversion of pimeloyl-ACP methyl ester to species smaller by 14-15.7 amu indicating loss of a methyl group and formation of pimeloyl-ACP (Fig. 4C). Hence, BioJ is a member of the carboxylesterase family with BioH-like activity. BioH is a rather promiscuous esterase and BioJ shares this property. BioJ cleaved two non-physiological substrates, adipoyl-ACP methyl ester; and suberyl-ACP methyl ester, having dicarboxylic acid moieties of C6 and C8, respectively (Fig. 4D). However, BioJ was unable to cleave malonyl-ACP methyl ester, the first dedicated intermediate of the biotin synthetic pathway (Fig. 4D).

Functional mapping of a catalytic triad required for BioJ activity

As a representative member of α , β -hydrolases (Sanishvili *et al.*, 2003), *E. coli* BioH features a conserved catalytic Ser82-Asp207-His235 triad (Fig. 5A). Modeling of the BioJ sequence on the *E. coli* BioH structure gave a putative catalytic triad (Fig. 5A) that seemed to share an almost identical 3D configuration with BioH (not shown) although the neighboring residues were markedly different (Fig. 5A). The predicted catalytic triad consisted of S151, D248 and H278, (Fig. 5A). To test function of the putative triad we constructed genes encoding three mutant BioJ proteins, S151A, D248A and H278A and

purified the proteins together with the wild type protein (Fig. S3). In contrast to the wild type BioJ protein (Fig. 5B) all three mutant enzymes failed to cleave pimeloyl-ACP methyl ester (Fig. 5C-E). Some hydrolysis activity was detected when excess BioJ S248A mutant enzyme (>6.8 pmol) was used (not shown). However, we believe that this result is not physiologically relevant given the high concentration of the enzyme required. The significance of the amino acid residues of the predicted catalytic triad was also verified *in vivo* by construction of a series of $\Delta bioH$ strains carrying a plasmid encoding BioJ (either the wild type or one of the three mutant proteins) and tested their abilities to grow on biotin-free plates (Fig. 5F). The strain expressing wild type BioJ gave robust growth even when the arabinose inducer levels was as low as 0.05% whereas no growth was seen for the strain carrying the empty vector (Fig. 5F). Expression of all three mutant BioJ proteins failed to allow growth of the $\Delta bioH$ strain indicating that the hydrolysis seen with excess BioJ S248A *in vitro* was not physiologically relevant.

The physiological role of BioJ in dethiobiotin synthesis

Although the lines of evidence given above indicated clearly that BioJ activity is required for conversion of the pimeloyl methyl ester moiety to the final product biotin, a further test was its ability to function in the *in vitro* system previously developed (Lin *et al.*, 2010) to assay the overall biotin synthesis pathway. Note that the penultimate intermediate, dethiobiotin (DTB) is assayed rather than biotin in order to avoid the complication of the extremely labile the BioB enzyme that catalyzes the last step of the pathway. Crude extracts of a $\Delta bioH$ strain were used and synthesis of DTB was bioassayed using the strain ER90 ($\Delta bioF bioC bioD$) (Lin *et al.*, 2010, Feng *et al.*, 2013a, Feng *et al.*, 2013b). Growth of the biotin auxotroph strain ER90 is visualized by deposition of a red formazan deposit (del Campillo-Campbell *et al.*, 1979) (Fig. 6). Biotin or DTB addition to the paper disk allowed growth of the indicator strain ER90 and 1-2 pmol could be detected (Fig. 6A). Addition of pimeloyl-ACP methyl ester to crude extracts prepared from the $\Delta bioH$ mutant strain (0.2-0.3 mg total protein) gave no detectable growth of the assay strain (Fig. 6B) whereas good growth was seen upon addition of purified BioJ to the reactions (Fig. 6C) which indicated DTB synthesis. In contrast, incubations containing any of the mutant BioJ proteins failed to allow ER90 growth (Fig. 6D). These results clearly illustrated the physiological role of BioJ in bacterial DTB biosynthesis.

