Molecular characterization of the *Borrelia burgdorferi in vivo*-essential protein PncA

Mollie W. Jewett,[1,2] Sunny Jain,[2] Angelika K. Linowski,[2] Amit Sarkar[1] and Patricia A. Rosa[1]

[1]Laboratory of Zoonotic Pathogens, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Hamilton, MT 59840, USA

[2]Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida (UCF), Orlando, FL 32827, USA

Correspondence
Mollie W. Jewett
Mollie.Jewett@ucf.edu

The conversion of nicotinamide to nicotinic acid by nicotinamidase enzymes is a critical step in maintaining NAD$^+$ homeostasis and contributes to numerous important biological processes in diverse organisms. In *Borrelia burgdorferi*, the nicotinamidase enzyme, PncA, is required for spirochaete survival throughout the infectious cycle. Mammals lack nicotinamidases and therefore PncA may serve as a therapeutic target for Lyme disease. Contrary to the *in vivo* importance of PncA, the current annotation for the *pncA* ORF suggests that the encoded protein may be inactive due to the absence of an N-terminal aspartic acid residue that is a conserved member of the catalytic triad of characterized PncA proteins. Herein, we have used genetic and biochemical strategies to determine the N-terminal sequence of *B. burgdorferi* PncA. Our data demonstrate that the PncA protein is 24 aa longer than the currently annotated sequence and that *pncA* translation is initiated from the rare, non-canonical initiation codon AUU. These findings are an important first step in understanding the catalytic function of this *in vivo*-essential protein.

INTRODUCTION

Human infection with the pathogenic spirochaete *Borrelia burgdorferi* following the bite of an infected tick can result in a multi-stage, inflammatory disorder called Lyme disease. Humans are incidental hosts for *B. burgdorferi* and, in nature, the spirochaete cycles between *Ixodes* sp. ticks and small rodents and birds. In order to survive in these diverse host environments, *B. burgdorferi* must employ molecular mechanisms for persistence and transmission (Rosa *et al.*, 2005; Tilly *et al.*, 2008).

The genome of *B. burgdorferi* is complex and segmented, consisting of a small, linear chromosome and, in the characterized type strain B31, at least 21 linear and circular plasmids (Casjens *et al.*, 2000; Fraser *et al.*, 1997). In general, the genes present on the chromosome encode proteins found in other bacterial species, whereas approximately 60 % of the genes found on the plasmids appear to encode proteins unique to *Borrelia* (Casjens *et al.*, 2000; Fraser *et al.*, 1997). Consistent with its reduced genome size, *B. burgdorferi* lacks many of the genes encoding metabolic enzymes required for *de novo* synthesis of

nucleotides, amino acids, fatty acids and enzyme cofactors, all of which are essential for growth and survival of the spirochaete throughout its infectious cycle. Consequently, *B. burgdorferi* must scavenge required nutrients from its host environments (Bono *et al.*, 1998; Byram *et al.*, 2004; Jewett *et al.*, 2007b, 2009; Lawrence *et al.*, 2009; Purser *et al.*, 2003; Tilly *et al.*, 2004).

The *pncA* gene (BBE22) present on linear plasmid 25 (lp25) encodes a nicotinamidase that hydrolyses nicotinamide to nicotinic acid for production of NAD$^+$ (Purser *et al.*, 2003). *B. burgdorferi* does not appear to harbour genes encoding enzymes required for *de novo* NAD$^+$ biosynthesis (Casjens *et al.*, 2000; Fraser *et al.*, 1997) and must therefore rely on salvage and interconversion of nicotinamide from the host for NAD$^+$ homeostasis. The *pncA* gene is dispensable for spirochaete growth in nutrient-rich *in vitro* culture medium. Spirochaetes lacking the entire lp25 plasmid are non-infectious in the mouse, but reintroduction of the *pncA* gene alone to spirochaetes lacking lp25 is sufficient to restore mouse infectivity to near-wild-type levels, demonstrating that the nicotinamidase activity encoded by *pncA* is essential for survival of *B. burgdorferi* in the mammalian environment (Purser *et al.*, 2003). Furthermore, *pncA* is critical for *B. burgdorferi* survival and replication in the tick (Grimm *et al.*, 2005; Strother & de Silva, 2005). However, the current annotation for the 5′ end of the *pncA* ORF does not include an obvious

ribosome-binding site proximal to the start codon and the predicted PncA protein is 20 aa shorter than *Escherichia coli* PncA. In addition, *B. burgdorferi* PncA lacks an N-terminal aspartic acid residue that completes the catalytic triad conserved among nicotinamidase enzymes of other bacterial species, suggesting that the encoded protein may be inactive (French *et al.*, 2010; Purser *et al.*, 2003). These observations are inconsistent with the known critical importance of PncA in the infectious cycle of *B. burgdorferi*. Therefore, herein we have characterized this *in vivo*-essential protein through genetic and biochemical identification of the N-terminal sequence of *B. burgdorferi* PncA.

METHODS

Bacterial clones and growth conditions. All low-passage *B. burgdorferi* clones are listed in Table 1 and were derived from strain B31 clone A3. *B. burgdorferi* was grown in liquid Barbour–Stoenner–Kelly (BSK) II medium supplemented with gelatin and 6 % rabbit serum (Barbour, 1984) and plated in solid BSK medium as described previously (Rosa & Hogan, 1992; Samuels, 1995). All spirochaete cultures were grown at 35 °C supplemented with 2.5 % CO_2. Kanamycin was used at 200 μg ml^{-1}.

Salmonella typhimurium wild-type strain LT2 and the isogenic *pncA*$^-$*nadB*$^-$ mutant (TT400 *sty* LT2 *pncA278*::Tn*10*d-cam *nadB215*::Tn*10*) (Zhu *et al.*, 1991) were kindly provided by S. J. Norris, University of Texas Health Science Center at Houston, TX, USA. All *S. typhimurium* strains were grown in Luria–Bertani (LB) medium, on LB agar or on M9 glucose agar (Sambrook & Russell, 2000). Strains transformed with pBSV2*TT constructs were supplemented with 50 μg kanamycin ml^{-1}.

All cloning and plasmid propagation was conducted in either *E. coli* Top10 (Invitrogen) or *E. coli* DH5α cells grown in LB medium or on LB agar supplemented with 50 μg kanamycin ml^{-1}.

Construction of pBSV2*TT vector. Vector pBSV2* (Bestor *et al.*, 2010), a derivative of pBSV2 (Stewart *et al.*, 2001), was generated by removal of a second *Eco*RI site present outside the multiple cloning site of pBSV2 by using targeted PCR mutagenesis (Bestor *et al.*, 2010). In order to prevent any potential readthrough transcription that might originate from the sequence upstream of the multiple cloning site, the Rho-independent transcription-terminator sequence for *bmpB* (Ramamoorthy *et al.*, 2005) repeated three times in tandem was cloned into the *Eco*RI site within the multiple cloning site of pBSV2*. Briefly, the forward and reverse sequences of the *bmpB* terminator repeated three times in tandem, each harbouring a 5′ *Mfe*I site and a 3′ *Eco*RI site, were synthesized as ssDNA oligos (Table 2, primers 1 and 2). To anneal the two ssDNA oligos, 2 nmol of each oligo was combined, heated to 95 °C for 10 min and cooled to room temperature over 90 min. The double-stranded terminator fragment was digested with *Mfe*I and *Eco*RI and ligated into *Eco*RI-cut pBSV2*, creating pBSV2*TT. Sequence analysis confirmed retention of a single *Eco*RI restriction site 3′ of the terminator sequence, which was used for subsequent cloning as described below.

