**University of South Bohemia in České Budějovice**

Faculty of Science

# Differential expression of genes in replicative (spiral) and persistent (biofilms) forms of the causative agent of human Lyme borreliosis

Bachelor Thesis

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## Annotation

The onset and development of post treatment Lyme disease syndrome (PTLDS) remains a constant challenge in the diagnosis and treatment of Lyme borreliosis (LB). The driving force behind this is the formation of persisters cells, which are able to hide within the host, "invisible" to the immune system and resistant to antibiotic treatment. Thus far, few types of persisters have been found to be formed by the *Borrelia burgdorferi* spirochete, with biofilms being one of them. Multiple atypical forms of spirochetes often are uneasy to be detected via the average testing methods, leading to many cases being misdiagnosed. Additionally, the formation of biofilms, as a resilient complex community of cells, has not only been observed in case of Lyme disease, but also in other illnesses worldwide. This thesis deals with the analysis of genes differentially expressed in biofilms in comparison to the replicating forms of spirochetes, with aim to better understand the formation of these morphotypes.

## Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.
České Budějovice, 11/05/2023

……………………………...
Sabrina Vranjes

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## List of abbreviation

| Abbreviation | Definition |
|---|---|
| ACA | Acrodermatitis chronica atrophicans |
| AFM | Atomic force microscope |
| ATL | Tissue lysis buffer |
| AW | Washing buffer |
| bb sl | Borrelia burgdorferi sensu lato |
| Bb ss | Borrelia burgdorferi sensu stricto |
| BmpA | basic membrane protein A |
| BSA | Bovine serum albumin |
| BSK | Barbour-Stoenner-Kelly |
| CDC | Centers for Disease Control and Prevention |
| cDNA | Circular DNA |
| CFS | Cystic Fibrosis Serum |
| Clp | Caseinolytic protease |
| dd$H_2O$ | Double-distilled water |
| dNTP's | Deoxyribonucleotide triphosphates |
| dps | Gene involved in DNA repair and protection |
| eDNA | Extracellular DNA |
| EDTA | Ethylenediaminetetraacetic acid |
| EM | Erythema migrans |
| EPS | Extracellular polymeric substance |
| GBP | Glucan-binding protein |
| glpD | Gene encoding glycerol-3-phosphate dehydrogenase |
| HipA | Serine/threonine kinase |
| HSP | heat shock protein |
| LB (medium) | Luria-Bertani medium |
| metG | Methionyl-tRNA synthetase |
| Osp | Outer surface protein |
| oxyR | Oxidative stress regulator |
| PCR | Polymerase Chain reaction |
| plsB | Glycerol-3-phosphate 1-O-acyltransferase |
| ps | phylostratum |
| psi | Pounds per square inch |
| psp | Phage shock protein |
| qRT-PCR | Quantitative real-time reverse transcription PCR |
| RB | Round bodies |
| RT-PCR | Reverse transcription PCR |
| TAE | Tris-acetate-EDTA |
| TBE | Tris-borate-EDTA |
| tkA | Translocase A |
| UV | ultraviolet |
| Lp | Linear plasmid |
| cp | Circular plasmid |

# Abstract

Lyme borreliosis is a multisystem illness caused by the spiral bacterium of the *Borrelia burgdorferi* sensu lato complex. The disease is wildly distributed in the Northern Hemisphere, with the number of infections increasing in recent years. Although most cases are successfully treated with antibiotics, in some cases the disease progresses into a persistent infection resulting in the development of post-treatment Lyme disease syndrome (PTLDS). This chronic form of the disease is, in large part, linked to the formation of persistent forms of spirochetes, which are able to evade the hosts immune system and resist antibiotic treatment through the differential regulation of their gene expression. Biofilm aggregates are a complex community of cells and constitute one type of persister formed by the *Borrelia* species. Currently there are no effective diagnostic or therapeutic protocols for the detection and elimination of the persistent forms in human patients.

This thesis aims to investigate and identify some genes differentially expressed in biofilms in comparison to replicative forms and demonstrate their difference at a transcriptome level. In this study we present the examples of the genes that are up-regulated in biofilm-dominated cultures and down-regulated in spirochetes. Additionally, we investigate the sequences of these genes with the aim to analyze their functions.

# Summary

The differential expression of genes in biofilms and replicating spirochetes of *Borrelia burgdorferi* sensu stricto was investigated *in vitro*. A comprehensive proteomics analysis, conducted at the Faculty of Sciences USB, was used as a basis for this study, from which proteins exhibiting differential expression between the two morphotypes were selected. Their corresponding nucleotide sequences were obtained from UNIPROT database, which allowed specific primers to be designed. The gene-specific primers were tested using RT-PCR with replicative spirochete and biofilm RNA as templates and analyzed by gel electrophoresis. Results revealed an overexpression of two genes BB_A40 and BB_0383 in biofilm-dominated cultures. The genes were sequenced and showed a high percentage of similarity with the nucleotide sequence obtained from the original proteins defined by proteomics analysis. These findings confirm the differential expression of genes upon biofilm formation, and also could be used to develop novel testing methods directed at biofilms. More research must be conducted in order to confirm these findings.

# Table of Contents

1. Introduction ... 1
1.1 *Borrelia Burgdorferi* Sensu Stricto ... 1
1.2 Lyme Borreliosis (LB) ... 1
1.2.1 Stages of Lyme Borreliosis ... 2
1.3 Persisting forms of *borrelia* ... 3
1.3.1 Granules/ Blebs ... 5
1.3.2 Cell Wall Deficient Forms (L-Forms) ... 6
1.3.3 Cystic Forms ... 6
1.4 Biofilms ... 7
1.5 Genetic Factors Involved In Persistence ... 9
2. Materials ... 11
3. Methods ... 14
3.1 *Borrelia* Cultivation ... 14
3.1.1 Preparation of Modified Kelly-Pettenkoffer (MKP) Medium for The Cultivation of *Borrelia* Cultures ... 14
3.2 Genomic DNA Isolation ... 15
3.3 RNA Isolation ... 16
3.4 Polymerase Chain Reaction (PCR) Analysis ... 17
3.4.1 Standard PCR ... 17
3.4.2 One-Step Reverse Transcription (RT) PCR ... 18
3.4.3 Preparation of Reactions And Procedure ... 18
3.5 Gel Electrophoresis ... 20
3.6 Cloning of PCR Products ... 21
3.6.1 Ligation of PCR Product ... 21
3.6.2 Transformation of Competent Cells ... 21
3.6.3 Preparation of Luria-Bertani (LB) Liquid Medium ... 22
3.6.4 Preparation of LB Agar Plates ... 22
4. Results ... 22
4.1 *Borrelia* Cultivation ... 22
4.2 RNA Isolation ... 23
4.3 Results Summary ... 24
4.3.1 Sequencing Results ... 27
4.3.2 Gene Bb_A40 ... 28
4.3.3 Gene Bb_0383 ... 29
5. Discussion ... 30
6. Literature: ... 33
7. Supplemented Materials ... 36
7.1 Sequences ... 36
7.2 Primer Pairs ... 47

# 1. Introduction

## 1.1 *Borrelia burgdorferi sensu stricto*

*Borrelia burgdorferi* sensu lato complex (*Bb* sl) is a group of corkscrew-shaped bacteria that belong to the family *Spirochaetocea*, class Spirochetes. It is a tick-borne pathogen that moves between a vertebrate host and tick vector. Today, *Bb* sl complex consists of 23 already described species widely distributed around the world. Five spirochete species within this complex are responsible for the major number of causes of human Lyme borreliosis (LB) worldwide. These include: *Borrelia garinii, Borrelia bavariensis, Borrelia afzelii, Borrelia burgdorferi* sensu stricto and *Borrelia mayonii*. Some other species, which pathogenicity to humans is still under investigation include: *Borrelia spielmanii, Borrelia valaisana, Borrelia bissettii, Borrelia kurtenbachii and Borrelia lusitaniae*. The other thirteen species from *Bb* sl complex were never detected in humans or isolated from samples of human origin. Because of this, until now they are considered to be non-pathogenic to humans.

*Borrelia burgdorferi* sensu lato spirochetes, known as Lyme borreliosis (LB) spirochetes, are primarily transmitted by ticks from *Ixodes ricinus* complex, which are the main vectors for the pathogen worldwide. The main Ixodes species of medical importance are *Ixodes scapularis* and *Ixodes pacificus* (North America, mainly USA) and *Ixodes ricinus* and *Ixodes persulcatus* (Europe and Asia).

## 1.2 Lyme borreliosis (LB)

Lyme borreliosis is a multisystem disorder caused by selected species of spirochetes from *B. burgdorferi* sl complex. Even though only 5 species were confirmed to be significantly pathogenic to humans, the number of diagnosed cases increases yearly. Before 2013, existing epidemiological evidence and conscientious estimates maintained the official figures of confirmed cases in Europe at 85,000 annually as well as around 30,000 each year in the United States [1]. The year of 2013 marked a watershed moment in the statistical outlook, as well as the scope of the disease. The Centers for Disease Control and Prevention, a globally respected epidemiological institute, issued a statement declaring that roughly 329,000 new LB cases were identified yearly in the U. S. between 2005 and 2010, tenfold greater than initially

thought [2, 3]. Furthermore, it was disclosed that approximately 476,000 LB infections were confirmed and treated every year in the U. S. from 2010 and 2018. In a motion on LB (2018/2774 (RSP)) of the European Parliament, which estimated the existence of around 850,000 new infections with LB annually, the growth in newly diagnosed cases in Europe was also emphasized [4].

The most common sign for LB is the appearance of round skin lesions referred to as erythema migrans (EM) in the early stage of the disease. EM can be caused by multiple species of the *Bb* sl complex and are often accompanied by flu-like symptoms. Once the bacterium disseminates via the bloodstream to the secondary sites of infection it can induce a whole array of clinical manifestations. *Borrelia* species differ in their organ tropisms and induce a wide range of signs and symptoms. The most prevalent musculoskeletal manifestation of *B. burgdorferi* s.s. exposure is Lyme arthritis. These transient or recurrent incidents of arthritis affect around 60% of patients with undiagnosed, early signs of infection - erythema migrans [5]. In Europe, where *B. garinii* and *B. afzelii* are far more prevalent than *B. burgdorferi* s.s., the number of LB patients suffering from arthritis drops to 5-25% [6]. Studies on the serotypes of European strains show a striking association between *B. garinii* infection and neuroborreliosis. It must be noted that both *Borrelia burgdorferi* s.s. and *Borrelia afzelii* can induce neurological symptoms as well, though not as often as *B. garinii* [7, 8]. *B. afzelii* appears to primarily affect the epidermis in humans, cause lymphadenosis benigna cutis, and acrodermatitis chronica atrophicans (ACA) [9, 10]. While not the only cause of ACA, *B. afzelii* predominates in the disease's etiology. In truth *B. garinii* was also identified in ACA cases [8, 11]. While ACA has seldom been documented in the U. S., it can be found in roughly 10% of LB cases in Europe [12]. In patients from Europe, a relationship between *B. bissettii* and cardiovascular LB symptoms was found [13,14].