BioJ activity is required for *F. novicida* viability

The physiological relevance of BioJ was tested using mutants of *F. novicida* in which an alanine substitution was made for the two putative catalytic triad residues other than the previously tested S151A mutation (Napier *et al.*, 2012). Growth of the new strains was tested in nutrient-limiting CHB and loss of any of the three triad residues clearly resulted in a severe growth defect relative to wild-type *F. novicida* (Fig. 7) as previously reported for the S151A strain (Napier *et al.*, 2012). Addition of either biotin (Fig. 7A) or pimelate (Fig. 7B) restored growth defect of all three catalytic residue point mutant strains. These results demonstrate the physiological importance of each of the BioJ Ser-Asp-His triad residues.

The BioJ catalytic residues are required for replication in macrophages and survival in mice

Given that loss of any of the three BioJ catalytic residues blocked growth in minimal medium, we expected that like the the S151A mutant the D248A, and H278A mutants might exhibit a severe replication defect in macrophages. We infected murine bone marrow-derived macrophages (mBMDM) with wild-type *F. novicida*, $\Delta bioJ$, and each of the three BioJ catalytic mutant strains (S151A, D248A or H278A) and found that 6 h post-infection, the wild-type strain had replicated almost 10-fold whereas the $\Delta bioJ$ and all three catalytic

triad mutants replicated only about 2-fold (Fig. 7C). However, when biotin was added to the medium at the time of infection, the $\Delta bioJ$, S151A, D248A, and H278A replication defects were significantly reversed (Fig. 7C) thereby illustrating the importance of biotin availability during infection and the functional importance of BioJ during intracellular replication.

Finally, we set out to determine whether the catalytic triad was required for *F. novicida* virulence during *in vivo* infection. We performed competition experiments in which 1:1 mixtures of the wild-type strain with the $\Delta bioJ$, S151A, D248A or H278A strains were used to infect mice. Forty-eight hours post-infection, the levels of the $\Delta bioJ$ strain were ~1-2 logs lower than the wild-type *F. novicida* in both spleen and liver (Fig. 7D). All three catalytic triad mutants displayed similar 1-2 log reductions in the spleen and liver levels (Fig. 7E-G). Taken together, these data demonstrate that BioJ is a novel *Francisella*-specific biotin gatekeeper enzyme that in the absence of free biotin is functionally required for bacterial replication in nutrient limited media and in macrophages, as well as for systemic survival in mice.

BioJ represents a new carboxyl-esterase subclade

Prior to this study, three enzymes, BioH, BioG and BioK, were known to catalyze the demethylation of pimeloyl-ACP methyl ester. Although the BioJ proteins were predicted to be members of the α , β -hydrolase family, they are evolutionarily distinct from *E. coli* BioH in that they are 50 aa longer than the *E. coli* protein and share essentially no sequence identity with BioH. Since, BioH, BioG, and BioK recognize the same physiological substrate (Me pimeloyl-ACP) despite their low sequence identity and share the same catalytic residues (Shapiro *et al.*, 2012), we compared the phylogenetic relationship of these isozymes with BioJ and found that BioJ homologues are restricted to *Francisella* species (Fig. 8 and Fig. S2). A minimum-likelihood phylogenetic tree was constructed using six BioJs, nine BioHs, four BioKs and seven BioGs. These proteins are categorized into four different sub-clades. Although the BioH, BioG and BioK proteins group into the same clan, CL0028, the BioHs belong to the α , β -hydrolase 6 family whereas BioGs and BioKs are members of the α , β -hydrolase 5 family. All the BioJ homologues group into domain Abhydrolase 3 of Clan CL0028. Based on the structure of *E. coli* BioH and homology modeling of the other proteins, all five groups of proteins seem structurally distinct (Fig. 8), which is consistent with the diversity observed in their phylogenetic evolution (Fig. 8). Given the fact that BioJ homologues formed a unique subclade and were evolutionary distant from all of the other groups of functionally related proteins (Fig. 8), we therefore propose that it is a new class of biotin synthesis carboxylesterase.

