Ticks and Tick-borne Diseases xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

The *folA* gene from the *Rickettsia* endosymbiont of *Ixodes pacificus* encodes a functional dihydrofolate reductase enzyme

James L. Bodnar, Sergio Fitch, Allison Rosati, Jianmin Zhong*

Department of Biological Sciences, Humboldt State University, 1 Harpst Street, Arcata, CA, 95521, USA

ARTICLE INFO

Keywords:
Rickettsia species phylotype G021
Ixodes pacificus
Dihydrofolate reductase
Recombinant protein
In vitro enzymatic assay

ABSTRACT

Although nonpathogenic bacterial endosymbionts have been shown to contribute to their arthropod host's fitness by supplying them with essential vitamins and amino acids, little is known about the nutritional basis for the symbiotic relationship of endosymbionts in ticks. Our lab has previously reported that *Rickettsia* species phylotype G021 in *Ixodes pacificus* carries all five genes for de novo folate synthesis, and that these genes are monophyletic with homologs from other *Rickettsia* species. In this study, the rickettsial folate synthesis *folA* gene, coding for dihydrofolate reductase, was PCR amplified, cloned into an expression vector, and overexpressed in *E. coli*. Bioinformatic analysis identified that the FolA protein of phylotype G021 has the conserved DHFR domain, NADP binding sites, and substrate binding sites of bacterial dihydrofolate reductase. SDS-PAGE results showed that recombinant rickettsial FolA protein was overexpressed in BL21(DE3) *E. coli* in its soluble form. Affinity chromatography was used to purify the protein, and in vitro enzyme assays were performed to assess the biochemical activity of dihydrofolate reductase. The specific activity of recombinant FolA from phylotype G021 was determined to be 16.1 U/mg. This study has revealed that *Rickettsia* species phylotype G021 of *I. pacificus* is capable of producing a functional enzyme of the folate biosynthesis pathway, addressing the nutritional interactions behind the symbiosis between *Rickettsia* species phylotype G021 and its host.

1. Introduction

Among invertebrate animals, arthropods are especially prone to establishing symbiotic relationships with intracellular bacteria (Rio et al., 2016; Zug and Hammerstein, 2015). These relationships contribute to the ecological success of arthropods by providing protection from natural predators (Tsuchida et al., 2010) and parasite induced mortality (Brownlie and Johnson, 2009), producing detoxifying enzymes for degradation of insecticides (Indiragandhi et al., 2007), increasing heat tolerance (Moran and Yun, 2015) and reproductive success (Himler et al., 2011), and conferring direct fitness benefits under conditions of nutritional stress (Brownlie and Johnson, 2009). There are many examples of nutrient provisioning by intracellular bacteria to arthropods with low nutrient diets (Baumann, 2005; Dale and Moran, 2006; Moran, 2006). In fact, the reduced genomes of arthropod symbionts have retained only the genes required for maintaining a symbiotic lifestyle: those involved in host fitness and indispensable molecular processes, such as DNA and protein synthesis (Dale and Moran, 2006; McCutcheon and Moran, 2011; Moran, 2006).

The western blacklegged tick, *Ixodes pacificus,* is a vector of *Borrelia burgdorferi* and *Anaplasma phagocytophilum*, the causative agents of Lyme borreliosis and anaplasmosis, respectively, in the western United States and western Canada (Lane et al., 1994; Reubel et al., 1998; Richter et al., 1996). *I. pacificus* is an ectoparasite and relies on a strict host blood diet that contains very low concentrations of nutrients and essential vitamins (Rio et al., 2016). *I. pacificus* feeds on vertebrate hosts three times in their life, each occurring before moving on to the next stage of the life cycle (Padgett and Lane, 2001). As revealed by the *I. scapularis* (a close relative of *I. pacificus*) genome project (NCBI Bioproject Accession number PRJNA34667), some of the genes required for synthesizing vitamins such as folate (vitamin B9) are not possessed by ixodid ticks. Remarkably, juveniles and adults of *I. pacificus* are capable of surviving for up to a year between blood meals (Padgett and Lane, 2001).