Cloning of *pncA* fragments. A DNA fragment containing approximately 1000 bp upstream of *pncA* and the annotated PncA ORF followed by a C-terminal FLAG-epitope tag with *Eco*RI and *Xba*I sites was PCR-amplified from *B. burgdorferi* genomic DNA using primers 3 and 4 (Table 2). The resulting DNA fragment was cloned into *Eco*RI/*Xba*I-cut pBSV2*TT, yielding pBSV2*TT *pncA*-FLAG N (native). Positive clones were analysed and verified by restriction digest and DNA sequence analysis, respectively. Two fragments of the *pncA* gene, differing in the extent of their 5′ sequence (*pncA*-FLAG A and *pncA*-FLAG B) and with *Eco*RI/*Xba*I ends, were generated by PCR from *B. burgdorferi* genomic DNA using 5′ primers 5 and 6, respectively, and 3′ primer 4 (Table 2). Individual PCR fragments were cloned into *Eco*RI/*Xba*I-cut pBSV2*TT, resulting in plasmids pBSV2*TT *pncA*-FLAG A and pBSV2*TT *pncA*-FLAG B (Fig. 2). Plasmids were analysed and verified by restriction digest and sequence analysis. The constitutive *flaB* promoter including a ribosome-binding site with *Eco*RI ends was amplified from *B. burgdorferi* genomic DNA using primers 7 and 8 and cloned into the *Eco*RI-cut

Table 1. Bacterial clones used in this study

Species/clone	Description	Reference
B. burgdorferi		
A3 68-1	Low-passage clone derived from B31 (lp25$^-$, lp56$^-$)	Lawrence *et al.* (2009)
+/pBSV2*TT *pncA*-FLAG A	Harbours the shuttle vector carrying promoterless *pncA*-FLAG A	This work
+/pBSV2*TT *flaB*$_P$-*pncA*-FLAG A	Harbours the shuttle vector carrying *pncA*-FLAG A under the control of the *flaB* promoter	This work
+/pBSV2*TT *pncA*-FLAG N	Harbours the shuttle vector carrying *pncA*-FLAG N under the control of the endogenous *pncA* promoter	This work
S. typhimurium		
pncAnadB mutant	LT2 *pncA278*::Tn*10*d-cam *nadB215*::Tn*10*	Purser *et al.* (2003); Zhu *et al.* (1991)
pncA-FLAG A	Harbours the shuttle vector carrying promoterless *pncA*-FLAG A	This work
pncA-FLAG B	Harbours the shuttle vector carrying promoterless *pncA*-FLAG B	This work
pncA-FLAG N	Harbours the shuttle vector carrying *pncA*-FLAG N under the control of the endogenous *pncA* promoter	This work
flaB$_P$-*pncA*-FLAG A	Harbours the shuttle vector carrying *pncA*-FLAG A under the control of the *flaB* promoter	This work
flaB$_P$-*pncA*-FLAG B	Harbours the shuttle vector carrying *pncA*-FLAG B under the control of the *flaB* promoter	This work

Table 2. Oligonucleotides used in this study

Functional restriction-enzyme sites added to the primer are underlined. The sequence for one copy of the *bmpB* terminator is indicated in bold. The sequence for the *B. burgdorferi* codon usage-optimized FLAG epitope is indicated in italics. The engineered ATG sequence in primer 5 is indicated in lower-case letters.

Number	Name	Sequence 5′–3′
1	bmpB termX3 FWD	CCCCCCC<u>CAATTG</u>**CTTTTTTGGCTGGCTATATTGCAGCCAAAAAAAGCTTTTCG**CT TTTTTGGCTGGCTATATTGCAGCCAAAAAAAGCTTTTCGCTTTTTTGGCTGGCTA TATTGCAGCCAAAAAAAGCTTTTCGG<u>AATTCC</u>G
2	bmpB termX3 REV	CG<u>GAATTC</u>**CGAAAAGCTTTTTTTGGCTGCAATATAGCCAGCCAAAAAAG**CGAAAA GCTTTTTTTGGCTGCAATATAGCCAGCCAAAAAAGCGAAAGCTTTTTTTGGCTG CAATATAGCCAGCCAAAAAAG<u>CAATTG</u>GGGGGGG
3	pncA 5′ *Eco*RI N	CG<u>GAATTC</u>CTATGCTATCCCCTTGTTCAAGTC
4	pncA FLAG *Xba*I 3′	GC<u>TCTAGA</u>TTA*TTTATCATCATCATCTTTATAATC*TATATTAAGCTTACTTTGGCTG
5	pncA 5′ *Eco*RI A	CG<u>GAATTC</u>atgGCACTTATTTTAATAGATATAC
6	pncA 5′ *Eco*RI B	CG<u>GAATTC</u>ATGCCAGTATCTAACAGTAATG
7	flaB prom 5′ *Eco*RI	CG<u>GAATTC</u>TGTCTGTCGCCTCTTGTGGCTTCC
8	flaB prom 3′ *Eco*RI	CG<u>GAATTC</u>GATTGATAATCATATATCATTCC

plasmids pBSV2*TT *pncA*-FLAG A and pBSV2*TT *pncA*-FLAG B in front of the promoterless *pncA* genes, resulting in plasmids pBSV2*TT *flaB*$_p$-*pncA*-FLAG A and pBSV2*TT *flaB*$_p$-*pncA*-FLAG B. Plasmids were analysed and verified by restriction digest and sequence analysis.

Nicotinamidase functional-complementation assay. The *S. typhimurium pncAnadB* mutant was made chemically competent and transformed by heat shock with plasmids pBSV2*TT *pncA*-FLAG A, B and N and plasmids pBSV2*TT *flaB*$_p$-*pncA*-FLAG A and B. Transformants were selected on LB plates containing kanamycin. Individual transformants were tested for nicotinamidase activity (resulting from synthesis of a functional *B. burgdorferi* PncA protein) by growth on M9 glucose agar supplemented with 1 mM MgSO$_4$, 0.0005 % thiamine, 0.1 % Casamino acids, 50 µg kanamycin ml^{-1} and 0.1 mM either nicotinamide or nicotinic acid. Growth on LB agar plus kanamycin was used as a positive control. The production of PncA–FLAG was determined by immunoblot analysis of total protein lysates using an anti-FLAG M2 monoclonal antibody (Sigma).

Functional analysis of pBSV2*TT *pncA*-FLAG A in mice. The pBSV2*TT *pncA*-FLAG A and pBSV2*TT *flaB*$_p$-*pncA*-FLAG A plasmids were each transformed by electroporation into A3 68-1 (Lawrence *et al.*, 2009) as described previously (Jewett *et al.*, 2007) and transformants were selected in solid BSK medium containing kanamycin and confirmed by PCR using primers 4 and 5 (Table 2). Total genomic DNA was prepared from PCR-positive clones and screened for the presence of *B. burgdorferi* plasmid content (Elias *et al.*, 2002). Clones that retained the plasmid content of the parent clone were used in further experiments. The University of Central Florida (UCF) is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care. Protocols for all animal experiments were prepared according to the guidelines of the NIH and approved by the UCF's Institutional Animal Care and Use Committee. Groups of six 6–8-week-old C3H/HeN female mice were needle-inoculated as described previously (Jewett *et al.*, 2009) with 1×10^4 spirochaetes. Mouse infection was assessed 3 weeks post-inoculation by reisolation of spirochaetes from ear, bladder and joint tissues as described previously (Elias *et al.*, 2002; Grimm *et al.*, 2004).