Discussion

Although *bioH* is not integrated into the biotin operon of *E. coli*, in numerous other bacteria *bioH* is found within the operon and is located immediately upstream of *bioC* (Rodionov *et al.*, 2002). Other bacteria lack BioH but have a gene (either *bioG* or *bioK*) encoding an α , β -hydrolase that is able to perform the BioH function (Rodionov *et al.*, 2002, Shapiro *et al.*, 2012). An extreme example is *Bacillus fragilis* which encodes a BioG-BioC fusion protein (Rodionov *et al.*, 2002, Shapiro *et al.*, 2012). *Francisella bioJ* is the first example of a biotin synthesis α , β -hydrolase encoded next to, but not within, a biotin operon. Our view is that the α , β -hydrolases of biotin synthesis are “wild cards” that can be traded among bacteria or developed *de novo* within a specific group of bacteria. The gene encoding a given type of α , β -hydrolase can either become domesticated as is the case for those located in a biotin operon or remain completely undomesticated as is the case of *E. coli bioH* which is neither in the operon nor coregulated with the operon genes. By these criteria *Francisella bioJ*

appears a partly domesticated gene in that it closely neighbors, but is not within, the biotin operon and seems to lack the sequence that putatively regulates the operon in response to biotin supply. BioJ like BioK is restricted to a specific group of bacteria, *Francisella* species and cyanobacteria, respectively, whereas BioH and BioG are found across a broad diversity of bacteria. The diversity of these α,β -hydrolases is in remarkable contrast to the very strongly conserved proteins responsible for synthesis of the biotin heterocyclic rings. A plausible explanation of this dichotomy is that ester hydrolysis is a simple reaction whereas ring formation requires much more complex chemistry. Indeed, it has been shown that a *P. aeruginosa* PAO1 esterase of unknown function can attain BioH activity by simple amino acid substitutions (Flores *et al.*, 2012).

The only biotin-modified *Francisella* protein detected by genome sequence analysis has all of the hallmarks of AccB, the biotinylated subunit of the essential acetyl-CoA carboxylase reaction that initiates fatty acid synthesis (Fig S5). This argues that the lack of biotin engenders an inability to make both membrane phospholipids and the lipid A component of the outer membrane. If so, it might be expected that supplementation with exogenous fatty acids could bypass biotin deficiency. However, in other gram-negative bacteria such as *E. coli* this strategy fails because the lipid A biosynthetic pathway cannot utilize exogenous fatty acids (Raetz *et al.*, 2007).

Francisella is unusual in that, despite the small genome size, it encodes two biotin protein ligase (BPL) genes whereas a single BPL gene seems the norm across biology (Beckett, 2009, Chapman-Smith & Cronan, 1999, Rodionov *et al.*, 2002). The *birA1* gene (FTN_0568) is annotated to encode a BPL lacking DNA binding activity, whereas *birA2* (FTN_0811) is a bifunctional regulator retaining BPL function like *E. coli* BirA (Table S3). The encoded proteins show only about 28% sequence identity and thus the genes seem unlikely to have arisen by gene duplication. Transposon insertions into *birA1* showed that the gene was necessary for bacterial replication *in vivo* and macrophage escape (Weiss *et al.*, 2007, Asare & Abu Kwaik, 2010). However, since ligation of biotin to its cognate proteins is an essential reaction (Chapman-Smith & Cronan, 1999), recovery of these insertions indicates that another enzyme, probably BirA2, provides BPL activity. Indeed, the lack of insertions into the *birA2* gene (Weiss *et al.*, 2007, Asare & Abu Kwaik, 2010) argues that it encodes the housekeeping BPL required for growth in the laboratory.

Another unusual feature of *Francisella* biotin metabolism is that supplementation of biotin-free minimal medium with pimelate allows growth of *bioJ* strains albeit to a lesser extent than does biotin. Since the BioF reaction requires that one of the pimelate carboxyl groups be in thioester linkage to ACP or CoA, *Francisella* must contain an enzyme that activates pimelate. *E. coli* Δ *bioH* strains can also grow on pimelate, but only when a pimeloyl-CoA synthetase, such as *Bacillus subtilis* BioW, is expressed (Lin *et al.*, 2010, Agarwal *et al.*, 2012). However, a search for *Francisella* genes encoding BioW homologues was unsuccessful indicating that these bacteria encode a novel pimeloyl-CoA synthetase.