Physiological changes occur in response to a blood meal including engorgement and molting. During the early phase of the blood meal, a period of intermolt growth is undertaken in which a new cuticle and visceral tissues are grown (Sonenshine, 1993). Based on this knowledge, research has been aimed at investigating the mechanisms by which the tick is capable of surviving on the limited nutrients available in blood, with such long periods between feedings, and growing new tissues before molting to the next life stage without an abundant

* Corresponding author at: Department of Biological Sciences, Humboldt State University, 1 Harpst Street, Arcata, CA, 95521, USA.
E-mail addresses: jlb264@humboldt.edu (J.L. Bodnar), sf935@humboldt.edu (S. Fitch), anr47@humboldt.edu (A. Rosati), jz15@humboldt.edu (J. Zhong).

https://doi.org/10.1016/j.ttbdis.2017.12.013
Received 4 August 2017; Received in revised form 7 December 2017; Accepted 16 December 2017

vitamin source. A major obstacle in understanding tick biology is the paucity of information concerning the functions of endosymbiotic bacteria. For example, little light has been shed on the nutritive exchanges choreographing the symbiotic architecture of endosymbionts in ticks.

A novel species of endosymbiotic *Rickettsia* classified as *Rickettsia* species phylotype G021 was discovered in *I. pacificus* ticks by PCR and sequencing. Phylotype G021 is grouped with the Spotted fever group rickettsiae with close relation to *R. buchneri*, *R. akari*, and *R. australis* (Phan et al., 2011). Our lab reported that each *I. pacificus* tick carries phylotype G021 (Cheng et al., 2013b), and that the bacterium is passed through inheritance and maintained through all four stages of tick development (Cheng et al., 2013a). Inferences have been made regarding the virulence of *Rickettsia* species phylotype G021 based on the ubiquitous prevalence and the 100% efficiency of transovarial transmission and transstadial passage of this species in *I. pacificus* compared to its fellow *Rickettsia* species phylotype G022 in *I. pacificus* (Cheng et al., 2013a; Cheng et al., 2013b). The authors suppose that phylotype G021 is nonpathogenic, but further characterization is required. Thus far, no data has been shown that would suggest that *Rickettsia* species phylotype G021 induces pathogenic effects in vertebrates. Furthermore, the closely related *R. buchneri* in *I. scapularis*, and *R. peacockii* show no indications of pathogenicity in vertebrates (Felsheim et al., 2009; Kurtti et al., 2015; Weller et al., 1998).

Although the function of the *Rickettsia* species phylotype G021 in *I. pacificus* is unknown, recent metabolic reconstructions carried out in our lab showed that all five genes of the folate (vitamin B9) biosynthetic pathway exist in the genome of *Rickettsia* species phylotype G021, including *folA, folC, folE, folkP*, and *ptpS* (Hunter et al., 2015). These are the genes necessary to complete folate synthesis (Hanson and Gregory, 2002; Pribat et al., 2009). These data, along with the nutrient poor diet, nature of life cycle, and lack of folate biosynthesis capabilities of *I. pacificus*, are convincing evidence of nutrient provisioning by phylotype G021.

In this report, we used rickettsial FolA as an indicator into the nature of the symbiosis between phylotype G021 and *I. pacificus* ticks, specifically the rickettsial folate biosynthesis in *I. pacificus*. The ability of *Rickettsia* species phylotype G021 to synthesize the essential vitamin B9 has been determined through bioinformatic gene annotation followed by isolation and biochemical characterization of the recombinant FolA protein. If it can be demonstrated that *Rickettsia* species phylotype G021 does indeed generate functional folate biosynthesis enzymes, further research may be aimed at in vivo evaluation of the specific benefits received by the *I. pacificus* ticks harboring them.

2. Materials and methods

2.1. Materials

N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) buffer was purchased from Acros Organics (Geel, Belgium). Dihydrofolic acid (DHF) and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Aldrich (St. Louis, MO). BSA was purchased from Promega (Madison, WI).

2.2. Tick collection and DNA extraction

Flat ticks collected from Humboldt county California (GPS coordinate: N 40 55.200 W 123 50.400) in March 2012 were identified by light microscopy using key features of the *Ixodes pacificus* species according to the standard morphological key (Furman and Loomis, 1984). The ticks were ground with a pestle (Fisher Scientific, Tampa, FL) in liquid nitrogen and boiled in sealed Eppendorf tubes with a heating block at 100 °C for 15 min in 100 µl of 0.7 M ammonium hydroxide, followed by heating at 100 °C for 10 min to remove the ammonia (Guy and Stanek, 1991). The extracted DNA was quantified using a Nanodrop ND-1000 spectrophotometer at OD_{260} (Thermo Fisher Scientific, Waltham, MA) and stored at −20 °C.