Purification and N-terminal sequencing of PncA–FLAG. In order to purify the PncA protein from *B. burgdorferi*, the pBSV2*TT *pncA*-FLAG N plasmid was transformed into A3 68-1 (Lawrence *et al.*, 2009), as described above. The production of

PncA–FLAG in *in vitro*-grown *B. burgdorferi* was confirmed by immunoblot analysis of total protein lysate from clone A3 68-1/ pBSV2*TT *pncA*-FLAG N using anti-FLAG M2 monoclonal antibody (Sigma). Clone A3 68-1/pBSV2*TT *pncA*-FLAG N was grown to stationary phase ($>1 \times 10^8$ spirochaetes ml^{-1}) in 250 ml BSK II medium. Spirochaetes were harvested by centrifugation at 8000 r.p.m. (JA-14 rotor) for 10 min and washed in 10 ml ice-cold HN buffer (50 mM HEPES, 50 mM NaCl; pH 7.6). The washed cells were resuspended and lysed in 10 ml Cell Lytic B (Sigma) and 1 ml protease inhibitor cocktail (Sigma). The cell lysate was centrifuged for 15 min at 21 000 *g* and 4 °C to remove the cellular debris and the supernatant was stored on ice. A 3 ml column containing 500 µl anti-FLAG M2 affinity resin (Sigma) was prepared according to the manufacturer's recommendations. The soluble protein lysate was loaded onto the column and the flow-through was collected. The flow-through was reloaded and collected three times. The column was washed with 20 column volumes of TBS (50 mM Tris, 150 mM NaCl; pH 7.6). Protein was eluted from the column by the addition of 6 ml 0.1 M glycine–HCl, pH 3.5. Six 1 ml fractions were collected into 1.5 ml microfuge tubes containing 25 µl 1 M Tris, pH 8.0. Protein samples were resolved by electrophoresis on an SDS-PAGE gel (12.5 % polyacrylamide) and blotted onto nitrocellulose. Immunoblots were hybridized with anti-FLAG M2 monoclonal antibody. PncA–FLAG protein from elution 1 was resolved by electrophoresis on an SDS-PAGE gel (12.5 % polyacrylamide) and blotted onto a PVDF membrane. The PVDF membrane was stained with Coomassie brilliant blue to visualize the PncA–FLAG protein. The PncA–FLAG protein fragment was cut from the PVDF membrane and sent to the Protein Sequencing Unit of the Protein Chemistry Section of the Research Technologies Branch, NIAID, NIH, Rockville, MD, USA, for N-terminal sequencing. The nucleotide and amino acid sequences for *pncA* have been submitted to GenBank under accession no. JN223414.

RESULTS

Additional upstream coding sequence is necessary for the nicotinamidase activity of *B. burgdorferi* PncA

The *pncA* gene of *B. burgdorferi* encodes a functional nicotinamidase enzyme that is critical for *B. burgdorferi*

survival in mice and ticks (Grimm et al., 2005; Purser et al., 2003). The current annotation of the pncA ORF (GenBank accession no. NP_045429) (Casjens et al., 2000; Fraser et al., 1997) does not have an obvious proximal ribosome-binding site, encodes a protein that is 20 aa shorter than homologues in other bacteria and lacks an N-terminal aspartic acid residue found in the catalytic triad of well-characterized nicotinamidase enzymes (Purser et al., 2003). In order to explore the hypothesis that the pncA ORF contains additional 5′ coding sequence that is necessary for its enzymic activity, we took advantage of a prediction made by Purser et al. (2003), who suggested that the actual translational start site of the B. burgdorferi PncA protein may be 48 nt upstream of the current annotation (Purser et al., 2003). This sequence does not contain an in-frame start codon; therefore, we engineered an in-frame ATG start codon upstream of the 48 nt flanking sequence preceding the pncA ORF, as well

as a 3′ FLAG-epitope tag. This fragment was cloned into the B. burgdorferi shuttle vector pBSV2*TT, creating plasmid pBSV2*TT pncA-FLAG A (Fig. 1a). Although this pncA gene probably did not use the endogenous mechanism of translational regulation because of the introduction of the engineered ATG start codon, it allowed us to test the function of the newly included upstream sequence in producing an active B. burgdorferi PncA enzyme. In addition, we cloned two other pncA genes into pBSV2*TT. The pncA ORF as currently annotated, along with a single nucleotide point mutation (T to A) to create an ATG start codon [the start codon for the pncA ORF is currently proposed to be TTG (Casjens et al., 2000; Fraser et al., 1997)] and including a 3′ FLAG-epitope tag sequence, was cloned to create pBSV2*TT pncA-FLAG B (Fig. 1a). The 1000 bp pncA upstream sequence, believed to carry the endogenous pncA promoter sequence, followed by the pncA ORF including
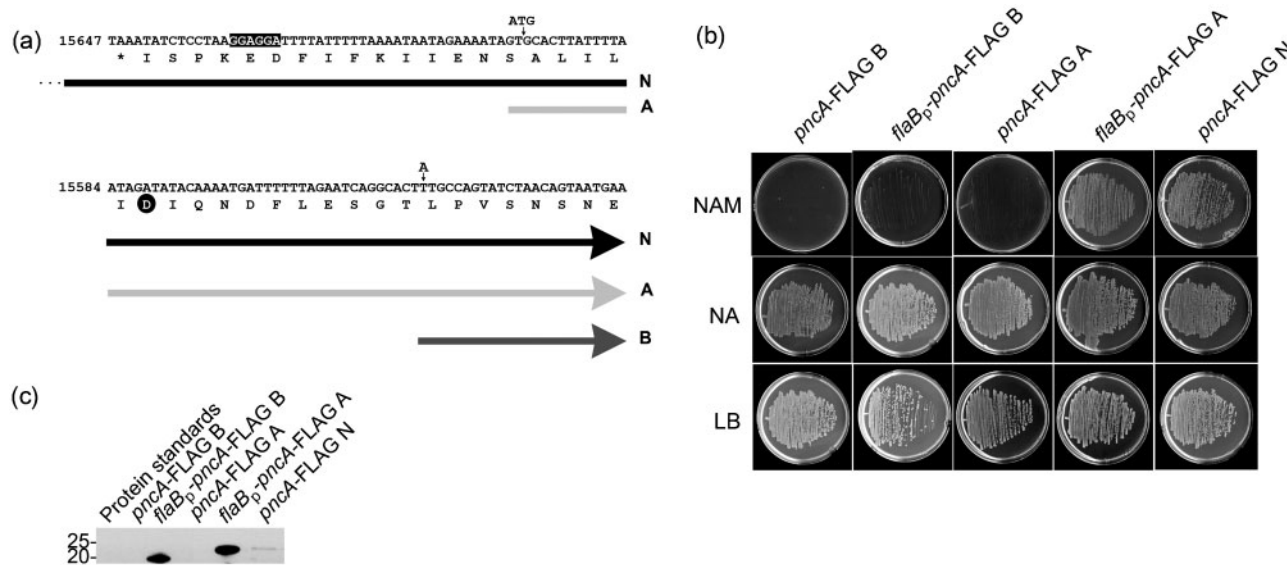
Fig. 1. Identification of the B. burgdorferi pncA sequence sufficient to encode a functional nicotinamidase enzyme. (a) Schematic representation of the three pncA-FLAG constructs used for functional complementation of an S. typhimurium pncAnadB mutant for growth on minimal medium containing 0.1 mM nicotinamide. The reverse complement of nt 15678–15558 on lp25 is shown, with the translated single-letter amino acids indicated below. The 5′ sequences of each pncA construct are represented by block arrows: pncA-FLAG N (N, black arrow; note that the 5′ end of this construct continues approximately 900 bp upstream of the sequence that is shown, as indicated by three dots), pncA-FLAG A (A, light-grey arrow) and pncA-FLAG B (B, dark-grey arrow). The putative ribosome-binding site sequence is highlighted in black. The aspartic acid residue conserved among the catalytic triad of PncA homologues is indicated by a black circle. The locations of the engineered ATG start codon for construct pncA-FLAG A, and the nucleotide point mutation (T to A) to create an ATG start codon for construct pncA-FLAG B, are indicated by small vertical arrows. (b) Functional-complementation assay for nicotinamidase activity of pncA-FLAG constructs. Representative data are shown for the overnight growth at 37 °C of the S. typhimurium pncAnadB mutant harbouring each pncA-FLAG construct (indicated above each column) on minimal agar containing 0.1 mM nicotinamide (NAM) or 0.1 mM nicotinic acid (NA). Rich agar (LB) was used as a positive control. All agar plates contained 50 μg kanamycin ml^{-1}. (c) Immunoblot analysis of PncA–FLAG production in the S. typhimurium pncAnadB mutant harbouring each pncA-FLAG gene. Total protein lysates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and probed for PncA–FLAG using an anti-FLAG M2 monoclonal antibody. Sizes of the protein standards are indicated in kDa. The predicted molecular masses for PncA–FLAG B and PncA–FLAG A are 21 and 24 kDa, respectively.