Experimental procedures

Strains and growth conditions

All *E. coli* strains (Table S1) were routinely grown at 37°C in liquid LB medium (Luria-Bertani medium containing 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl per liter) or plates of LB agar. The biotin-free M9 minimal media containing 0.1% vitamin-free Casamino Acids and 0.2% glycerol as sole carbon source was used to test for biotin requirements. Antibiotics were supplemented as follows (in μ g/ml): sodium ampicillin, 100 and kanamycin sulfate, 50. All *F. novicida* strains were grown in TSB/0.2% cysteine or Chamberlain's Medium (CHB) (Teknova) (Chamberlain, 1965). Generation of the

kanamycin (kan)-marked *FTN_0818* deletion strain, the *FTN_0818* clean deletion strain and the *FTN_0818* serine point mutant strain (FTN_0818-S151A) were reported previously [Napier, 2012]. The aspartic acid (FTN0818-D248A) and histidine point mutant strains (FTN0818-H278A) were constructed by overlapping PCR using primers encoding a single amino acid substitution and using a kan-cassette for selection. These constructs were transformed into the *FTN_0818* clean deletion mutant strain followed by selection on kanamycin plates (30 µg/ml). All strains mentioned above were verified by PCR and sequencing (Eurofins EWG Operon).

Site-directed mutagenesis

Site-directed mutagenesis was performed as described by Zheng *et al.* (Zheng *et al.*, 2004). The PCR reaction system (25 µl) included the following components: 2.5 µl of 10x *pfx* buffer (Invitrogen), 0.5 µl of 50 mM MgSO₄ (Invitrogen), 0.5 µl of 40 mM dNTP mix (10 mM each), 1.0 µl of forward/reverse primers (*Fphi*(S151A)-F plus *Fphi*(S151A)-R, 10 pmol/µl) (Table S2), 1.0 µl of pBAD322-*Fphi* (or pET28-*Fphi*) as template (10 ng/µl), 0.5 µl of Platinum *pfx* (2.5 U/µl, Invitrogen), and 17.0 µl of distilled sterilized H₂O. The reaction was done using the program consisting of a denaturing cycle at 95°C for 5 min; 20 cycles comprised of 95°C for 50 s, 55°C for 50 s, and 68°C for 6.5 min and a final step of 8 min at 68°C. To remove the residual template plasmid, the gel purified PCR products were digested for overnight with DpnI (20 U/µl, New England Biolabs) at 37 °C. Subsequently, the processed PCR products were transformed into chemically competent cells of Topo 10 (Invitrogen) and the inserts of purified plasmids were confirmed by direct DNA sequencing. The two mutant plasmids pBAD322-*Fphi* (S151A) and pET28-*Fphi* (S151A) were transformed into the Δ *bioH* strain STL24 (Agarwal *et al.*, 2012, Lin *et al.*, 2010) and strain BL21(DE3), respectively.

Plasmids and DNA manipulations

F. philomiragia (ATCC 25017) genomic DNA was purchased from ATCC for PCR amplification of *Fphi_1796* (*bioJ*). Since *F. philomiragia* genomic DNA has a very low GC content (32.0%) (Zeytun *et al.*, 2012), Phusion High-Fidelity DNA Polymerase (New England Biolabs) was required. The gel-purified PCR products (QIAGEN) were cloned into the XmaI-SphI digested low copy, arabinose-inducible expression vector pBAD322 and BamHI-XhoI T7 promoter-driven expression vector pET28(a) (Table S2) to give plasmids pBAD322-*Fphi* and pET28-*Fphi* (Table S1). All plasmid constructs were verified by direct DNA sequencing.

Protein expression and purification

The N-terminal hexahistidine tagged BioJ protein (wild type version plus three versions of mutants) was expressed in BL21(DE3) carrying pET28-*Fphi* (Table S1). IPTG was added to 0.3 mM to 500 ml bacterial cultures at an OD_{600 nm} of 1.0 followed by incubation at 30°C for 6 h. The cells were pelleted by centrifugation (4200 g, 20 min), washed three times with ice-cold PBS buffer (101.4 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, 12% glycerol, pH 7.4) and resuspended in PBS buffer containing 25 mM imidazole. Following lysis in a French pressure cell and removal of bacterial debris by centrifugation (16,000×g for 60 min), the clarified supernatant was loaded on a nickel chelate resin (Qiagen) for no less than 2 h. After washing the column with ten column volumes of PBS buffer containing 50 mM imidazole, the BioJ proteins were eluted with 150 mM imidazole, dialyzed against PBS buffer and then concentrated by ultra-filtration (10 kDa cut-off, Amicon Ultra) (Feng & Cronan, 2010). The protein purity was visualized by gradient SDS-PAGE (4-20%), and further confirmed by liquid chromatography quadruple time-of-flight

(qTOF) mass spectrometry of tryptic peptides as described previously (Feng & Cronan, 2011a, Feng & Cronan, 2011b).