### 1.2.1 Stages of Lyme borreliosis

The progression of LB follows three main stages: 1) early localized (acute) stage, 2) early dissemination stage and 3) the late dissemination stage.

The early acute stage is characterized by unique EM skin lesions at the site of the tick bite experienced by approximately 70-80% of patients [15]. EM usually appears after an incubation period of 3-32 days (mostly within 7 to 10 days) post infection with the rash expanding to approximately 7cm diameter [16]. In the absence of EM this stage of the disease

can easily go unnoticed, rendering treatment options unavailing. In some cases, the infection remains local with minor inherent symptoms - in others the spirochetes disseminate hematogenously further into the host and instigates the second stage of LB.

The early dissemination stage occurs weeks to months after initial infection and may include the formation of smaller secondary EM lesions independent from the location of the tick bite. Further migration of the bacterium through the bloodstream and/ or lymphatic system and eventual invasion of distal organs gives rise to a multitude of clinical manifestations. These include carditis if spread to the heart tissue, meningitis accompanied by facial palsy if infection is established in the lining of the brain, arthritis, ophthalmic symptoms and a variety of neurologic symptoms [17]. The ability of *Borrelia* to penetrate the distant sites of secondary colonization proves essential for the evasiveness of the disease.

The final stage of the illness - late dissemination stage, develops months to years after the onset of infection. At this point the disease has fully spread throughout the body and manifests Lyme arthritis, acrodermatitis chronica atrophicans and neurological symptoms such as numbness in extremities, insomnia and behavioral changes [4, 5]. Within six months of the original infection, Lyme arthritis, which occurs in roughly 60% of patients, ranks as the second-most common symptom of this stage [18]. The arthritis is oligoarticular, asymmetrical and monoarticular, proving to be particularly debilitating, as it most frequently affects the knee joints.

Most cases of Lyme borreliosis, if detected early, can be successfully treated with a 2- to 4-week course of antibiotics. However, in some cases the bacteria are able to survive this treatment and evading the host's immune response, resulting in post-treatment LD syndrome (PTLDS). The International Lyme and Associated Disease Society attributes this to the likelihood of the infection going unnoticed and the inaccuracy of conventional laboratory testing methods [19]. The etiology of PTLDS is unknown, however several illness-causing mechanisms have been hypothesized, the main one being microbial persistence.

## 1.3 Persisting forms of *Borrelia*

Several different morphologies of spirochetes have been identified: spiral-shaped spirochetes, round bodies, blebs or cystic forms. The presence of these diverse morphologies may

contribute to the spirochete ability to evade the host immune system and survive in the host tissues [20].
The term "persister" was first coined by Bigger  and stems from its precursor "survivor", which referred to a small population of bacterial *Staphylococcus* that survived penicillin treatment in 1944  [7, 8]. Unfortunately, research on these cell types was limited by their relative rarity and it took several decades before reaching clinical relevance. Ultimately, Moyed and Bertrand encountered an atypical culture of *Escherichia coli*, which produced a substantial amount of persister cells, shedding light on the feasibility of these unusual phenotypes among the scientific community. Persister cells are unique in their ability to survive antibiotic treatment without having acquired this trait by genetic inheritance, which sets them apart from resistant mutants [22]. Their formation is instigated by hostile changes to their environment, when they undergo a series of morphological changes that lead in their survival. The underlying mechanism by which persisters forms were traced back to regulatory genes, facilitating several parallel processes responsible for the suppression of metabolic activity [23]. Although persister formation leads to a state of suppressed metabolic activity comparable to cell death, these pleomorphic forms are reversible, meaning that as soon as external conditions become favorable again, they can transform back into active, replicating spirochetes and cause a relapse of the infection.

There is evidence that *Borrelia* uses a variety of tactics to bypass host defenses and create long-lasting infections. These strategies include active immune suppression, induction of immunological tolerance, and the utilization of phase and antigenic diversity, for example. Spirochetes can also evade the host's immune system by hiding in the extracellular matrix and not using host tissues for replication or growth. The successful survival highlights *Borrelia's* adaptability and persistence, rendering eradication difficult and attributing to therapeutic shortcomings. Multiple studies have shown that *Borrelia* can establish persistent infections in diverse hosts, including humans. The hosts' immune responses, antibiotic treatment, and infection routes can all impact the ability of spirochetes to persist. More research of the mechanisms underlying survival strategies, discovering of their potential in therapeutic practice, could lead to better methods for treating persistent infections.

Indeed, persisters encompass a multi-drug tolerant subpopulation of bacteria that have the ability to change in response to environmental fluctuations. This adaptability to hostile pressure proves essential in the *Bb* life cycle, as they move from tick vectors to reservoir

hosts, facilitating their adjustment to differing blood pH and temperature during transfer. Spirochete forms are highly heterogenous, differing on the environment in which they reside (*in vivo* or *in vitro*), which further challenges their eradication. According to recent research [1, 2] heterogeneous persisters have varying susceptibilities to a wide range of antibiotics. *In vitro* studies of three morphological forms of *Borrelia* demonstrated that five antimicrobials routinely used to treat LB patients (doxycycline, amoxicillin, tigecycline, metronidazole, and tinidazole) had varying efficacy in eliminating spirochetes, round bodies, and biofilm-like colonies [25]. Though all five medications demonstrated considerable mortality of the spirochete forms (85-90%) and round bodies (68-90%), none of these treatments were able to suppress replicative colony formation by over 55%. Differential expression of genes, such as downregulation of the outer surface protein A (OspA) tied to innate immune response prevention, and upregulation of outer surface protein C (OpsC) associated with the deactivation of host complement proteins, further helps spirochetes to go unnoticed [8, 9]. At least four types of persisters are known to be formed by the *Borrelia* spirochetes: granules/ blebs, cystic forms, biofilm aggregates and cell wall deficient L-forms/ round bodies (RBs).

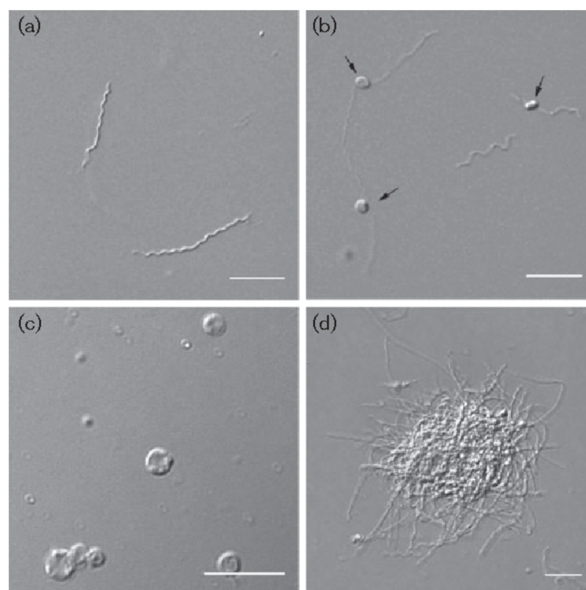


*Figure 1. Different morphologies of Borrelia burgdorferi: (a) spirochetes, (B) blebs on spirochetes, (c) $H_2O$-induced round bodies and (d) biofilms. Adapted from Leena, 2015 [27].*

### 1.3.1 Granules/ Blebs

Reversible pleomorphism, or the capacity to exist in several morphologies, is a characteristic mechanism of *Borrelia*. Aging of a culture has been linked to the formation of round bodies (RB) or blebs, whose genesis coincides with the demise of motile spirochetes [28]. Previously, the transition of the pathogen's motile form into a spherical body was

assumed to signal a decline in viability. However, it has recently been demonstrated that under favorable conditions, round bodies can still move and transform to the reproductive spiral form.

The looped or round-shaped forms arises when a replicative spirochete shakes or curls around itself liberating granules from the periplasmic sheath, forming detachable granules, capable of multiplying, and in some cases transmitting the disease [29]. These are thought to occur as an intermediate stage between spirochetes and cysts/ RBs. They are comprised of an expanded envelope most commonly on the apex or lateral side of the spirochete harboring a folded protoplasmic cylinder within [27]. Meriläinen and colleagues discovered that granule formation could be instigated by introduction of $H_2O$-induced RBs into a normal culture medium. Several stimuli have been linked to the generation of these pleomorphic forms, including antibiotic treatment and complement factors, supporting the trend of their covariance with increased environmental stress . However, it is still unclear whether persisting forms, such as RBs generated in response to antibiotic treatment or RBs that arise in an aged culture, share the same characteristics.

### 1.3.2 Cell wall deficient forms (L-forms)

Another atypical form of *Borrelia,* formed in response to unfavorable conditions, are L-forms, also referred to as spheroplasts or cell wall deficient forms. They were first reported by Klieneberger, who named them after her place of work "Listner Institute" [19]. As their name may suggest, they lack a cell wall. Without a wall the bacteria lose their "markers", essential for detection by host immune system. Not only does this make them imperceptible to the hosts defense system, it also renders antibiotics ineffective. Similar to the granules, L-forms can revert back to their spiral form once the conditions become favorable [31].

### 1.3.3 Cystic forms

In 2008 Miklossy and colleagues first observed atypical cystic forms of *Borrelia* utilizing immunohistochemical methods and dark field AFM [29]. They result from adverse conditions through ring-shaped, loop, spherule and end knot formation. Emergence occurs stepwise when the spirochete curls up into a globule and forms a sheath around itself, making it highly resistant to antibiotic treatment. Miklossy and colleagues discovered responsive correlation

between this outer sheath and anti-OspA antibodies, verifying their superior immunity [29]. Although limited data has been acquired on the occurrence of granular forms, they have been located in skin lesions, spinal fluid and on a silver stained hippocampus section in patients with Lyme neuroborreliosis [22]. Brorson and Brorson and Mursic with colleagues demonstrated that spiral forms inoculated in CSF transformed into cysts in less than 24h [14, 15]. Further studies were successful in the cultivation of cystic forms both *in vitro* and *in vivo,* both showing the efficacy of cultivation under a nutritional deficit. CSF-induced blebs reverted to their reproductive spiral forms once transferred to Barbour-Stoenner-Kelly (BSK) medium. Alternatively, cystic forms were obtained by reduction of rabbit serum (a key source of fatty acids) in BSK medium, and readily converted back into their motile analogues once nutrient levels were restored [34]. Cyst generation in response to antibiotics: ceftriaxone, penicillin and doxycycline was also reported, despite continuous access to nutrient-rich BSK medium [35].