a 3′ FLAG-epitope tag sequence were cloned to create pBSV2*TT *pncA*-FLAG N (native) (Fig. 1a). Plasmids pBSV2*TT *pncA*-FLAG A and pBSV2*TT *pncA*-FLAG B both lacked promoter sequences; therefore, the *B. burgdorferi flaB* promoter including a ribosome-binding site sequence, which has been shown to function well in *E. coli* (Elias *et al.*, 2003; Stewart *et al.*, 2001), was added in order to drive transcription of the *pncA*-FLAG gene, creating expression plasmids pBSV2*TT *flaB*$_p$-*pncA*-FLAG A and pBSV2*TT *flaB*$_p$-*pncA*-FLAG B.

A genetic approach involving functional complementation of a *Salmonella* mutant lacking nicotinamidase activity was employed to investigate the sequence necessary to produce an enzymically active *B. burgdorferi* PncA protein. This approach was used successfully for the initial characterization of BBE22 (PncA) as a nicotinamidase (Purser *et al.*, 2003). The plasmids carrying the different *B. burgdorferi pncA* constructs were transformed into an *S. typhimurium pncAnadB* mutant that is unable to grow on minimal medium supplemented with nicotinamide, due to a lack of nicotinamidase activity (Purser *et al.*, 2003). *Salmonella* mutants harbouring the promoterless *pncA*-FLAG A and *pncA*-FLAG B genes were unable to grow in the presence of nicotinamide alone and did not produce detectable PncA–FLAG protein (Fig. 1b, c). However, upon introduction of the *flaB* promoter, *flaB*$_p$-*pncA*-FLAG A allowed growth of the *Salmonella* mutant on nicotinamide and production of an estimated 24 kDa PncA–FLAG protein (Fig. 1b, c), indicating that this construct harboured sufficient coding sequence to produce an active nicotinamidase enzyme. In contrast, despite the presence of a promoter to drive transcription of the *pncA* gene, *flaB*$_p$-*pncA*-FLAG B did not allow growth of the *Salmonella* mutant on nicotinamide (Fig. 1b), although a protein corresponding to the predicted size of the current annotation for PncA (21 kDa) was detected by immunoblot analysis using anti-FLAG antibodies (Fig. 1c). The *pncA* sequence of *flaB*$_p$-*pncA*-FLAG B, which follows the current annotation of the *pncA* ORF, starts 30 bp downstream of the GAT codon encoding a putative catalytic aspartic acid residue (Fig. 1a), without which a functional PncA enzyme may not be produced, even in the presence of a constitutive promoter. The plasmid pBSV2*TT *pncA*-FLAG N, presumed to harbour the endogenous *pncA* promoter region, allowed growth of the *Salmonella* nicotinamidase mutant on nicotinamide (Fig. 1b). Interestingly, *Salmonella* mutants expressing *pncA*-FLAG N produced a PncA–FLAG protein of similar size to that produced by *Salmonella* mutants expressing *flaB*$_p$-*pncA*-FLAG A, albeit at a lower level, perhaps due to a difference between the strengths of the *pncA* and *flaB* promoters in *Salmonella*. These data suggested that *pncA* promoter-driven expression of *pncA*-FLAG N in *Salmonella* was sufficient to produce a functional *B. burgdorferi* PncA enzyme to allow growth of the *Salmonella* mutant on nicotinamide alone. In addition, these data indicate that native *B. burgdorferi* PncA may be

3 kDa larger than the protein as currently annotated. All *Salmonella* transformants were able to grow on minimal medium supplemented with nicotinic acid, which bypasses the requirement for nicotinamidase activity, as well as rich medium (LB) (Fig. 1b). Together, these data demonstrate that the *pncA* ORF that includes an additional 48 nt of upstream coding sequence, when driven by the *flaB* promoter, is sufficient to produce a PncA protein with functional nicotinamidase activity and suggest that the native PncA protein harbours additional upstream sequence relative to the current annotation for the *pncA* ORF.

Expression of *pncA*–FLAG A restores mouse infectivity to spirochaetes lacking lp25

Our data indicated that a *pncA* gene including an engineered ATG start codon and 48 nt of additional sequence upstream of the annotated start of translation of the *pncA* ORF, when driven by the *flaB* promoter, produced nicotinamidase activity in a *Salmonella* functional-complementation assay (Fig. 1). The nicotinamidase activity of PncA is critical for *B. burgdorferi* mouse infectivity (Purser *et al.*, 2003). The *pncA* gene alone, under the control of its endogenous promoter, has been shown to restore near-wild-type levels of mouse infectivity to spirochaetes lacking the entire lp25 plasmid (Purser *et al.*, 2003). Therefore, we sought to determine the ability of pBSV2*TT *flaB*$_p$-*pncA*-FLAG A to complement attenuated lp25$^-$ spirochaetes *in vivo*. *B. burgdorferi* clone A3 68-1, lacking lp25 and lp56, was transformed with either plasmid pBSV2*TT *pncA*-FLAG A (promoter-negative) or pBSV2*TT *flaB*$_p$-*pncA*-FLAG A (promoter-positive) and groups of mice were inoculated with 1×10^4 spirochaetes carrying either plasmid. Three weeks post-inoculation, the number of mice infected in each group was determined by serology and reisolation of spirochaetes from tissues. Introduction of pBSV2*TT *flaB*$_p$-*pncA*-FLAG A into spirochaetes lacking the entire lp25 plasmid was sufficient to restore mouse infectivity, as five of six mice inoculated with these spirochaetes became infected. These data suggest that spirochaetes harbouring pBSV2*TT *flaB*$_p$-*pncA*-FLAG A were able to produce active nicotinamidase enzyme *in vivo*. Those spirochaetes harbouring pBSV2*TT *pncA*-FLAG A, which lacks a promoter to drive *pncA* expression, remained non-infectious, as none of six mice became infected. These data indicate that expression of an additional 48 nt of upstream coding sequence along with the *pncA* ORF resulted in *in vivo* production of a PncA protein that harbours sufficient activity to restore infectivity to spirochaetes lacking lp25. In addition, these data, along with the functional-complementation data in the *Salmonella pncAnadB* mutant, demonstrated that the presence of the FLAG-epitope tag on the C terminus of the PncA proteins did not affect the nicotinamidase activity of the functional enzymes.