Size exclusion chromatography and chemical cross-linking assays

The purified recombinant BioJ protein was subjected to gel filtration analyses using a Superdex 75 column (Pharmacia) run on an Äkta fast protein liquid chromatography system (GE Healthcare) as described previously (Feng & Cronan, 2010, Feng & Cronan, 2011b). The column effluent was monitored at a flow rate of 0.46 ml/min in PBS buffer (10 mM Na₂HPO₄, 2 mM KH₂PO₄, 20 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The peak was collected and analyzed by gradient SDS-PAGE (4-20%).

To further test the solution structure of *Francisella* BioJ, chemical cross-linking with ethylene glycol bis-succinimidylsuccinate (Pierce) was performed (Feng *et al.*, 2013b). In each chemical cross-linking reaction (20 µl in total), the purified BioJ protein (~10 mg/ml) was separately mixed with different concentrations of cross-linker (0, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10 and 20 µM), and incubated for 30 min at room temperature. All the reaction products were separated using gradient SDS-PAGE (4-20%).

Enzymatic assays

The *in vitro* enzymatic activity of BioJ and its derivatives was determined using the protocol established for the *E. coli* BioH with appropriate modifications (Agarwal *et al.*, 2012, Flores *et al.*, 2012, Shapiro *et al.*, 2012). The enzymatic reaction was reconstituted in the 50 mM HEPES buffer (pH 7.0) containing 5% glycerol. In each reaction with the total volume of 10 µl, BioJ (~14 µg/ml, 3.6 µM) was mixed with 150 µM of its physiological substrate pimeloyl-ACP methyl ester (or a shorter or longer homologue) and incubated at 37°C for 1 h. The generated products of these reactions were loaded into 20% PAGE gel containing 2.5 M urea and then run at 130V for 2.5 h.

MALDI TOF/TOF mass spectrometry

The mixture of BioJ-catalyzing reaction (30 µl in total) was subjected to dialysis in buffer of 20 mM ammonium acetate (2L) overnight at 4 °C (Feng *et al.*, 2013b). After ammonium acetate was evaporated under a stream of nitrogen (Feng & Cronan, 2009), the dried protein samples then were dissolved in methanol and mixed with 50 mg/mL 2,5-dihydroxybenzoic acid (DHB) matrix (Sigma, St. Louis, MO) in 50% acetonitrile and 0.1% trifluoroacetic acid in a 1:10 ratio and deposited on a standard stainless steel target. The mass spectra were collected in positive ion mode on an UltrafleXtreme MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a frequency tripled Nd:YAG solid state laser using the FlexControl 1.4 software package (Bruker Daltonics). Following external calibration, 2000 spectra were acquired at 500 Hz using a randomized raster, summed, and saved for analysis. Data processing was done using the FlexAnalysis 3.4 software package (Bruker Daltonics). Spectra were smoothed and a baseline correction was applied using the built-in features of the software package (Feng *et al.*, 2013b).

Reconstitution of the *in vitro* DTB synthesis system and the DTB bioassay

To test the potential role of BioJ in biotin biosynthesis, we reconstituted an *in vitro* system that included the crude extract of strain STL24 (*E. coli* Δ*bioH*) plus the various BioJ proteins as described by Lin *et al.* (Lin *et al.*, 2010). The production of biotin was visualized using the biotin auxotrophic strain ER90 (Δ*bioF bioC bioD*)-based biotin bioassay previously developed (Lin *et al.*, 2010, Feng *et al.*, 2013a, Feng *et al.*, 2013b).