## 1.4 Biofilms

Biofilms represent a complex community of cells permanently attached to an interface, substratum or to each other, immersed in a matrix of extracellular polymeric substances (EPS), which they secrete themselves. [20]. Oxygen and nutrients are acquired from their surroundings via channel-like structures embedded in the extracellular material. The EPS typically contains a plethora of vital components, ranging from polysaccharides and proteins to divalent metals and DNA [36]. The predominant building block, however, is a non-sulfated mucopolysaccharide, commonly referred to as alginate. High amounts of this compound were detected in biofilms formed by multiple bacterial species, including *Leptospira biflexa, Treponema denticola* and others from the order Spirochaetales [20]. Furthermore, it is occasionally used as a marker for the detection of biofilm colonies, alongside complementary contents such as calcium, and extracellular DNA (eDNA). It must be noted that this method is limited by the chemical versatility in the structure of mature biofilms as well as between species, rendering it unreliable [20]. K. Jefferson managed to compile a series of genes across multiple bacterial species essential for the formation of biofilms (cause genes), as well as ones differentially expressed in mature biofilms (effect genes). The expression of these genes was found to assist the conglomerate in its adhesive abilities, stress response, metabolism, motility and division [37]. Wilson and Devine demonstrated that surface proteins found in certain

strains of bacteria, such as glucan-binding-protein (GBP) in *Streptoccuc muans*, induce the production of polysaccharides, and thus aid biofilm generation [38].

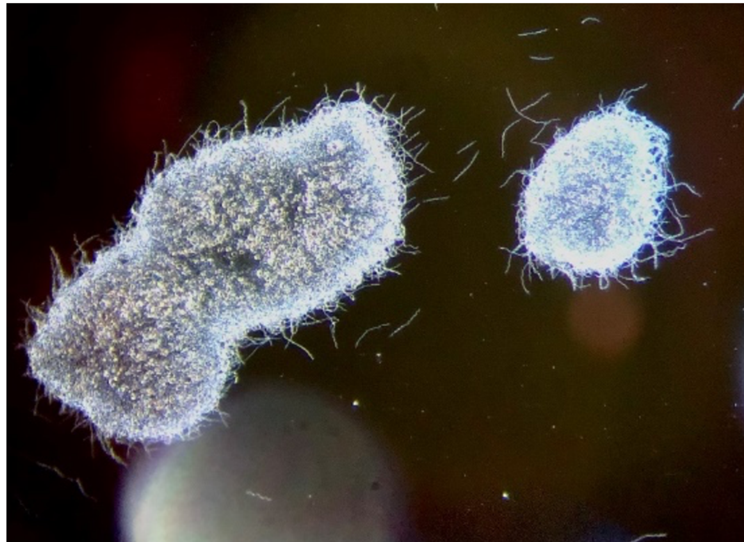

*Figure 2. Age-induced Borrelia burgdorferi biofilm agglomerates captured by dark field microscopy (pictured by author).*

The formation of biofilms can be partially traced back to Darwin's theory of evolution and explained by the constant strive of living organisms towards a nutrient-rich environment, suitable for replication, with the ultimate goal being survival and propagation. Seeing as the body provides ideal conditions for many multicellular organisms, there is a constant effort for its invasion, provoking the adaptation of microbe colonization strategies in order to evade immune detection. The formation of biofilms provides one such possibility, as the metamorphosis into planktonic mode of growth conceals the threat from the immune system's radar. The immediate extracellular environment of the EPS facilitates the bacteria's adhesion to host tissue and serves as a protective layer, shielding it from unfavorable conditions, such as non-physiological pH, extreme temperature changes, xenobiotics/ antimicrobials, high concentrations of metals and evasion of phagocytes [20]. It's worth noting that the phagocytosis of biofilms may in fact impose greater damage on surrounding tissue than on the agglomerate itself. Studies performed on *staphylococci* and *enteroccoci* record EPS synthesis in direct response to osmotic pressure and iron depravation - conditions similar to those encountered within a host [24, 25]. Alternatively, *Escherichia coli* gene expression, regulating biofilm formation showed increased activity when nutrients were reduced, in comparison to controlled analogues [41].

In contrast to other persisting forms, biofilms aren't purely a designated defense mechanism, nor are they exclusively a colonization strategy. As demonstrated by K. Jefferson, their

genesis can also be driven by communal behavior or occur as a default stage of microbial development [20]. Nevertheless, it must be kept in mind that the driving factors in the appearance of biofilm communities may vary between species [7, 22, 26].

In terms of *Borrelia,* the generation of biofilms is typically achieved through culture aging or implementation of environmental stress, and starts with the initial phase of cell-cell and cell-surface aggregation, followed by progressive aging and suppression of metabolic activity [20]. This leads researchers to consider the establishment of *Borrelia* biofilms primarily as a stage of microbial development, as well as a mechanism of defense. Indeed, this sessile community of cells constitutes a key agent in the resilience of LB, its continuous reoccurrence and ultimately the establishment of a chronic infection. The immune-privileged nature of biofilms with emphasis on their extracellular matrix, renders them especially problematic to eradicate [22].

As for today detection of biofilms, as well as other persisting forms of *Borrelia*, is not as reliable as techniques available for detection of spiral forms. This along with the absence of biofilm-targeted antibiotics enables them to go undetected for months-years and reside within the human body as a ticking time bomb [19].

## 1.5 Genetic factors involved in persistence

The phenomenon of persistence in *Borrelia* continues to puzzle researchers in its phenotypic adaptability in reaction to environmental change. When a subpopulation of bacteria encounter stimuli from a hostile environment, they go through a series of changes, dynamically altering their gene expression. During the span of their life, they are found to continuously switch between two available phenotypes: susceptible and persistent, strictly enforced by epigenetic factors. The susceptible state represents replicative, active forms of the bacteria in their characteristic spiral shape, able to actively induce a state of inflammation within the host. In contrast, persistent forms embody dormant pleomorphisms, capable of hiding from the host's immune system and resistant to harsh conditions [43]. Although literature on the exact mechanism of their metamorphosis remains limited, contiguous studies on persistence in viruses and fungi have provided insight into the series of changes leading to persistence formation and survival. Aside from the gene expression and epigenetic adaptations, such as changes in DNA modification and protein signaling, alterations also played a part in the

generation of persistent cells [43]. Zhang and colleagues located permutations of several persister genes ( Ying-Yang model) by means of mutagenesis [43]. Mutations causing an increase in persistent traits include: *metG* encoding methionyl-tRNA synthetase, *glpD* encoding glycerol-3-phosphate dehydronase, *hipA* encoding toxin and *tktA* encoding transketolase A [43]–[45]. Mutations conferring decreased bacterial persistence were found to be: *relA*, *ubiF*, *phoU* and *sucB* [43], [46]–[48]. Additional pathway linked to persistence include: stringent response (*dksA, relA*), signaling pathways (*oxyR, pspBC, tnaA, flu; comE/ comC*), DNA repair and protection (*xerC, xerD, recA, recB, lexA, dps*), macromolecular degradation, anti-oxidative stress (catalase, superoxide dismutase) and reserve energy production (*tgs1, ubiF, plsB, sucB, glpD*) [7, 28]. It must be noted that the approach used to identify these permutations (mutagenesis) is limited to non-essential dominant genes, having a noticeable effect on the resulting phenotype, and proves ineffective in identification of multi-determinant phenotypes. Furthermore, keeping in mind that *in vivo* persisters differ from *in vitro* persisters, a certain degree of variability should be considered when investigating their "genetic fingerprint". Potential overlaps can be investigated via RNA sequencing analysis or single-cell techniques for better understanding of the differential gene expression patterns of *Borrelia*.

Additionally Feng and colleagues (2015) conducted an extensive analysis on differentially expressed genes observed in *Bb* treated with amoxicillin and doxycycline, the two most commonly used antibiotics for the treatment of LB [24]. According to their findings a total of 675 differentially expressed genes were observed in the doxycycline tolerant persisters and 516 in those unsusceptible to amoxycillin. Interestingly, the genes upregulated in the doxycycline treated group included Clp Protease (ClpP), the most upregulated protein, and heat shock proteins (HSP). The intracellular protease ClpP can detect and destroy misfolded proteins through the assistance of the ClpX, C, or A subunits. Since doxycycline interferes with bacterial protein production by attaching to its 30S ribosomal subunit thereby misfolding the protein, ClpP upregulation might be a reaction to this. The HSP chaperones provide support to proteins, assuring proper folding, and assist in the refolding of broken proteins, which may be vital for the survival of persisters. The authors discovered several up-regulated genes that are linked to DNA repair, which potentially could support DNA stability upon antibiotic strain. Perhaps the most surprising finding was that the majority of proteins being down-regulated both in amoxicillin and doxycycline, were outer membrane proteins. This may help *Bb* withstand antibiotic treatment by reducing the requirement for protein synthesis.

A shortage in these lipoproteins could in fact strengthen the inner membrane through up-regulation of membrane proteins. This is critical, as doxycycline and amoxicillin both work by inhibiting protein synthesis.

Earlier we conducted our own proteomic analysis and compared the protein profiles of three forms of *Borrelia* – active replicative, biofilms and cystic forms. Our findings provided a ground for further investigation into the driving factors behind appearance of alternative spirochetes morphologies and persistence, as well as possible detection strategies. The main goal of this project was to analyze the data obtained by proteomics analysis and to identify differentially expressed genes, characteristic and unique to *Borrelia* biofilms, that can be used for the development of reliable and sensitive molecular detection of persisting forms.

# 2. Materials

*Table 1. Materials used in this study.*

| **Borrelia cultivation** | |
|---|---|
| **MKP medium** | 14.4% rabbit serum |
| **Persteril- solution of ethaneperoxoic acid, hydrogen peroxide and sulfuric acid** | 0.5% solution |
| **DNA isolation** | |
| **Genomic DNA isolation kit** | DNeasy® Blood & Tissue Kit (250), (Qiagen) |
| **PCR products purification (columns)** | Centrifugal Filter Units DNA extraction from agarose (Millipore) |
| **RNA isolation** | |
| **RNA isolation kit** | Direct-zol™ RNA Miniprep, Zymo-Spin™ Column, (Zymo research) |

| **PCR** | |
| --- | --- |
| **RT-PCR kit** | • ONETAQ® One-Step RT-PCR Kit, (New England Biolabs)<br>• SuperScript™ IV One-Step RT-PCR System, (ThermoFisher Scientific) |
| **Gel electrophoresis** | |
| **50x TAE buffer** | 50mM EDTA, 200mM Tris-HCl |
| **10x TBE buffer** | 25mM EDTA, 450mM boric acid,1.3M tris base |
| **Agarose (DNA ELFO)** | 0.8-2% agarose (Serva) for DNA electrophoresis in 1xTAE buffer |
| **2x Bromophenol Blue, Xylene Cyanol FF** | RNA Gel Loading Dye (2x), (Thermo Fisher Scientific) |
| **6x CoralLoad PCR loading dye supplemented with SYBR Gold** | HotStarTaq® *Plus* Master Mix Kit, (Qiagen)<br>SYBR Gold (10000x) (Molecular probes) |
| **PCR Marker** | Gene Ruler 100 bp Plus DNA Ladder, (Thermo Fisher Scientific) |
| **DNA Cloning** | |
| **Cloning kit for sequencing** | TOPO™ TA Cloning™ Kit for Sequencing, without competent cells, (Invitrogen) |
| **DH5α chemically competent cells** | |
| **Ampicillin** | working concentration 50μg/mL |
| **(S.O.C.) Super Optimal broth with Catabolite repression medium (Invitrogen)** | Self-prepared or ready to use (Amresco®) |
| **LB broth** | |
| **Agar** | |