The PncA protein of *B. burgdorferi* uses the non-canonical initiation codon AUU: expression and sequencing of the native PncA protein

Our data demonstrate that the addition of 48 nt of upstream coding sequence to the *pncA* ORF resulted in production of a *B. burgdorferi* PncA protein with functional nicotinamidase activity in a *Salmonella* mutant functional-complementation assay (Fig. 1b), as well as during an active *B. burgdorferi* mouse infection (see above). However, the *pncA* construct used in these experiments harboured an engineered ATG start codon and was under the control of the constitutive *flaB* promoter. Therefore, we sought to define experimentally the N-terminal amino acid sequence of the PncA protein expressed in *B. burgdorferi* under the control of its endogenous promoter. To this end, plasmids pBSV2*TT *pncA*-FLAG N and pBSV2*TT alone were each transformed into *B. burgdorferi* clone A3 68-1. Positive transformants with the plasmid content of the parent were identified and used for further studies. Native PncA–FLAG protein was produced in and purified to homogeneity from *B. burgdorferi* using anti-FLAG agarose beads (Fig. 2a, b). No FLAG epitope-tagged protein was

detected in or purified from *B. burgdorferi* carrying the *Borrelia* shuttle vector alone (data not shown). Purified PncA–FLAG was separated by SDS-PAGE, transferred to a PVDF membrane and N-terminally sequenced using Edman degradation. A single peptide was detected with the amino acid sequence MFKIIENSALILIDIQNDF (Fig. 2c). This N-terminal amino acid sequence demonstrates that the PncA protein is 24 aa longer than currently predicted and includes the critical aspartic acid residue (Fig. 3), thereby completing the catalytic triad that is conserved among nicotinamidase enzymes. Furthermore, these data revealed that the *B. burgdorferi* PncA protein uses the non-canonical initiation codon AUU, which is located 5 bp downstream of a putative ribosome-binding sequence (Fig. 2c). Together, these data identify the translational start site of the *B. burgdorferi* PncA protein.

DISCUSSION

Nicotinamidase enzymes play a critical role in maintaining NAD^+ homeostasis and have been found to contribute to important biological processes in both prokaryotes and
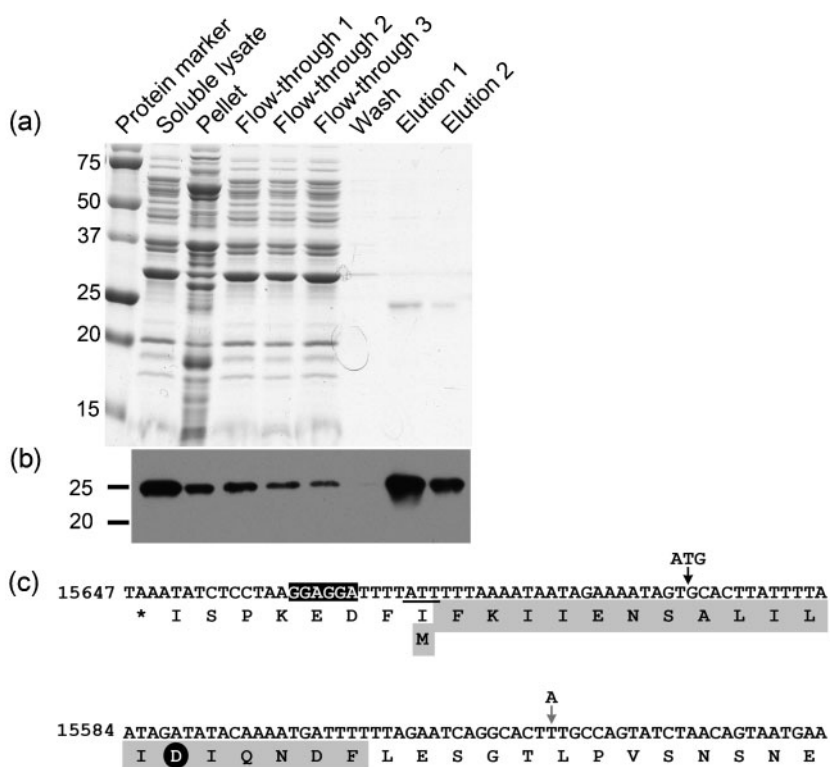
Fig. 2. Purification and N-terminal sequence analysis of PncA–FLAG protein from *B. burgdorferi*. (a) Coomassie-stained SDS-PAGE gel of protein fractions collected during purification of the PncA–FLAG protein from *B. burgdorferi*. (b) Immunoblot of the protein fractions collected during purification of the PncA–FLAG protein from *B. burgdorferi* using antibodies against the FLAG-epitope tag. Numbers to the left of panels (a) and (b) indicate the masses in kDa of the protein standards. (c) Reverse complement of nt 15647–15525 from lp25. The corresponding translated single-letter amino acid sequence is shown below the nucleotide sequence. The sequence of the first 20 aa of native purified PncA–FLAG as determined by Edman degradation and N-terminal sequencing is highlighted in grey. The putative ribosome-binding site sequence is highlighted in black. The non-canonical translational start codon of native purified PncA–FLAG (ATT) is underlined. The aspartic acid residue conserved among the catalytic triad of PncA homologues is indicated by a black circle. The locations of the engineered ATG start codon and translational start site for construct *pncA*-FLAG A, and the nucleotide point mutation (T to A) to create an ATG start codon and translational start site for construct *pncA*-FLAG B (which reflects the current proposed translational start site for the *pncA* ORF), are indicated by vertical arrows (black and grey, respectively).
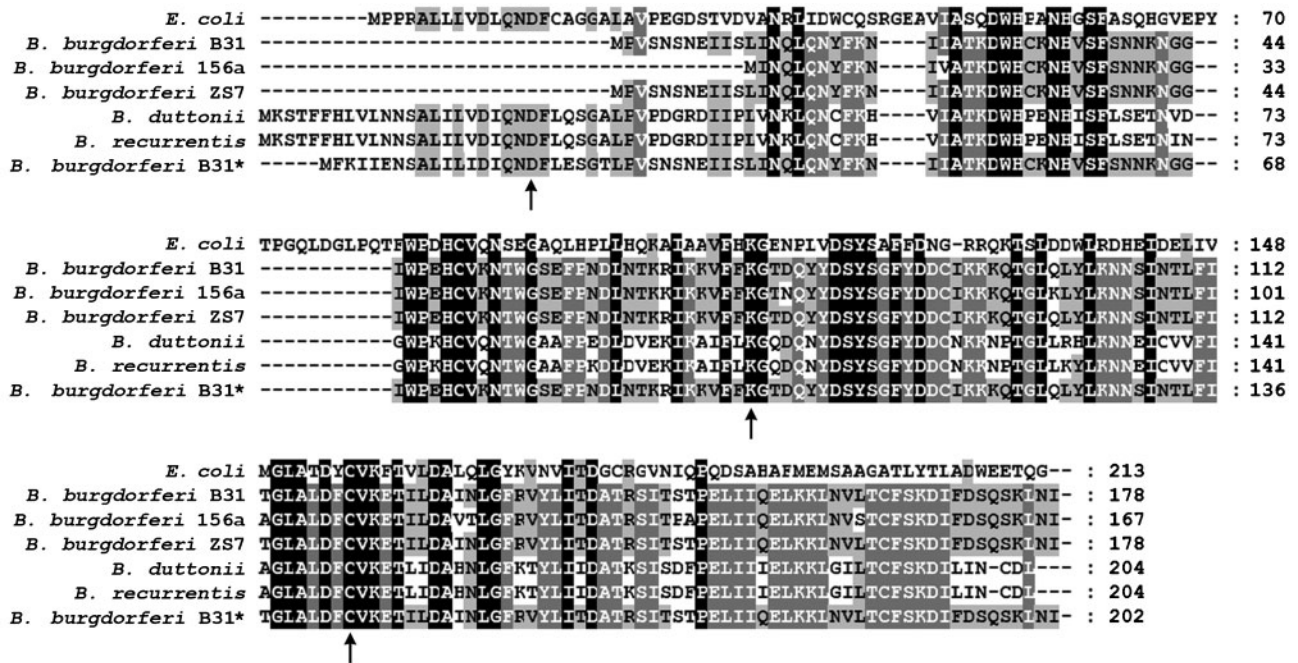
Fig. 3. Alignment of the PncA homologues from *E. coli* and *Borrelia* spp. Shown is an amino acid alignment of the PncA proteins, according to current annotation, from *E. coli* (GenBank accession no. AAC74838), Lyme disease spirochaetes *B. burgdorferi* B31 (NP_045429), *B. burgdorferi* 156a (YP_002477119.1) and *B. burgdorferi* ZS7 (YP_002455325.1), relapsing fever spirochaetes *B. duttonii* (YP_002223541.1) and *B. recurrentis* (YP_002223408.1), and the PncA protein sequence for *B. burgdorferi* B31 defined herein (*) (JN223414). Amino acids identical to the consensus sequence are shaded. Amino acid residues conserved in the catalytic triad of nicotinamidase enzymes are indicated by arrows. Dashes represent spaces introduced for optimal sequence alignment. Amino acid sequences were aligned using the CLUSTAL W algorithm in the MEGALIGN program from the DNASTAR Lasergene suite.