First, the $\Delta bioH$ cell-free extracts were prepared as described by Lin and coworkers (Lin *et al.*, 2010) with some modifications. Strain STL24 (*E. coli* $\Delta bioH$) was cultivated at 37 °C to 0.8 OD₆₀₀ in 200 ml of M9 minimal medium containing 1 nM biotin. To remove excess of biotin, the bacterial cells were washed twice in M9 media and sub-cultured into 1 liter of M9 minimal medium at 37 °C for 5 hours to derepress expression of the *bio* operon by starvation for biotin. The pelleted cells were lysed by passage through a French pressure cell in assay buffer at 17,500 p.s.i. and then centrifuged at 20,000g for 30 min to obtain the supernatant. The soluble fraction was further purified through ammonium sulfate precipitation (85% of saturation followed by 1 h of centrifugation at 10,000×g (small molecules and ACP species remain soluble in such ammonium sulfate solutions. To remove ammonium sulfate and any remaining small molecules, the solubilized precipitate was subjected to dialysis using Slide-A-Lyzer Dialysis Cassette G2 of 10 kDa cut-off (Thermo Scientific) against 1x PBS assay buffer at 4°C overnight. Bradford assays were adopted to determine the protein concentration of the crude extracts using the BioRad Protein Assay Dye Reagent (Thermo Scientific).

The *in vitro* system for biotin synthesis was developed as Lin and coauthors (Lin *et al.*, 2010) reported with minor changes. The components for were () are included as follows: 1 mg of $\Delta bioH$ cell-free crude extract protein, 10 mM MgCl₂, 5 mM dithiothreitol, 0.1 mM pyridoxal-5'-phosphate (PLP), 1 mM L-alanine, 1 mM KHPO₄, 1 mM NADPH, 1 mM ATP, 1 mM glucose-6-phosphate, 1 mM S-adenosyl-L-methionine, 60 μM pimeloyl-ACP methyl ester and 0.5 μM (~20 μg/ml) of a purified BioJ protein in 100 μl total volume. The reactions were incubated at 37 °C for ~3 h and quenched by immersion in boiling water for 15 min.

Biotin production was visualized using the reduction of 2,3,5-triphenyl tetrazolium chloride to the insoluble red formazan resulting from bacterial growth (Lin *et al.*, 2010, del Campillo-Campbell *et al.*, 1979, Feng *et al.*, 2013a, Feng *et al.*, 2013b),

Growth curves of *Francisella*

Bacteria were subcultured to an OD₆₀₀ of 0.03 in TSB/0.2% cysteine or Chamberlain's Medium (CHB) (Teknova) (Chamberlain, 1965) for *F. novicida*, while LVS cultures were grown in modified Mueller-Hinton (MH) broth described previously (Su *et al.*, 2007, Llewellyn *et al.*, 2011, Napier *et al.*, 2012). Subcultures were read hourly using a SynergyMX BioTek plate reader (Applied Biosystems). Biotin (0.25 μM) (Merck KGaA) or pimelate (200 μg/mL) (Sigma-Aldrich) was added when appropriate.

Macrophage preparation and infections

Bone marrow-derived macrophages (mBMDMs) were prepared as described previously (Su *et al.*, 2007, Llewellyn *et al.*, 2011, Napier *et al.*, 2012). Briefly, bone marrow was collected from the femurs of mice. Bone marrow cells were plated into sterile petri dishes and incubated in DMEM supplemented with 10% heat-inactivated FBS and 10% macrophage colony-stimulating factor (M-CSF)-conditioned medium (collected from M-CSF-producing NIH3T3 cells). Bone marrow cells were incubated at 37°C with 5% CO₂ and harvested after 6 days. All mBMDMs were incubated before and during infection in 24 well plates at 37°C with 5% CO₂. For infection, mBMDMs were seeded at 5 × 10⁵/well and incubated overnight at 37°C with 5% CO₂. mBMDMs were infected at a multiplicity of infection (MOI) of 10:1 (Su *et al.*, 2007, Llewellyn *et al.*, 2011, Napier *et al.*, 2012).