*Table 2. Laboratory supplies used in this study.*

| | **Type** |
|---|---|
| **Centrifuges** | Centrifuge 5415 C (Eppendorf)<br>Centrifuge 5415 D (Eppendorf)<br>Centrifuge 1415 R (Eppendorf)<br>Centrifuge Universal 32 R (Hettlich centrifuge) |
| **Electrophoresis** | SHU6 (Sigma Aldrich)<br>OVL Easycast™ B2 (Thermo Scientific) |
| **Power supply** | Consort |
| **Dark field microscope** | Leica DM 1000 LED (Leica) |
| **PCR cycler** | Mastercycler *personal* (Eppendorf) |
| **Thermomixer** | Thermomixer (Eppendorf) |
| **NanoDrop** | NanoDrop® ND-1000, (Thermo Scientific) |
| **Photosystem for gel documentation** | Kodak |
| **Biosafety Laminar Flow cabinet** | Gelaire |
| **PCR box** | DNA/RNA UVC/T-M-AR (Biosan) |
| **Vortex** | Heidolph REAX top |
| **Thermostat** | Biological Thermostat 120 |
| **Shaker** | Stuart Scientific |
| **Magnetic Stirrer** | Sigma |
| **UV illuminator** | HeroLab UVT-20M |
| **Thermostat** | Set for:<br>34°C (*Bb* cultivation)<br>37 °C (*E. coli* growing conditions) |

# 3. Methods

## 3.1 Borrelia cultivation

3.1.1 Preparation of modified Kelly-Pettenkoffer (MKP) medium for the cultivation of borrelia cultures.

3.1.1.1 Preparation of basic MKP medium

*Table 3. Ingredients used for the preparation of basic MKP medium (200mL).*

| Reagent | Mass [g] |
|---|---|
| CMRL-1066 (10x) without Glutamine | 1.94 |
| Neopeptone | 0.6 |
| HEPES | 1.2 |
| Citric Acid | 0.14 |
| D (+) Glucose | 0.6 |
| Pyruvic Acid | 0.16 |
| N-acetyl glucosamine | 0.08 |
| Sodium Bicarbonate | 0.4 |

All powdered ingredients were added to 100mL of $ddH_2O$ and mixed until fully dissolved (20-30 minutes). The pH was adjusted to 7.6 by 10N NaOH solution. The total volume was adjusted to 200mL using $ddH_2O$ and sterilized by vacuum filtration through 0.22-micron filter. Sterile medium was transferred to 50mL tubes and stored at -20℃ (up to 3 months).

3.1.1.2 Preparation of complete MKP medium

*Table 4. Amount of reagents used in complete MKP medium.*

| Reagents | Volume [mL] |
|---|---|
| Basic MKP medium | 200 |
| 7% Gelatin | 40 (freshly autoclaved) |
| Rabbit serum | 14.4 |
| 35% BSA | 7 |

All components were mixed by stirring. The medium was sterilized by vacuum filtration through 0.22-micron filter and transferred to 50mL tubes. Complete medium was stored at +4°C (up to 1 month).

The starting culture was initiated from the frozen bacteria stock when 200 µL of spirochete culture stock was added to 10 mL of MKP complete medium.
For passaging of *Borrelia*, 1mL of a not contaminated well grown culture was transferred to a 15mL tube containing 9-12mL of complete MKP medium under the sterile conditions. The samples were grown at 34°C. Throughout the cultivation the cultures were continuously examined under a dark field microscope. Inspections monitored the growth and development of the cultures as well as its purity over time until ready to be further used for DNA or RNA isolation. Biofilm formation was triggered by aging of the cultures for three weeks longer than the replicative forms.

To prepare for DNA or RNA isolation, cultures were centrifuged to collect the cells at 9,000rpm for 20min at 4°C. Supernatant was discarded and remaining pellet was re-suspended in 1mL of 1x PBS solution and transferred to 1.5mL Eppendorf tube to wash the cells. The sample was centrifuged at max speed for 15min, and supernatant was discarded. The obtained cells were stored for later use at -20°C.

## 3.2 Genomic DNA isolation

Genomic DNA was isolated by Qiagen DNeasy blood and tissue kit strictly following the provided protocol:

1. Add 180 µL of ATL buffer and re-suspend by vortexing
2. Add 20 µL proteinase K and mix thoroughly by vortexing
3. Incubate at 56°C until tissue is completely lysed (15-30min)
4. Remove from incubator and vortex for 15 seconds
5. Add 200 µL of ATL buffer to sample, and mix thoroughly
6. Add 200 µL of ethanol and vortex thoroughly
7. Incubate at 70°C for 10 minutes (optional)
8. Pipet all the mixture into a DNeasy mini spin column placed in a 2mL collection tube.

9. Centrifuge at 8,000 rpm for 1 minute and discard flow-through and collection tube
10. Place in new collection tube, add 500 µL of AW1 buffer and centrifuge at 8,000 rpm for 1 minute
11. Discard flow-through and collection tube
12. Place the column in fresh collection tube and add 500 µL of AW2 buffer, centrifuge at 8,000 rpm for 3 minutes
13. Discard flow-through and collection tube
14. Place mini spin column in a sterile 1.5 mL Eppendorf tube and add 50 µL of $H_2O$ to elute the genomic DNA
15. Incubate for 1 minute at room temperature
16. Centrifuge for 1 minute at 8,000 rpm and discard mini spin column
17. Store at 4°C until further use

After isolation, concentration of genomic DNA was measured using NanoDrop spectrophotometer. A total of three measurements was taken per each sample and the average value was calculated.

## 3.3 RNA isolation

RNA was isolated from *Borrelia burgdorferi* sensu stricto cells of both biofilm and spiral pleomorphic forms according to the supplied protocol and was used as a template in RT-PCR analysis. Briefly:

1. Add 300 µL of buffer 1 and re-suspend until mixture is homogenous
2. Add equal volume of ethanol and mix thoroughly by vortexing
3. Transfer the mixture into Zymo-Spin™ IICR Column in a new collection tube and centrifuge at 7,500 rpm for 1 minute
4. Transfer column into new collection tube and discard flow-through
5. Add 400 µL of RNA Wash Buffer and centrifuge at 7,500 rpm for 1 minute
6. In a separate Eppendorf prepare mixture of 75 µL digestion buffer and 5 µL DNase and mix by gentle inversion
7. Add the mix directly onto column matrix and incubate at room temperature for 15 minutes

8. Add 400 μL RNA PreWash to the column and centrifuge at 7,500 rpm for 1 minute
9. Discard flow-through and repeat step 8 one more time
10. Add 700 μL RNA Wash Buffer to the column and centrifuge at 7,500 rpm for 1 minute
11. Transfer the column into a sterile 1.5 mL Eppendorf tube and add 50 μL DNase/RNase-Free Water directly to column matrix to elute the RNA
12. Incubate at room temperature for 1 minute
13. Centrifuge sample at 7,500 rpm for 1 minute and discard Zymo-spin column
14. The eluted RNA must be stored at -80°C

To analyze the quality and integrity of RNA an aliquot of the purified RNA (5 μL) was denatured at 70°C for 10 minutes and loaded onto 0.8% Agarose gel on TBE buffer for direct analysis. A 2x Brompheol Blue, Xylene Cyanol FF loading dye supplemented with SybrGold was used for this analysis.

RNA was analyzed using NanoDrop, the concentration of the RNA was measured equal RNA concentrations for each sample were obtained by diluting. Diluted samples were aliquoted and stored at -80°C until further use.

## 3.4 Polymerase chain reaction (PCR) analysis

### 3.4.1 Standard PCR

Polymerase chain reaction (PCR) is a method of molecular and genetic analysis used to amplify particular segments of DNA between two regions of a specific sequence called primers. This technique requires the use of DNase/RNase-free water, Taq polymerase, nucleotides (dNTPs), buffer mix, primers and DNA templates. Each PCR reaction was conducted in a total volume of 20 μL.

Table 5. Components of one PCR reaction (20μL).

| Contents | Volume [μL] |
|---|---|
| 2x PCR Master Mix* | 10 |
| 0.1mM primer Forward | 1 |
| 0.1 mM primer Reverse | 1 |
| $H_2O$ | variable |
| Template DNA< 200 ng/reaction | variable |

### 3.4.2 One-Step Reverse Transcription (RT) PCR

RT-PCR is a modification of standard PCR involving the conversion of RNA into its complementary DNA sequence (cDNA) by reverse transcriptase enzyme. The cDNA molecule serves as a template for further amplification through PCR. Primers were designed specifically for certain areas of the *Borrelia* genome (genes) suspected to be under-regulated in spiral forms and up-regulated in biofilms.

### 3.4.3 Preparation of reactions and procedure

All the reagents necessary to perform the one-step RT-PCR were included in the kit. Two different kits were used, closely following their corresponding protocol:

#### 3.4.3.1 ONETAQ® One-Step RT-PCR Kit, (New England Biolabs)

1. Thaw system components and mix by vortexing
2. Mix reagents, except for RNA, in sterile RNase-free conditions (PCR box):

Table 6. Contents of one RT-PCR reaction (50μL).

| Reagents | Volume [μL] |
|---|---|
| *OneTaq* One-Step Reaction Mix (2x) | 25 |
| *OneTaq* One-Step Enzyme Mix (25x) | 2 |
| Gene-specific Forward Primer μM | 2 |
| Gene-Specific Reverse Primer μM | 2 |
| Nuclease-free $H_2O$ | 19-x |
| Total RNA (up to 1 μg) | x |

* PCR Master Mix contains: Taq DNA Polymerase, mixture of dNTPs and PCR buffer substituted with both KCl and $(NH_4)_2SO_4$.