eukaryotes, including bacterial pathogenesis (Anderson *et al.*, 2003; Balan *et al.*, 2008; Evans *et al.*, 2010; Frothingham *et al.*, 1996; Ghislain *et al.*, 2002; Hunt *et al.*, 2007; Kim *et al.*, 2004; Minard & McAlister-Henn, 2010; Purser *et al.*, 2003; Raynaud *et al.*, 1998; Silva *et al.*, 2009; van der Horst *et al.*, 2007; Vrablik *et al.*, 2009; Wang & Pichersky, 2007). Because of the absence of nicotinamidases in mammals, these enzymes may be potential drug targets for mammalian pathogens (Sauve, 2008), including the Lyme disease spirochaete *B. burgdorferi*.

B. burgdorferi lacks the enzymes for *de novo* biosynthesis of NAD$^+$ and must therefore rely on salvage of nicotinamide from the host for survival (Casjens *et al.*, 2000; Fraser *et al.*, 1997; Purser *et al.*, 2003). The *B. burgdorferi* nicotinamidase, PncA, serves an essential physiological function required for survival of *B. burgdorferi* throughout its infectious cycle (Grimm *et al.*, 2005; Purser *et al.*, 2003). However, in conflict with the essential *in vivo* role of PncA, the current annotation of the *pncA* ORF suggests that the encoded protein may lack catalytic activity due to the absence of an N-terminal aspartic acid that is conserved in the catalytic triad of nicotinamidase enzymes from other species (French *et al.*, 2010; Purser *et al.*, 2003). Using a genetic approach involving functional complementation of

a *Salmonella* mutant lacking nicotinamidase activity, we have found that addition of 48 nt of upstream flanking sequence to the annotated *pncA* ORF results in production of an active nicotinamidase enzyme when expressed under the control of the *flaB* promoter (Fig. 1). In contrast, the *pncA* ORF as currently annotated, similarly expressed alone under the control of the *flaB* promoter, did not yield nicotinamidase activity (Fig. 1). Furthermore, the plasmid carrying the *pncA* ORF along with 48 nt of additional upstream sequence under the control of the *flaB* promoter was capable of restoring mouse infectivity to spirochaetes lacking the entire lp25 plasmid. Recently, recombinant *B. burgdorferi* PncA, expressed in and purified from *E. coli*, was found to have poor catalytic activity (French *et al.*, 2010). Although the authors acknowledged that the current annotation of the *pncA* ORF may be inaccurate and stated that additional upstream sequence was added to the *pncA* gene in their expression plasmid, it remains unclear whether sufficient sequence was used to express enzymically active PncA (French *et al.*, 2010). We have identified a *pncA* sequence that is sufficient to encode an active nicotinamidase enzyme, which supports *B. burgdorferi* survival in the mouse. These data, as well as the *in vivo* requirement for *pncA*, raise the possibility of using exogenous expression of *pncA* in spirochaetes lacking

lp25 as an *in vivo* genetic selection mechanism for shuttle-vector maintenance without the need for antibiotic selection. The requirement for *pncA in vivo* is absolute, rather than the conditional requirement for antibiotic resistance, making *flaB*$_{\text{p}}$-*pncA* a novel selection cassette with application in genetic studies *in vivo*.

Purification of native PncA from *B. burgdorferi* using a C-terminal FLAG-epitope tag followed by N-terminal sequencing determined that translation of *pncA* initiates from the rare, non-canonical initiation codon AUU, which is 72 nt upstream of the current annotation for the start codon of the PncA protein (Fig. 2). The identified PncA start codon is 5 nt downstream of the putative ribosome-binding site and produces a 203 aa protein that harbours the N-terminal aspartic acid residue conserved in the catalytic triad of PncA homologues (Fig. 3). This is the first report of a *B. burgdorferi* gene using AUU as the initiation codon. Bioinformatic analysis suggests that AUU is the initiation codon for the *pncA* ORFs of all Lyme disease spirochaetes and that these ORFs are currently annotated incorrectly (Fig. 3). In contrast, the relapsing fever spirochaetes *Borrelia recurrentis* and *Borrelia duttonii* appear to initiate translation of *pncA* using a canonical AUG initiation codon and are predicted to produce 204 aa PncA proteins, which include the conserved N-terminal aspartic acid residue (Lescot *et al.*, 2008) (Fig. 3). The few genes demonstrated to use an AUU initiation codon to initiate protein synthesis are bacterial *infC*, which encodes initiation factor 3 (IF-3) (Hu *et al.*, 1993; Liveris *et al.*, 1993; Pon & Gualerzi, 1986; Sacerdot *et al.*, 1996), *E. coli pcnB*, which encodes poly(A) polymerase (PAPI) (Binns & Masters, 2002), *orfB* of bacterial transposable IS*3* element family member IS*911* (Prère *et al.*, 2011), gp26* of bacteriophage T4 (Nivinskas *et al.*, 1992) and a small number of eukaryotic genes (Ivanov *et al.*, 2008). Genes *infC* and *pcnB* are the only two examples in which AUU is the definitive initiation codon; the other examples use AUU as an alternative initiation codon in addition to a more conventional initiation site (Binns & Masters, 2002).