Mouse infections

For mouse infections, female C57BL/6 mice (6-8 wk) (Jackson Laboratory, Bar Harbor, ME) were housed under specific pathogen-free housing at Emory University. Experimental

studies were performed in accordance with the Institutional Animal Care and Use Committee guidelines. Competitive index [CI = (mut output/WT output)/(mut input/WT input)], as described previously (Su *et al.*, 2007, Llewellyn *et al.*, 2011, Napier *et al.*, 2012). Statistical analysis for CI experiments described previously (Weiss *et al.*, 2007).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Genetic organization of biotin synthesis-related genes and working model for the *Francisella* biotin biosynthesis pathway

A. Comparison of the *Francisella* biotin operon genome neighborhood to that of *E. coli*.

Note that the two *Francisella* genomes are inverted due to opposite ORF numbering systems

B. Scheme of the proposed *Francisella* biotin synthesis pathway

Fig. 2 (Cronan)

Fig. 2.

Expression of *Francisella philomiragia* *bioJ* (Fphi_1796) complements growth of an *E. coli* $\Delta bioH$ strain

A. Expression of *Francisella philomiragia* *bioJ* (Fphi_1796) allows growth of the *E. coli* $\Delta bioH$ strain on biotin-free medium

B. Growth curves of the $\Delta bioH$ strain with various expression levels of plasmid-borne *bioJ* (Fphi_1796). The growth curves were automatically plotted using a Bioscreen C instrument. The *E. coli* $\Delta bioH$ strain was STL24 (Table S1). Designations: Vec, empty vector pBAD322; Fphi, *F. philomiragia* *bioJ*; Ara, arabinose. Glycerol was the carbon source in the absence of arabinose.

Fig. 3.

Characterization and verification of *Francisella* BioJ

A. Size exclusion chromatography profile of purified BioJ

The separation was conducted using a Superdex 75 column eluted at 0.46 ml/min. The insert gel is a gradient SDS-PAGE (4-20%) analysis of BioJ collected from the peak fractions. The molecular weights of the calibration proteins kit (Biorad) are shown by the ruler at the top. The standard proteins were bovine serum albumin (67 kDa), ovalbumin (43 kDa), ribonuclease A (13.7 kDa), aprotinin (6.5 kDa) and vitamin B12 (1.35 kDa), respectively. Chemical cross-linking analyses of fatty acid metabolism FadR, a protein of known dimeric structure (**B**) and BioJ (**C**). FadR provides a control for the efficacy of cross-linking. Ethylene glycol bis-succinimidylsuccinate was the cross-linker. M: Molecular weight.

D. MS identification of the purified BioJ protein

The tryptic peptides that match the BioJ sequence are given in bold and underlined type.

Fig. 4 (Cronan)

Fig. 4.

Francisella BioJ is a functional carboxylesterase

A. Schematic diagram of the enzymatic reaction catalyzed by *Francisella* BioJ

B. Enzymatic assays for *Francisella* BioJ hydrolysis of pimeloyl-ACP methyl ester to pimeloyl-ACP

The enzymatic reactions (10 μ l total volume) were performed at 37°C and contained 150 μ M pimeloyl-ACP methyl ester as substrate. The reaction mixtures were analyzed by 20% PAGE containing 2.5 M urea. The BioJ-reaction times (right-hand six lanes of **Panel B**) were 2, 5, 10, 15, 20 and 30 minutes, respectively. CK denotes a reaction containing all components except BioJ. The triangle over the right-hand seven lanes of the bottom panel represents BioJ levels in an inverse dilution series (0.034, 0.068, 0.17, 0.34, 0.7, 1.7 and 3.4 pmol).

C. Matrix-assisted laser desorption/ionization mass spectroscopic analysis of the BioJ reaction

Upon overexpression of ACP in *E. coli* three forms of the protein are found due to titration of the deformylase and methionine amino peptidase that process the amino termini of nascent proteins. Form 1 is the form isolated when expression is from the chromosomal *acpP* gene. Abbreviations: Met, methionine; Me, methyl ester.

D. Substrate specificity of BioJ

The minus signs denote no addition of BioJ enzyme whereas the plus signs denote addition of the protein.