3. Add RNA template and start reactions in thermocycler as follows:

*Table 7. PCR steps, temperature and time for ONETAQ One-Step RT-PCR Kit.*

| Step | Temperature | Time | Cycles |
|---|---|---|---|
| Reverse Transcription | 48°C | 15-30 minutes | 1 |
| Initial Denaturation | 94°C | 1 minute | 1 |
| Denaturation<br>Annealing<br>Extension | 94°C<br>50-65°C<br>68°C | 15 seconds<br>30 seconds<br>1 minute/ kb | 40 |
| Final Extension | 68°C | 5 minutes | 1 |
| Hold | 4°C | ∞ | 1 |

### 3.4.3.2 SuperScript™ IV One-Step RT-PCR System, (ThermoFisher Scientific)

1. Defrost components from kit and mix by vortexing
2. Mix all reagents, except for RNA templates, in sterile conditions

*Table 8. Components of one RT-PCR reaction (50µL)*

| Components | Volume [µL] |
|---|---|
| Platinum™ SuperFi™ RT-PCR Master Mix (2x) | 25 |
| Forward Primer (10 µM) | 2.5 |
| Reverse Primer (10 µM) | 2.5 |
| SuperScript™ IV RT Mix | 0.5 |
| Template RNA (0.01 pg to 1 µg total RNA) | x |
| Nuclease-free water | variable |

3. Add RNA templates and negative control ($H_2O$) to reaction tubes
4. Place reactions in pre-heated thermal cycler and run program

*Table 9. PCR steps, temperature and time for SuperScript IV One-Step RT-PCR kit.*

| Step | Temperature | Time | Cycles |
|---|---|---|---|
| Reverse transcription | 45-60°C | 10 minutes | 1 |
| RT initial denaturation | 98°C | 2 minutes | 1 |
| Denaturation<br>Annealing<br>Extension | 98°C<br>55-72°C<br>72°C | 10 seconds<br>10 seconds<br>30 seconds/kb | 35-40 |
| Final extension | 72°C | 5 minutes | 1 |
| Hold | 4°C | ∞ | 1 |

In the case of both kits, completed reactions were immediately placed on ice and prepared for further analysis by gel electrophoresis.

## 3.5 Gel Electrophoresis

Gel electrophoresis is a method used to separate DNA/RNA fragments by size in agarose gel. Migration occurs due to negatively charged phosphorus groups in DNA/RNA being pulled by an electric current through the gel to the positive pole. It allows for detection of presence and size of the PCR product.

Results of every PCR and RT-PCR reaction were examined by gel electrophoresis: 1% agarose gel was prepared by dissolving 2g of agarose in 200 mL of 1x TAE buffer (0.001 M EDTA, 0.04 M Tris-acetate) by boiling and left to cool before transferring it into casting tray for gel to set. CoralLoad 6x concentrated loading dye was added to each sample at a ratio of 1:6. It contains two marker dyes (orange and red) that allow for the estimation of DNA/RNA migration distance and one dye to visualize the PCR product under UV light. The samples were then loaded onto gel and run for 20-40 minutes at 100V. The gel was removed and inspected under UV light. All results were documented.

PCR products were cut from gel and purified. Gel pieces were transferred to the provided columns with collection tubes. Samples were centrifuged at 5,000 rpm for 10 minutes and immediately placed on ice, the concentration of the obtained purified PCR product was measured before the cloning step or direct PCR product sequencing.

## 3.6 Cloning of PCR products

Purified PCR product was cloned into a pCR-4 TOPO plasmid followed by the transformation of the competent cells. TOPO™ TA Cloning™ Kit for Sequencing was used to conduct this procedure in accordance with the provided protocol:

### 3.6.1 Ligation of PCR Product

1. In a separate DNase/RNase-free Eppendorf prepare mixture for ligation reaction

*Table 10. Reagents used in preparation of ligation mixture (6μL).*

| Reagent | Volume [μL] |
|---|---|
| Vector mix | 1 |
| Salt solution | 1 |
| PCR product | 1.5- 4* |
| DNase/RNase-free water | variable |

2. Mix by inversion and incubate at room temperature for 15-20 minutes

### 3.6.2 Transformation of competent cells

1. Add 2 μL of ligation reaction to provided One Shot TOP10 chemically competent *E. coli* cells and mix by inversion
2. Leave on ice for 20-30 minutes
3. Incubate at 42°C for 30 seconds
4. Add 250 μL of SOC medium in sterile conditions
5. Incubate in shaker at 37°C and 200 rpm for 1 hour. Spread 10-50 μL from transformation reaction on pre-warmed LB/ampicillin plates
6. Incubate the plates at 37°C overnight without shaking

* Volume dependant on concentration of PCR product [ng/ μL].

### 3.6.3 Preparation of Luria-Bertani (LB) liquid medium

*Table 11. Composition of LB medium (1L).*

| Reagent | Concentration [%] | Amount [g] |
|---|---|---|
| Tryptone | 1.0 | 10 |
| Yeast Extract | 0.5 | 5 |
| NaCl | 1.0 | 10 |

1. Dissolve all components in 950 mL deionized water
2. Adjust pH of solution to 7.0 with NaOH
3. Bring volume up to 1 L with deionized water
4. Autoclave on liquid cycle for 20 minutes at 15 psi
5. Allow solution to cool to 55°C before adding the antibiotic
6. Store at 4°C

### 3.6.4 Preparation of LB agar plates

1. Add 15 g/ L agar to LB medium
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. Let LB/agar to cool to 55°C and add ampicillin to the final concentration of 50 µg/ mL. Mix well, avoid bubbles.
4. Pour into 10-cm petri dishes
5. Let solidify, invert and store at 4°C until use.

# 4. Results

## 4.1 Borrelia cultivation

*Borrelia burgdorferi* sensu stricto was grown in MKP medium until the density $10^5$ cell/ mL for spiral (active) forms of borrelia or left to grow until 99% of population were represented as biofilms (in average 3 weeks). Throughout the cultivation the cultures were continuously examined under a dark field microscope.

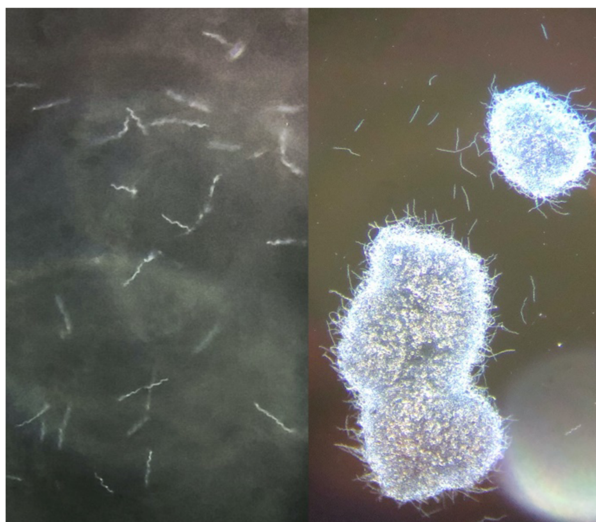

*Figure 3. Cultures of replicative spiral forms (left) and biofilm aggregates (right) observed under dark field microscope.*

## 4.2 RNA isolation

For each morphological form, two cultures were selected for both spiral forms (A) and biofilm aggregates (B) RNA extraction. The concentration of each sample of isolated RNA was measured by NanoDrop and adjusted to an equal value (15 ng/µL) to ensure an equimolar identity for each.

*Table 12. Dilution procedure for isolated RNA samples used.*

| Sample | $C_{initial}$ [ng/µL] | $C_{final}$ [ng/µL] | Dilution Factor |
|---|---|---|---|
| $B_1$ | 15.3 | 15.3 | 1 |
| $B_2$ | 45.3 | 15.3 | 2.96 |
| $A_1$ | 17.2 | 15.3 | 1.12 |
| $A_2$ | 30.8 | 15.3 | 2 |

The intactness of the isolated RNA was controlled by the presence of all ribosomal RNA via gel electrophoresis.

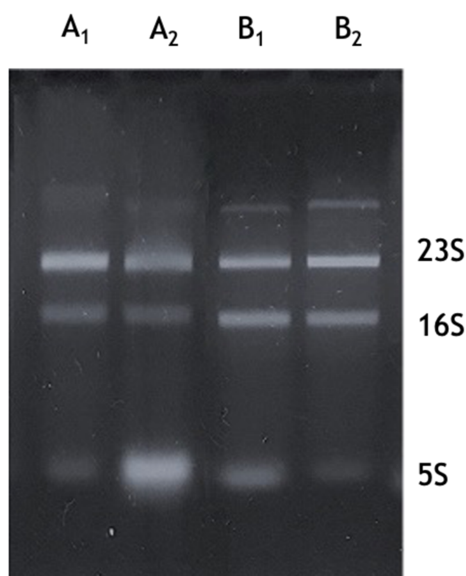


*Figure 4. RNA aliquots suspended on 0.8% agarose gel, run on TBE buffer. (A) - RNA isolated from replicative spirochetes, (B) - RNA obtained from biofilm cultures.*

## 4.3 Results Summary

Based on the findings of the protein profile comparisons in biofilms and spiral forms of borrelia (Figure 6) the most representative proteins displaying an up-regulation in biofilms and down-regulation in spiral forms were selected. The heat map was provided to us after completion of proteomics analysis.

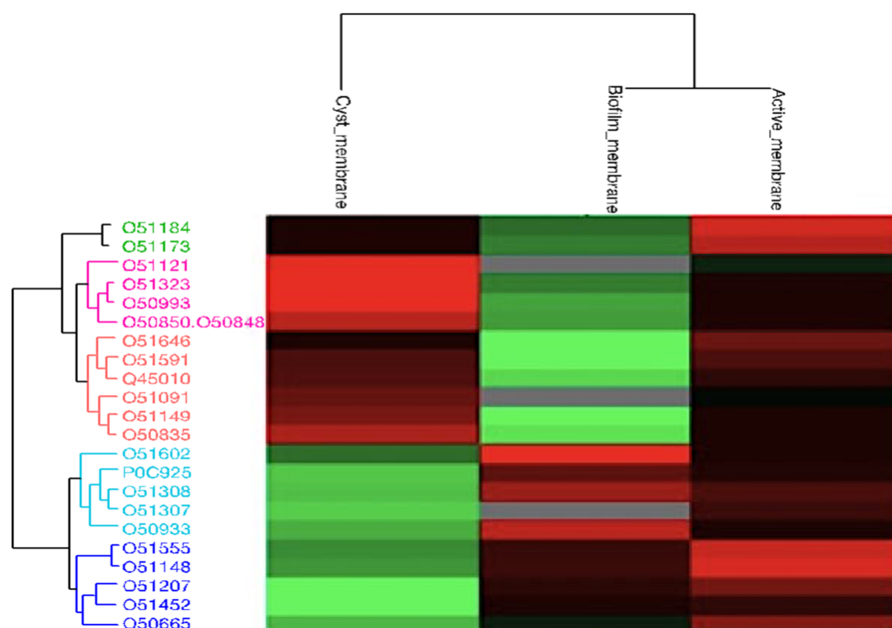


*Figure 5. Proteomic profile of upregulated and downregulated surface proteins in Bb cysts, biofilms and active spirochetes.*

The candidates were characterized using UNIPROT and their corresponding nucleotide sequences were obtained from GenBank. All the genes investigated are provided in the supplemented materials. Using the program PrimerBlast from GenBank database, primers were designed. The primers were used to cover and amplify a maximal length of a given gene. All the combinations tested are available in the supplemented materials. Those that displayed promising results are summarized below (Table 13).