Given the few characterized examples of AUU-initiated proteins, the biological significance of having an AUU start codon is unclear. Characterization of AUU-initiated translation of the *E. coli pcnB*-encoded PAPI suggests a possible role for the non-canonical start codon in the control of protein production (Binns & Masters, 2002). PAPI catalyses the template-independent addition of adenosine monophosphate to the 3′ ends of RNA species. Overproduction of *E. coli* PAPI is deleterious for cell growth (Binns & Masters, 2002; Cao & Sarkar, 1992a, b). Using experimentally modified genes, the efficiency of translation with AUU as an initiation codon has been found to be 1–13 % of that of AUG-initiated translation due to IF-3 initiation codon-mismatch discrimination and destabilization (Sacerdot *et al.*, 1996; Sussman *et al.*, 1996). Because of the reduced level of translation resulting from AUU initiation, the AUU initiation codon of *pcnB* is believed to be the mechanism that limits PAPI synthesis

(Binns & Masters, 2002). We have not found over-production of *B. burgdorferi* PncA to be cytotoxic, nor has this been reported in the literature. No increase in *B. burgdorferi* PncA production or change in spirochaete growth rate was observed when the AUU sequence was mutated to a canonical AUG (A. K. Linowski & M. W. Jewett, unpublished data). Elucidation of the role, if any, of the AUU initiation codon in translational control of *pncA* is the focus of future studies.

B. burgdorferi is an A+T-rich organism with a G+C content of only 30 mol% (Casjens *et al.*, 2000; Fraser *et al.*, 1997). The codon-usage frequency of AUU in the *B. burgdorferi* B31 genome is reported to be 55.7 %, compared with 18.1 % for AUG (http://www.kazusa.or.jp/codon/). Further bioinformatics analysis is required to determine the frequency of AUU codons as putative initiation codons, rather than encoding an isoleucine residue. Our findings stress the importance of critical evaluation of genome annotation of ORFs of interest and highlight the possibility that additional AUU-initiated ORFs in the *B. burgdorferi* genome may remain to be identified.

Together, our data have defined experimentally the complete amino acid sequence of the *B. burgdorferi* PncA protein. These findings provide a foundation for determination of the structure and catalytic function of this *in vivo*-essential protein that may contribute to rational design of nicotinamidase enzyme inhibitors that could serve as novel antimicrobials for the treatment of Lyme disease.

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REFERENCES

Anderson, R. M., Bitterman, K. J., Wood, J. G., Medvedik, O. & Sinclair, D. A. (2003). Nicotinamide and PNC1 govern lifespan extension by calorie restriction in *Saccharomyces cerevisiae*. *Nature* **423**, 181–185.

Balan, V., Miller, G. S., Kaplun, L., Balan, K., Chong, Z. Z., Li, F., Kaplun, A., VanBerkum, M. F., Arking, R. & other authors (2008). Life span extension and neuronal cell protection by *Drosophila* nicotinamidase. *J Biol Chem* **283**, 27810–27819.

Barbour, A. G. (1984). Isolation and cultivation of Lyme disease spirochetes. *Yale J Biol Med* **57**, 521–525.

Bestor, A., Stewart, P. E., Jewett, M. W., Sarkar, A., Tilly, K. & Rosa, P. A. (2010). Use of the Cre-*lox* recombination system to investigate the lp54 gene requirement in the infectious cycle of *Borrelia burgdorferi*. *Infect Immun* **78**, 2397–2407.

Binns, N. & Masters, M. (2002). Expression of the *Escherichia coli pcnB* gene is translationally limited using an inefficient start codon: a second chromosomal example of translation initiated at AUU. *Mol Microbiol* **44**, 1287–1298.

Bono, J. L., Tilly, K., Stevenson, B., Hogan, D. & Rosa, P. (1998). Oligopeptide permease in *Borrelia burgdorferi*: putative peptide-binding components encoded by both chromosomal and plasmid loci. *Microbiology* **144**, 1033–1044.

Byram, R., Stewart, P. E. & Rosa, P. (2004). The essential nature of the ubiquitous 26-kilobase circular replicon of *Borrelia burgdorferi*. *J Bacteriol* **186**, 3561–3569.

Cao, G. J. & Sarkar, N. (1992a). Identification of the gene for an *Escherichia coli* poly(A) polymerase. *Proc Natl Acad Sci U S A* **89**, 10380–10384.

Cao, G. J. & Sarkar, N. (1992b). Poly(A) RNA in *Escherichia coli*: nucleotide sequence at the junction of the *lpp* transcript and the polyadenylate moiety. *Proc Natl Acad Sci U S A* **89**, 7546–7550.

Casjens, S., Palmer, N., van Vugt, R., Huang, W. M., Stevenson, B., Rosa, P., Lathigra, R., Sutton, G., Peterson, J. & other authors (2000). A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi*. *Mol Microbiol* **35**, 490–516.

Elias, A. F., Stewart, P. E., Grimm, D., Caimano, M. J., Eggers, C. H., Tilly, K., Bono, J. L., Akins, D. R., Radolf, J. D. & other authors (2002). Clonal polymorphism of *Borrelia burgdorferi* strain B31 MI: implications for mutagenesis in an infectious strain background. *Infect Immun* **70**, 2139–2150.

Elias, A. F., Bono, J. L., Kupko, J. J., III, Stewart, P. E., Krum, J. G. & Rosa, P. A. (2003). New antibiotic resistance cassettes suitable for genetic studies in *Borrelia burgdorferi*. *J Mol Microbiol Biotechnol* **6**, 29–40.

Evans, C., Bogan, K. L., Song, P., Burant, C. F., Kennedy, R. T. & Brenner, C. (2010). NAD$^+$ metabolite levels as a function of vitamins and calorie restriction: evidence for different mechanisms of longevity. *BMC Chem Biol* **10**, 2.

Fraser, C. M., Casjens, S., Huang, W. M., Sutton, G. G., Clayton, R., Lathigra, R., White, O., Ketchum, K. A., Dodson, R. & other authors (1997). Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. *Nature* **390**, 580–586.

French, J. B., Cen, Y., Vrablik, T. L., Xu, P., Allen, E., Hanna-Rose, W. & Sauve, A. A. (2010). Characterization of nicotinamidases: steady state kinetic parameters, classwide inhibition by nicotinaldehydes, and catalytic mechanism. *Biochemistry* **49**, 10421–10439.

Frothingham, R., Meeker-O'Connell, W. A., Talbot, E. A., George, J. W. & Kreuzer, K. N. (1996). Identification, cloning, and expression of the *Escherichia coli* pyrazinamidase and nicotinamidase gene, *pncA*. *Antimicrob Agents Chemother* **40**, 1426–1431.

Ghislain, M., Talla, E. & François, J. M. (2002). Identification and functional analysis of the *Saccharomyces cerevisiae* nicotinamidase gene, PNC1. *Yeast* **19**, 215–224.

Grimm, D., Tilly, K., Byram, R., Stewart, P. E., Krum, J. G., Bueschel, D. M., Schwan, T. G., Policastro, P. F., Elias, A. F. & Rosa, P. A. (2004). Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals. *Proc Natl Acad Sci U S A* **101**, 3142–3147.

Grimm, D., Tilly, K., Bueschel, D. M., Fisher, M. A., Policastro, P. F., Gherardini, F. C., Schwan, T. G. & Rosa, P. A. (2005). Defining plasmids required by *Borrelia burgdorferi* for colonization of tick vector *Ixodes scapularis* (Acari: Ixodidae). *J Med Entomol* **42**, 676–684.

Hu, W. S., Wang, R. Y., Shih, J. W. & Lo, S. C. (1993). Identification of a putative *infC-rpmI-rplT* operon flanked by long inverted repeats in *Mycoplasma fermentans* (incognitus strain). *Gene* **127**, 79–85.

Hunt, L., Holdsworth, M. J. & Gray, J. E. (2007). Nicotinamidase activity is important for germination. *Plant J* **51**, 341–351.

Ivanov, I. P., Loughran, G. & Atkins, J. F. (2008). uORFs with unusual translational start codons autoregulate expression of eukaryotic ornithine decarboxylase homologs. *Proc Natl Acad Sci U S A* **105**, 10079–10084.