Fig. 5 (Cronan)

Fig. 5. Identification of a catalytic Ser-Asp-His triad motif required for BioJ function

A. Comparison of the putative active site of BioJ with the known catalytic residues of BioH.

The key residues are in purple. *S. marcescens*: *Serratia marcescens*

Enzymatic assays of BioJ (**Panel B**) and the single mutant S151A (**Panel C**), D248A (**Panel D**) and H278A proteins (**Panel E**) by the conformationally-sensitive electrophoretic mobility shift assay.

Minus denotes no addition of BioJ (or a mutant protein) whereas the triangle on the right hand represents the protein levels in a inverse dilution series (0.034, 0.068, 0.17, 0.34, 0.7, 1.7, 3.4 pmol). The enzymatic reaction (10 μ l total volume) contained 150 μ M methyl pimeloyl-ACP. The reaction mixture was separated using 20% PAGE containing 2.5 M urea.

F. *In vivo* functional analyses of the triad residue BioJ mutant proteins (S151A, D248A & H278A)

The plates of were incubated at 37 $^{\circ}$ C for ~20 h after streaking. The host strain was the *E. coli* Δ *bioH* strain STL24. Designations: Ara, arabinose; Fphi, *F. philomiragia bioJ* ; Vec, medium copy arabinose-inducible plasmid pBAD322.

Fig. 6.

Bioassay of BioJ function in the overall biotin synthesis pathway

A. Both biotin and dethiobiotin (DTB) support growth of the biotin auxotrophic strain ER90 on biotin-free minimal medium

B. Evidence for the absence of biotin in cell extracts prepared from the *E. coli* $\Delta bioH$ strain Strain STL24 ($\Delta bioH$) (Lin *et al.*, 2010) was used to prepare cell extracts and the biotin indicator strain was ER90 ($\Delta bioF \Delta bioC \Delta bioD$). Either biotin or DTB as positive control or the cell extract (10 mg/ml) was spotted on the paper disk as described (Lin *et al.*, 2010). The bioassay was performed using strain ER90. The plates were kept at 30°C for ~20 h. The red formazan deposit formed by reduction of the tetrazolium indicator denotes growth of the biotin auxotrophic strain ER90 supported by either biotin or DTB.

C. Scheme of the *in vitro* DTB synthesis system

D. Restoration of DTB synthesis to the $\Delta bioH$ extract by addition of pimeloyl-ACP methyl ester and BioJ

Either the purified wild type BioJ protein or the mutant BioJ versions (S151A, D248A and H278A) was added to 20 $\mu\text{g/ml}$ (0.5 μM). The reaction on the upper left quadrant contained nearly all the components required for DTB synthesis with the exception of BioJ (or its mutant) enzyme whereas samples spotted on the upper right quadrant lacked cell extract, only contained both the wild type and mutant BioJ proteins. The reactions of the lower left quadrant contained all the components required for DTB synthesis. Designations: plus, addition of BioJ protein (or its mutants); minus: no addition of BioJ protein (or its mutants) or cell extracts.

Fig. 7.

The BioJ Ser-Asp-His catalytic triad is essential for growth of *F. novicida* in minimal media and for efficient replication in macrophages (and systemic survival in mice)

The BioJ catalytic triad is required for growth of *F. novicida* in minimal medium, and can be alleviated by the addition of biotin (A) or pimelate (B). Wild-type strain U122 of *F. novicida* and the three catalytic point mutants of BioJ (S151A, D248A and H278A) are grown in Chamberlain's medium (CHB) with/without biotin or pimelate. The OD₆₀₀ was measured every hour.

C. The catalytic triad of BioJ is required for replication in macrophages lacking exogenous biotin.

D-G. The BioJ catalytic triad residues are required for systemic survival in mice. Mice were infected sub-cutaneously with a 1:1 mixture of wild-type strain 122 of *Francisella* with $\Delta bioJ$ (D), S151A (E), D248A (F) or H278A (G). At 48 h post infection the, spleens and livers were harvested to quantify bacterial levels, and the competitive index (CI) was calculated. Statistical analysis was described previously (Weiss, et al., 2007). * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

Fig. 8.

Phylogenetic tree and structural modeling of the bacterial biotin synthesis gatekeeper enzymes

The modeled structure of each member except BioH which has a known structure is shown on the right. All BioJ homologues are found in *Francisella* species are given in red.