*Table 13. Primer combinations designed for Bb replicating spirochetes and biofilms that showed promising results.*

| Forward primer | Sequence (5'- 3') | Reverse Primer | Sequence (5'- 3') | Size of Primer [bp] | Annealing temperature [°C] |
|---|---|---|---|---|---|
| **Gene BB_0383** Basic Membrane protein A | | | | | |
| F2 | AAGGTAGTCTTGGG AGCGAA | R2 | AAATCTGAGCCCGCA TCCTT | 199 | 55.3 |
| F2 | AAGGTAGTCTTGGG AGCGAA | R1 | GCAACGCTTCTACCA GCTTC | 527 | 55.3 |
| **Gene BB_A40** Uncharacterized protein | | | | | |
| F5 | TACCCGGTATTTGA GAGCCAC | R3 | GCAGGACCTAATGCT AAAGCG | 380 | 56.1 |

All primers were used in RT-PCR reactions with an equal quantity of RNA of both biofilms and spiral forms of borrelia as a template. The majority of the reactions revealed no difference in amplification between the two forms of borrelia. The following instances depict the multitude of reactions that proved to be ineffective.

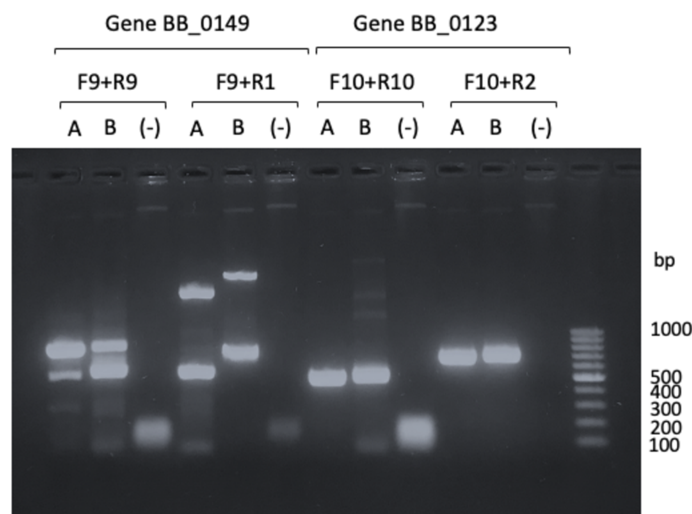


*Figure 6. Results showing no difference in the expression of the genes BB_0149 and BB_0123 in both spiral forms as well as biofilms. (A) - spiral RNA, (B)- biofilm RNA and (-) - negative control.*

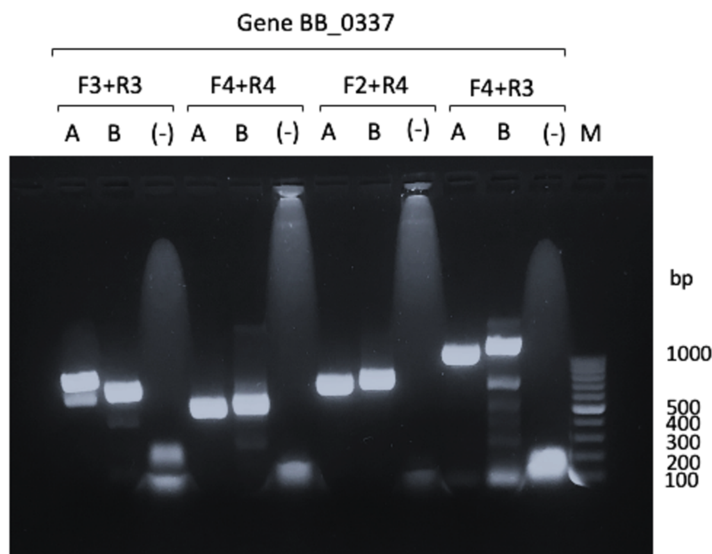


*Figure 7. Results displaying no difference in the expression in both morphological states for the gene BB_0337.*

We chose to reduce the number of PCR cycles from 40 to 25 in order to lessen the possibility of non-specific amplification of PCR products during lengthy PCR operations This modification was carried out to enhance the specificity and accuracy of our results by decreasing the likelihood of false-positive signals arising from the amplification of non-target sequences.

Several genes revealed positive results, displaying an amplified PCR product only in the case of RNA from biofilms used as a template. No result of amplification was found in the case of replicating forms of *borrelia.*

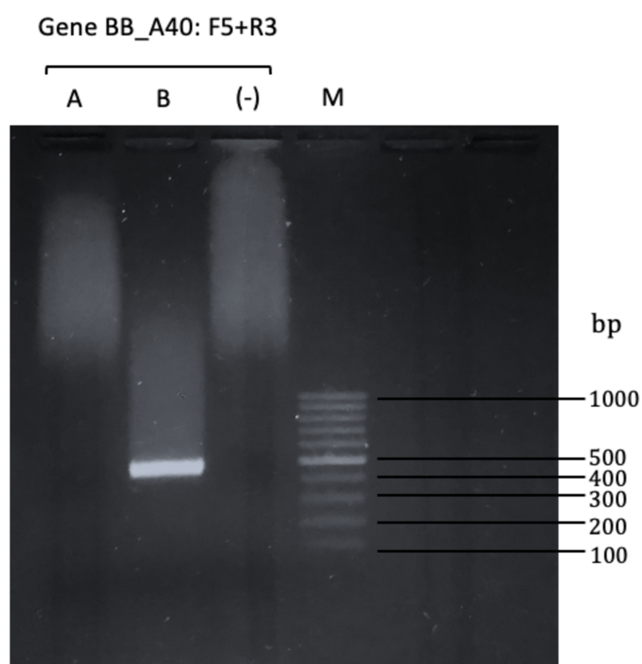


*Figure 8. PCR results with primer combination F5, R3 (380bp) of the gene BB_A40. A - represents the spirochete RNA, B - biofilm RNA, (-) - denotes the negative control and M – marker (100bp).*

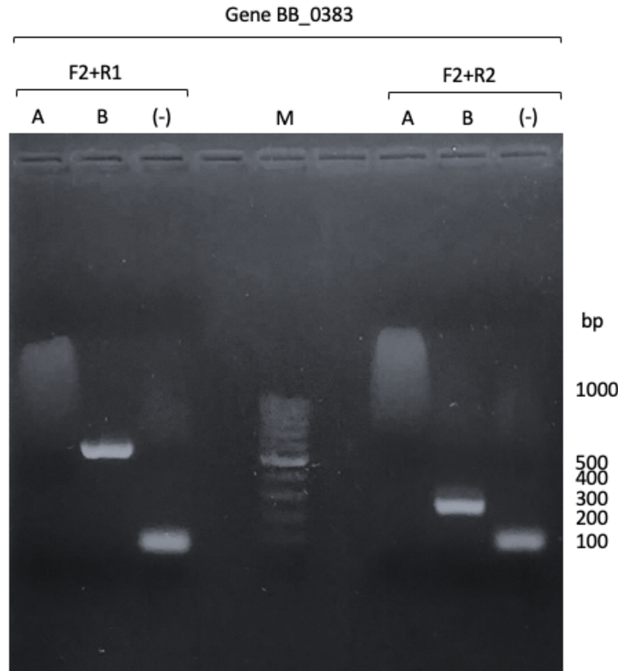


*Figure 9. PCR results with primers F2, R1 (527 bp) pictured left and F2, R2 (199 bp) pictured right, of the gene BB_0383.*

Pictured above are the results of gel electrophoresis of PCR amplification of two genes that exhibited the expected upregulation of expression in biofilms and downregulation in replicative spiral forms of *Bb* ss. The results observed for gene BB_A40 were successfully repeated in three replicates.

The preliminary results obtained by standard PCR should be confirmed by qRT-PCR. This was not conducted within the project due to the lack of time.

### 4.3.1 Sequencing results

Once PCR products were obtained and analyzed on agarose gel, they were cut from the gel and purified for sequencing. Direct sequencing of the PCR product was conducted at the first stage in order to confirm the sequence of interest, following by the cloning of the PCR product into pCR-4 TOPO vector to control the accuracy of the whole sequence. The vector possesses a pre-defined set of primers, which facilitated the acquisition of an accurate identity of the insert. (Figure 11)

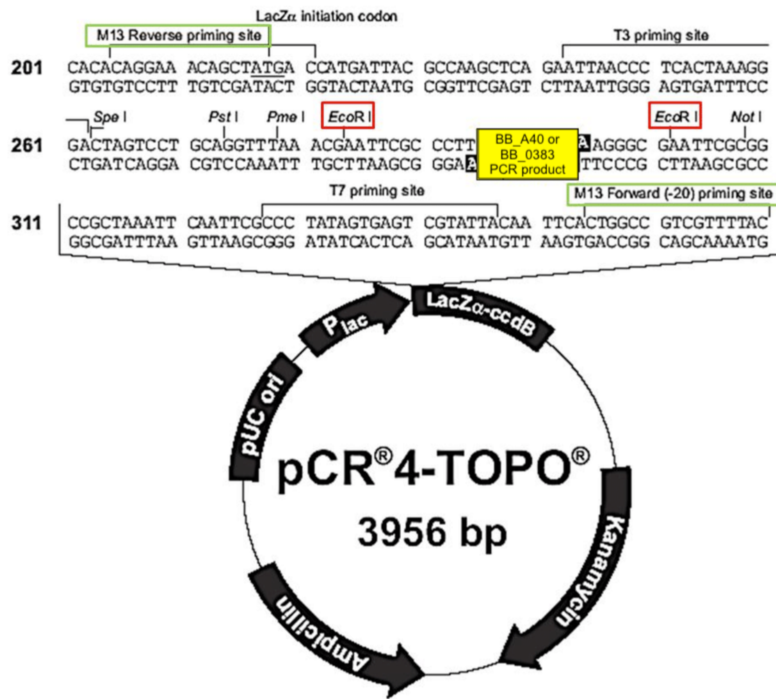


*Figure 10. Graphical representation of pCR 4-TOPO® vector map (Invitrogen).*

### 4.3.2 Gene BB_A40

The PCR product from gene BB_A40 yielded the following nucleotide sequence.

**BB_A40**

Nucleotide sequence

TATTTTGCTCAAAAAGGCGGACTTAAAAGCTCTTCTGCTGATAAGTTTGAAAATT
ATCAAGCTTTAGATTTTTGCTACAAATGCGGGGTTAAGCTTATTGTTAACGGTTCA
AATTTGCAAATAGCAAAAGGCGGTGGTAGTGATCTTTATGGGGTTTGTGTAGACT
TTGACGATTTTTCAAGAACCGGCACGGTTGTTCCAATCACTTGTAGTTTTGAATGT
GTTTTGATTACTAAAGACAAAACCATCAAAGCAGAAGACAAATTAATAATAAAC
AGCGAAGGGGTTTTAGAAAAATCTAGCAAAAATGCATCTGTTATTCACGCTTTAG
CATTAGGTCCTGCA

This result was compared with the original sequence obtained from GenBank, using the SeqMan module from DNASTAR. A total of 549 nucleotides were compared. Of these, 345 were identical with no gaps or ambiguous nucleotides present.