Jewett, M. W., Lawrence, K., Bestor, A. C., Tilly, K., Grimm, D., Shaw, P., VanRaden, M., Gherardini, F. & Rosa, P. A. (2007). The critical role of the linear plasmid lp36 in the infectious cycle of *Borrelia burgdorferi*. *Mol Microbiol* **64**, 1358–1374.

Jewett, M. W., Lawrence, K. A., Bestor, A., Byram, R., Gherardini, F. & Rosa, P. A. (2009). GuaA and GuaB are essential for *Borrelia burgdorferi* survival in the tick-mouse infection cycle. *J Bacteriol* **191**, 6231–6241.

Kim, S., Kurokawa, D., Watanabe, K., Makino, S., Shirahata, T. & Watarai, M. (2004). *Brucella abortus* nicotinamidase (PncA) contributes to its intracellular replication and infectivity in mice. *FEMS Microbiol Lett* **234**, 289–295.

Lawrence, K. A., Jewett, M. W., Rosa, P. A. & Gherardini, F. C. (2009). *Borrelia burgdorferi bb0426* encodes a 2′-deoxyribosyltransferase that plays a central role in purine salvage. *Mol Microbiol* **72**, 1517–1529.

Lescot, M., Audic, S., Robert, C., Nguyen, T. T., Blanc, G., Cutler, S. J., Wincker, P., Couloux, A., Claverie, J. M. & other authors (2008). The genome of *Borrelia recurrentis*, the agent of deadly louse-borne relapsing fever, is a degraded subset of tick-borne *Borrelia duttonii*. *PLoS Genet* **4**, e1000185.

Liveris, D., Schwartz, J. J., Geertman, R. & Schwartz, I. (1993). Molecular cloning and sequencing of *infC*, the gene encoding translation initiation factor IF3, from four enterobacterial species. *FEMS Microbiol Lett* **112**, 211–216.

Minard, K. I. & McAlister-Henn, L. (2010). Pnc1p supports increases in cellular NAD(H) levels in response to internal or external oxidative stress. *Biochemistry* **49**, 6299–6301.

Nivinskas, R., Vaiskunaite, R. & Raudonikiene, A. (1992). An internal AUU codon initiates a smaller peptide encoded by bacteriophage T4 baseplate gene 26. *Mol Gen Genet* **232**, 257–261.

Pon, C. L. & Gualerzi, C. O. (1986). Mechanism of translational initiation in prokaryotes. IF3 is released from ribosomes during and not before 70 S initiation complex formation. *FEBS Lett* **195**, 215–219.

Prère, M. F., Canal, I., Wills, N. M., Atkins, J. F. & Fayet, O. (2011). The interplay of mRNA stimulatory signals required for AUU-mediated initiation and programmed −1 ribosomal frameshifting in decoding of transposable element IS*911*. *J Bacteriol* **193**, 2735–2744.

Purser, J. E., Lawrenz, M. B., Caimano, M. J., Howell, J. K., Radolf, J. D. & Norris, S. J. (2003). A plasmid-encoded nicotinamidase (PncA) is essential for infectivity of *Borrelia burgdorferi* in a mammalian host. *Mol Microbiol* **48**, 753–764.

Ramamoorthy, R., McClain, N. A., Gautam, A. & Scholl-Meeker, D. (2005). Expression of the *bmpB* gene of *Borrelia burgdorferi* is modulated by two distinct transcription termination events. *J Bacteriol* **187**, 2592–2600.

Raynaud, C., Etienne, G., Peyron, P., Lanéelle, M. A. & Daffé, M. (1998). Extracellular enzyme activities potentially involved in the pathogenicity of *Mycobacterium tuberculosis*. *Microbiology* **144**, 577–587.

Rosa, P. A. & Hogan, D. (1992). Colony formation by *Borrelia burgdorferi* in solid medium: clonal analysis of *osp* locus variants. In *Proceedings of the First International Conference on Tick-Borne Pathogens at the Host–Vector Interface*, pp. 95–103. Edited by U. G. Munderloh & T. J. Kurtti. St Paul, MN: University of Minnesota.

Rosa, P. A., Tilly, K. & Stewart, P. E. (2005). The burgeoning molecular genetics of the Lyme disease spirochete. *Nat Rev Microbiol* **3**, 129–143.

Sacerdot, C., Chiaruttini, C., Engst, K., Graffe, M., Milet, M., Mathy, N., Dondon, J. & Springer, M. (1996). The role of the AUU initiation codon in the negative feedback regulation of the gene for translation initiation factor IF3 in *Escherichia coli*. *Mol Microbiol* **21**, 331–346.

Sambrook, J. & Russell, D. W. (2000). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Samuels, D. S. (1995). Electrotransformation of the spirochete *Borrelia burgdorferi*. In *Methods in Molecular Biology*, pp. 253–259. Edited by J. A. Nickoloff. Totowa, NJ: Humana Press, Inc.

Sauve, A. A. (2008). NAD$^+$ and vitamin B3: from metabolism to therapies. *J Pharmacol Exp Ther* **324**, 883–893.

Silva, R. M., Duarte, I. C., Paredes, J. A., Lima-Costa, T., Perrot, M., Boucherie, H., Goodfellow, B. J., Gomes, A. C., Mateus, D. D. & other authors (2009). The yeast PNC1 longevity gene is up-regulated by mRNA mistranslation. *PLoS One* **4**, e5212.

Stewart, P. E., Thalken, R., Bono, J. L. & Rosa, P. (2001). Isolation of a circular plasmid region sufficient for autonomous replication and transformation of infectious *Borrelia burgdorferi*. *Mol Microbiol* **39**, 714–721.

Strother, K. O. & de Silva, A. (2005). Role of *Borrelia burgdorferi* linear plasmid 25 in infection of *Ixodes scapularis* ticks. *J Bacteriol* **187**, 5776–5781.

Sussman, J. K., Simons, E. L. & Simons, R. W. (1996). *Escherichia coli* translation initiation factor 3 discriminates the initiation codon *in vivo*. *Mol Microbiol* **21**, 347–360.

Tilly, K., Grimm, D., Bueschel, D. M., Krum, J. G. & Rosa, P. (2004). Infectious cycle analysis of a *Borrelia burgdorferi* mutant defective in transport of chitobiose, a tick cuticle component. *Vector Borne Zoonotic Dis* **4**, 159–168.

Tilly, K., Rosa, P. A. & Stewart, P. E. (2008). Biology of infection with *Borrelia burgdorferi*. *Infect Dis Clin North Am* **22**, 217–234, v.

van der Horst, A., Schavemaker, J. M., Pellis-van Berkel, W. & Burgering, B. M. (2007). The *Caenorhabditis elegans* nicotinamidase PNC-1 enhances survival. *Mech Ageing Dev* **128**, 346–349.

Vrablik, T. L., Huang, L., Lange, S. E. & Hanna-Rose, W. (2009). Nicotinamidase modulation of NAD$^+$ biosynthesis and nicotinamide levels separately affect reproductive development and cell survival in *C. elegans*. *Development* **136**, 3637–3646.

Wang, G. & Pichersky, E. (2007). Nicotinamidase participates in the salvage pathway of NAD biosynthesis in *Arabidopsis*. *Plant J* **49**, 1020–1029.

Zhu, N., Olivera, B. M. & Roth, J. R. (1991). Activity of the nicotinamide mononucleotide transport system is regulated in *Salmonella typhimurium*. *J Bacteriol* **173**, 1311–1320.

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