2.3. Amplification of the rickettsial folA gene by polymerase chain reaction (PCR)

The complete coding sequence for the *folA* gene of phylotype G021 was amplified using PCR. Primers were designed using sequence data of the *folA* gene previously obtained in our lab (Hunter et al., 2015). The open reading frame (ORF) was amplified using the following primers: forward primer 5′-GAAT**GACAAATGTC**AAATGAAAAATAGAAAAATC ATCGGTATAATGG−3′; reverse primer 5′-GATT**GACACGAGTC**TTACC TCCTTTTAGTAAATTTATAAATCTGATAATTA−3′ (Elim Biopharmaceuticals, Hayward, CA), both containing the *Psh*AI restriction enzyme recognition site (shown in bold). The amplification reaction contained 50 ng genomic DNA from *I. pacificus,* 1X EconoTaq Plus Green Master Mix (0.1 units/µl EconoTaq DNA polymerase, 50 mM Tris-HCL, pH 9.0, 50 mM NaCl, 0.1 mg/ml BSA, 200 µM dNTPs, 1.5 mM $MgCl_2$) (Lucigen, Middleton, WI), and 1 µM forward and reverse primers in 25 µl of total volume. The PCR cycling conditions consisted of a single cycle at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, primer annealing at 50.3 °C for 1 min and extension at 72 °C for 1 min 20 s. The PCR included a final extension cycle at 72 °C for 7 min. The amplified product was subjected to electrophoresis in 1X TAE buffer (40 mM Tris, pH 8.6, 20 mM acetate, 1 mM EDTA) using a 1% agarose gel and visualized by ethidium bromide (0.5 µg/ml) staining. The gel image was documented using an AlphaImager HP (Alpha Innotech, San Leandro, CA).

2.4. Construction of expression plasmid and DNA sequencing

The pET-41a(+) expression vector (EMD Millipore, Billerica, MA) was used for cloning and subsequent protein expression. This plasmid expresses recombinant proteins under control of a T7 lac promoter for high levels of expression, attaches an N-terminal GST fusion tag for affinity purification, and has a kanamycin resistance gene for transformant selection.

The amplified rickettsial *folA* gene PCR product was gel purified and ligated into the linearized, dephosphorylated pET-41a(+) expression vector using T4 DNA ligase (New England Biolabs, Ipswich, MA). The ligation reaction was transformed into NovaBlue competent cells (EMD Millipore, Billerica, MA) according to the manufacturer's protocol. Transformed cells were plated on LB–kanamycin (50 mg/L) and incubated overnight at 37 °C. Individual colonies were inoculated in LB broth plus 50 mg/L kanamycin. Plasmid DNA was then purified using the Wizard *Plus* SV Minipreps DNA Purification System (Promega, Madison, WI), according to the manufacturer's centrifugation protocol. The purified clone DNA was quantified by Nanodrop at OD_{260} and stored at −20 °C.

folA clone DNA was sequenced at Elim Biopharmaceuticals (Elim, Hayward, CA) with vector specific forward and reverse primers (Forward primer: 5′-AAGAAACCGCTGCTGCTAAA−3′; Reverse primer: 5′-AAGCTTGTCGACGGAGCT-3′), whose sequences are located upstream and downstream of the target gene, respectively. Sequences were uploaded into CodonCode Aligner (CodonCode Corporation, Centerville, MA) and the sequences with the highest quality were chosen for analysis. The GenBank accession number of the *folA* gene of phylotype G021 is KT225568.

To serve as a control for the subsequent in vitro enzymatic assay, the *gltA* gene of phylotype G021 was also PCR amplified and cloned in the pET-41a(+) expression vector. The construct was confirmed by DNA sequencing at Elim Biopharmaceuticals (Elim, Hayward, CA). The GenBank accession number of the *gltA* gene of phylotype G021 is ALS88439.