*Table 14 .Summary of results obtained from the alignment of the original sequence and the observed sequence.*

| Number of selected sites: | 549 |
|---|---|
| Number of identical sites: | 345 |
| Base composition (all sites): | 34.5% A \| 13.8% C \| 20.5% G \| 31.3% T |
| Transition/ Transversion ratio: | 0.000 |
| Percentage of homology: | 99.7% |

### 4.3.3 Gene BB_0383

The results obtained from the sequencing analysis provide clear evidence that the gene of interest, namely bb_0383, is present.

**bmpA BB_0383**

Nucleotide sequence

ATGAATAAAATATTGTTGTTGATTTTGCTTGAGAGTATTGTTTTTTTATCTTGTAGT
GGTAAAGGTAGTCTTGGGAGCGAAATTCCTAAGGTATCTTTAATAATTGATGGAA
CTTTTGATGATAAATCTTTTAATGAGAGTGCTTTAAATGGCGTAAAAAAAGTTAA
AGAAGAATTTAAAATTGAGCTTGTTTTAAAAGAATCCTCATCAAATTCTTATTTAT
CTGATCTTGAAGGGCTTAAGGATGCGGGCTCAGATTTAATTTGGCTTATTGGGTA
TAGATTTAGCGATGTGGCCAAGGTTGCGGCTCTTCAAAATCCCGATATGAAATAT
GCAATTATTGATCCTATTTATTCTAACGATCCTATTCCTGCAAATTTGGTGGGCAT
GACCTTTAGAGCTCAAGAGGGTGCATTTTTAACGGGTTATATTGCTGCAAGACTT
TCTAAAACAGGTAAAATTGGATTTTTAGGGGGAATAGAAGGCGAGATAGTAGAT
GCTTTTAGGTATGGGTATGAGGCTGGTGCTAAGTATGCTAATAAAGATATAAAGA
TATCTACTCAGTATATTGGTAGTTTTGCTGACCTTGAAGCTGGTAGAAGCGTTGCA
ACTAGAATGTATTCTGATGAGATAGACATTATTCATCATGCTGCAGGCTTTGGAG
GAATTGGGGCTATTGAGGTTGCAAAAGAACTTGGTTCTGGGCATTACATTATTGG
AGTTGATGAAGATCAAGCATATCTTGCTCCTGACAATGTAATAACATCTACAACT
AAAGATGTTGGTAGAGCTTTAAATATTTTTACATCTAACCATTTAAAAACTAATA
CTTTCGAAGGTGGCAAATTAATAAATTATGGCCTTAAAGAAGGAGTTGTGGGGTT
TGTAAGAAATCCTAAAATGATTTCCTTTGAACTTGAAAAAGAAATTGACAATCTT
TCTAGCAAAATAATCAACAAAGAAATTATTGTTCCATCTAATAAAGAAAGTTATG
AGAAGTTTCTTAAAGAATTTATTTAA

In conclusion, out of all the 11 genes tested 2 exhibited expected and promising results: genes BB_A40 and BB_0383. Unfortunately, gene BB_A40 is currently uncharacterized and therefore not much information is available on it, and it might be an interesting subject for following studies.

Gene BB_0383 is known to encode the recombinant basic membrane protein A (BmpA), alternatively known as immunodominant antigen P39. This protein is the product of *B. burgdorferi* gene BmpA and is one of *B. burgdorferi* 's main antigens that plays a key role in Lyme arthritis. It was also shown that BmpA could stimulate the secretion of several specific chemokines and induce Lyme arthritis when the cells are exposed *in vitro* to recombinant *Borrelia burgdorferi* basic membrane protein A [49]. This protein is presently being investigated for its potential in serological testing techniques. It has predominantly been detected in individuals with persistent Lyme borreliosis infections, especially those exhibiting neurological and musculoskeletal manifestations [50]. Prior research has indicated that the gene is located within a 6.3-kilobase fragment originating from *B. burgdorferi* Sh-2-82, embedded in the recombinant plasmid pSPR33 [51].

# 5. Discussion

Lyme borreliosis (LB) is the most prevalent tick-borne disease induced by the spirochetes from *Borrelia burgdorferi* sensu lato complex on a global scale. The skin, joints, heart, and nervous system represent just a few of the various organ systems that are affected by the illness. As the infection develops, the bacteria disseminate throughout the host tissues, hiding from the host immune response. The majority of diagnosed patients respond well to early treatment with antibiotics. Nevertheless, despite targeted antibiotic therapy, persistent forms of *Borrelia* remain a threat to approximately 10-20% of patients diagnosed with LB [52]. The long-lasting persistence of LB symptoms and the failures of the antibiotic treatment of LB are comparable to other biofilm-associated chronic infections. Through impairing immune function, hindering antimicrobial therapy, and disseminating planktonic cells responsible for the distribution of infection within the body, biofilms are able to preserve resident bacteria and perpetuate chronic infections. This enables bacterial cells to not only become extremely resilient against antibiotic therapy but also their eradication from the host. Since bacteria can form biofilms in host tissues as well as on foreign objects - including catheters, implants, and

prosthetic devices, many serious illnesses affecting humans, animals, and plants become increasingly difficult to treat. This is especially prevalent in Cystic fibrosis, which is often complicated by chronic *Pseudomonas aeruginosa* biofilm infections in the lung, Prosthetic joint infections attributed to *Staphylococcus aureus* biofilms and Chronic wound infections, wherein biofilms formed by various bacterial species interfere with the healing process [53]–[55]. The main aim of our project was to identify differentially expressed genes involved in biofilm formation, which could potentially serve as markers for detecting these dormant morphotypes in chronic Lyme borreliosis patients.

*Borrelia* biofilm aggregates, which constitute microbial communities held together by a self-secreted matrix of extracellular polymeric substance, predominantly composed of polysaccharides, proteins, and nucleic acids, are more resilient to antibiotics proven effective against replicating *B. burgdorferi* spirochetes and round body forms. Antibiotic resistance and the recurrence of Lyme borreliosis have long been attributed to biofilm-like aggregates in *Borrelia*, both *in vitro* and *in vivo*, and continue to puzzle researchers in their complexity and resilience to this day. Additionally, an organization of exopolysaccharides among distinct *Borrelia* species was recently demonstrated by histochemical tests, raising the question whether biofilm formation is a physiological feature shared among all *Borrelia* species [56].

It is important to note that a fundamental understanding of the interconnected structural, enzymatic, and regulatory factors needed to facilitate biofilm development is imperative for the development of measures aimed at combating all biofilm-related illnesses. Therefore, identification and analysis of bacterial genes, proteins, adhesins, and other components involved in the development of biofilm aggregates can shed light on the mechanisms underlying the persistence and resilience of these morphotypes, which is an essential step in the development of preventative medications or measures to treat biofilm-related illnesses.

Although the mechanism, by which biofilm colonies form has previously been explored, this morphotype has not yet been compared to replicating spirochetes at the transcriptome level [36]. The recent work by Orak and colleagues (2023) was published only after our study was completed. Using RNAseq profiling, they were able to describe and compare the transcriptomes of *B. burgdorferi* spirochetes, round bodies, blebs, and biofilm-dominated cultures. Their findings revealed distinct transcriptional profiles for each morphological state, particularly highlighting that despite their physical distinctions, round bodies and spirochetes

share similar expression patterns. Contrary, blebs and biofilms displayed seemingly different transcriptomes that substantially differed from those of spirochetes and spherical bodies.

The recent findings indicate that the transition of *Borrelia* spirochetes to biofilms involves a significant downregulation of expression processes on the primary chromosome and a notable shift in the expression of genes from this chromosome onto plasmids. This suggests that *Borrelia* may rely on specific plasmid-residing genes for biofilm formation and maintenance, which is in accordance to our findings concerning the increased expression of gene BB_0383 in biofilms, described to be a plasmid-residing gene [49]. These plasmid-borne genes were also revealed to be young evolutionary genes that arose in the phylostratum 6 (ps6) ancestor of the *Borreliaceae* family. The exact role of the genes specific to *Borreliaceae* remains largely obscure, despite their abundance. This statement corresponds with our findings regarding the expression of uncharacterized protein of the gene BB_A40. Numerous virulence genes associated with immune evasion and tissue adhesion in Lyme borreliosis have been traced back to this evolutionary period, implying that the biofilm morphotype could indeed play a crucial function in the dissemination and persistence of *B. burgdorferi* within the mammalian host.

The authors of this study stress the importance of functional characterization of the large pool of unstudied *Borreliaceae*-specific genes that were discovered during transcriptomic comparison of various morphological forms of *Borrelia* spirochetes, as this subset undoubtedly contains genes involved in the pathogenesis of LB that are yet to be identified. This statement highlights the fundamental importance of the primary aim of our project.

The findings of this project represent just the beginning of important research on differentially expressed genes in various morphological variants of *Borrelia*. Further work should involve comparing proteomics and transcriptomics data, identifying accurate markers for distinct pleomorphic forms of spirochetes based on gene and protein expression levels by conducting qRT-PCRs, examining the expression of relevant recombinant proteins, and generating antibodies. This project should serve as an initial step towards identifying markers that can facilitate the detection of latent forms of *Borrelia* in patients with chronic Lyme borreliosis, for which conventional diagnostic tests have failed to identify the pathogenic agent causing the disease.

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# 7. Supplemented Materials

## 7.1 Sequences

**gene BB_0383**

GenBank accession number: Q45010

aa sequence

MNKILLLILLESIVFLSCSGKGSLGSEIPKVSLIIDGTFDDKSFNESALNGVKKVKEE
FKIELVLKESSSNSYLSDLEGLKDAGSDLIWLIGYRFSDVAKVAALQNPDMKYAII
DPIYSNDPIPANLVGMTFRAQEGAFLTGYIAAKLSKTGKIGFLGGIEGEIVDAFRY
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EVAKELGSGHYIIGVDEDQAYLAPDNVITSTTKDVGRALNIFTSNHLKTNTFEGGK
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NCBI Reference Sequence: NZ_CP019767.1

Nucleotide sequence from GenBank

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**gene BB_0389**

GenBank accession number: Q59191

aa sequence

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KAQFGGQRLGEMEVWALEAYGAAHTLQELLTVKSDDMSGRVKIYENIVKGVPT
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NCBI Reference Sequence: NZ_CP019767.1

Nucleotide sequence from GenBank

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CTATTGAAACCCCTGAAGGGCCAAATATTGGACTTATTGTTTCTTTGGCTACTT
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ATGGAGTGGTGACGGACCAATTAGAATATTTATCTGCTATTGACGAAGAGAA
AAAGTGTATTGCTCAGGCTAATGCTGCTTTTAATTCTAATGGAAAGTATCTTG
AAGATTTAGTTTCTGTTAGAATTTCTGGTGATTATACTACAACAAGTCCCACA
AATATAGACTATATGGACGTTTCTCCTAGGCAGCTAATTTCAGTATCTTCGGC
GTTAATTCCTTTTCTTGAGCACAATGATGCAAATCGAGCTCTTATGGGTTCTAA
TATGCAAAGACAAGCAGTACCTTTGCTTTTCCCTAAGCCTCCTATTGTTGGTA
CGGGTATGGAAAGCGTTGTTGCAAAGGATTCAGGAGTAGTTGTTAAGGCTAA
AAGAAGTGGGGAAGTTATTCTTGCAACAAGTAGTAAGATAGTTGTTAAACCTT
TTGAGGCAGAGAATGCTAAAGATTTAGATGAATATCATATTGTTAAGTATGAA
AGGACAAATCAAGACACTTGTTTTAATCAATCCGTTTTAGTTAAAGAGGGTC
AAAAAGTTGAAAGGGGCGAGATAATAGCTGACGGTCCTGCTACTAGATATGG
AGAACTTGCTCTTGGTAATAATTTATTGCTAGGAGTTATTCCTTGGAATGGATT
TAATTATGAGGATGCTATATTAATTTCTGATAGAATTGTAAAGGAAGATCTTT
ATACATCTATTCATATCAAAGAATTTAGCATAGAGGTAAGAGAAACTAAAC
TTGGTCCTGAGAAAGTTACAGGAGATATACCTAATGTTAGTGAAAAGATATTA
AATAAATTGGATGAAAATGGGATTATACGGATAGGAACTTATGTAAAGCCCG
GTGATATTCTGGTTGGTAAAGTTACTCCAAAGTCAGAAGGAGACATTACTCCT
GAATTTAGACTGTTAACTTCCATTTTTGGAGAAAAAGCAAAAGATGTTAAAA
ATAATTCATTAAAAGTTCCTCATGGTACTGAAGGTACAGTTATTGATGTTCAA
AGGATTACCAAAGAGGATGTTGGTAATCTTTCTCCTGGAGTTGAGGAGATACT
TAAAGTTTATGTTGCCAAAAAAAGGAAGCTTAAAGAGGGCGATAAAATGGCT
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TGCCTTATCTTGCAGACGGAACCCCTCTTGATATATGCTTAAATCCTTTGGGA
GTTCCATCTAGAATGAATATCGGACAGTTAATGGAATCTCAATTAGGCCTTGC
TGGTAAATATCTTGGTGAATCTTATAATGTTCCTGTTTTTGAATCTGCTACAAA
TGAACAAATTCAGGAAAAATTAAAAACTGCTGGATTTAATCCAACTTCTAAAG
AAATTTTATATGATGGTTATACAGGAGAGCCGTTCGAAAATGAAGTAATGGTT
GGGGTGATTTACATGCTTAAACTACACCATCTTGTTGATGATAAAATGCACGC
AAGATCAACAGGCCCATATTCTCTTGTTTCTCAGCAACCTCTTGGAGGAAAGG
CTCAATTTGGTGGGCAAAGACTTGGAGAAATGGAGGTTTGGGCTCTTGAAGCT
TATGGTGCGGCGCACACCCTTCAAGAACTTTTAACAGTTAAATCTGATGATAT
GTCAGGCAGAGTTAAAATATATGAAAATATAGTAAAAGGCGTTCCTACTAAT

GTATCAGGGATTCCTGAGTCTTTTAATGTGCTAATGCAAGAGCTTAGAGGGCT
TGGACTTGATTTGTCAATTTATGATGATGCTGGGAATCAGGTTCCTTTGACAG
AAAAAGAAGAAGAATTGATTAATAAAAGCTAG

**gene BB_0123**

GenBank accession number: O51149
aa sequence
MAIITMKSLLEAGVHFGHQVKRLDPRMKRFIFSERNEIHILDLQKTLQGIKDSYEL
VQRVIKDGKKVLFVGTKKQASEIIEQEARRSDMPYVNNRWLGGMLSNFNTIRKS
VQKLKKLEKMEVDGTFDMISKKEISQLNREKSKLAKNLTGIKDMETLPGAIFIIDP
KREQIAINEARKLKIPIISVVDTNCNPDVIDCPIPGNDDAIRSVALFTKIISDAILESD
KEVGIQIIENLNEED LMKEIEIKNDKSDSIEERGE.

NCBI reference sequence: NZ_CP019867.1
Nucleotide sequence from GenBank
TTGGCAATTATTACTATGAAGAGCCTGTTAGAGGCCGGAGTTCATTTTGGCCA
TCAAGTAAAAAGGCTTGATCCTAGAATGAAAAGATTTATTTTTTCTGAGAGAA
ATGAAATACATATTTTAGATCTTCAAAAAACTTTGCAGGGTATTAAAGATTCT
TATGAACTTGTTCAAAGGGTAATAAAAGATGGCAAAAAGGTGCTTTTTGTTGG
AACCAAAAAGCAAGCTAGTGAGATAATAGAACAAGAAGCAAGAAGAAGTGA
TATGCCATATGTAAACAATAGATGGCTTGGGGGCATGCTTTCTAATTTTAATA
CGATTAGAAAATCTGTTCAAAAATTAAAAAAGCTAGAAAAGATGGAAGTTGA
TGGAACTTTTGACATGATAAGCAAAAAAGAGATTTCACAACTTAATCGTGAA
AAATCAAAATTAGCTAAAAATTTAACAGGCATCAAGGACATGGAAACACTTC
CTGGTGCTATTTTTATCATTGATCCTAAGCGAGAGCAGATAGCTATTAATGAG
GCTAGAAAATTAAAAATTCCCATTATTTCTGTGGTTGATACTAATTGTAATCC
AGATGTTATTGATTGTCCAATTCCTGGCAATGATGATGCGATTCGCTCTGTT
GCTTTGTTTACTAAAATAATATCTGATGCTATTTTAGAAAGTGATAAAGAGGT
TGGTATTCAAATAATTGAAAATTTGAATGAAGAAGATTTGATGAAAGAAATT
GAAATTAAAAACGATAAAAGTGATTCTATTGAAGA AAGGGGAGAGTAA

**gene BB_0149**

GenBank accession number: O51173

aa sequence

MASGFFVPGLESKYNTKEIRESMLKSDKAKIDSSFKKLESLEQEKSAWQLINRKIS
TLNSLAKELTSLNSPFNLMSGNSSNSEVLTLSTRYGSKNETHKLIVDQIASADVFL
SSNFDPKKVTIPEGDYIFLVGKKEINVKSNGNIDLLVKDINNKGKGFLSAKIVKSD
KNGNSRFVLQSLKEGKENKLVIKGEGLSFAKQIGILSELKTNFNPNLSDIVVNQSSS
NNKLAFENNGLVLNPLSEVSIEIPEDIEITSRSKIKFEVKYFDTGLEEPDSKIIFNPGG
ATFKDAKVESEDSVVDLGSDLKTPLEKKYIQMNMVKICSKEGSLELPLINISNNFE
EVEVDVGALSNLEEINIENKANNKVIVISNVEIFDPKNRDGHLPINAKSFAENAKIK
FDGVDVERDSNVINDLVPNVTLSLKKPSSDMVEAKIEPDYEGIKRVLLDFIGAYNE
VLAEINIVSSNEDQPNNQKSNIVEELTYLSDSQKEEAYKNLGILRSEFLLKNLKSKL
ESIIFKPYVTSDPNFSIINQMGVFTNSISSSGGLSRYLRLDEKKFDESIRNNIDNVREL
FLYDLNGDRVYDNGIAKMLGDCLSPLVASGGVIYNKIKNYDLKIFNQKNKVEDY
KKKYEDRERKVEGELNTLDFTVKRMKDQENTLKAFDF NQRNK.

NCBI Reference Sequence: NZ_CP019767.1

Nucleotide sequence from GenBank

TTGGCATCAGGATTTTTTGTTCCTGGACTTGAGAGTAAGTACAATACTAAAGA
AATTCGTGAATCCATGCTTAAGTCCGATAAGGCTAAAATTGATTCTTCTTTTAA
GAAACTTGAATCTTTAGAGCAAGAGAAAAGTGCCTGGCAGTTAATTAATAGA
AAAATCTCTACTTTAAATTCTCTTGCAAAAGAACTTACATCACTCAACAGTCC
TTTTAATCTAATGTCAGGAAATTCTAGTAATAGCGAAGTTTTAACTTTGTCTAC
TAGATATGGATCTAAGAATGAGACTCATAAATTAATTGTTGATCAAATAGCGT
CAGCTGATGTGTTTTTGTCTTCAAATTTTGATCCTAAAAAAGTTACAATCCCAG
AGGGAGATTATATATTTTTAGTTGGCAAGAAAGAAATTAATGTAAAAAGTAA
TGGCAACATTGATTTACTTGTGAAGGATATTAATAACAAGGGAAAGGGCTTTT
TATCTGCAAAAATAGTGAAAAGTGATAAAAAATGGAAATAGTCGTTTTGTTTTG
CAATCCTTAAAGGAGGGCAAAGAAAACAAGCTTGTTATCAAAGGGGAGGGAT
TGTCTTTTGCTAAGCAAATTGGAATTTTAAGTGAGCTTAAAACCAATTTTAATC
CTAATCTTTCAGATATTGTTGTAAATCAATCTAGCAGCAACAATAAACTTGCT
TTTGAGAACAATGGTCTTGTTTTAAATCCGCTTTCAGAAGTATCAATTGAAATT
CCTGAAGATATTGAAATTACATCTAGGAGTAAGATTAAATTTGAAGTTAAGTA

TTTTGATACAGGCTTGGAAGAGCCTGATAGTAAGATTATTTTAATCCCGGAG
GGGCTACATTTAAGGATGCAAAAGTTGAGAGTGAAGATAGTGTAGTTGATCTT
GGATCTGATTTAAAAACCCCTTTGGAAAAAAAATATATTCAAATGAATATGGT
TAAAATATGTAGCAAGGAAGGTTCTTTGGAGCTTCCTTTAATAAATATTTCAA
ATAATTTTGAAGAAGTTGAAGTTGATGTTGGAGCTCTTTCTAATTTGGAAGAA
ATAAATATTGAAAATAAAGCAAATAATAAAGTAATTGTGATTAGCAATGTTG
AAATTTTTGATCCAAAAAATAGAGATGGTCATTTGCCAATAAATGCTAAAAGT
TTTGCTGAAAATGCAAAAATTAAATTTGATGGAGTAGATGTTGAGAGAGATTC
AAATGTTATAAATGATTTGGTTCCAAATGTGACATTAAGTTTAAAAAAACCCT
CAAGTGATATGGTTGAGGCTAAAATTGAACCTGATTATGAGGGGATTAAGAG
GGTTCTTTTAGATTTTATTGGTGCTTATAATGAGGTTCTTGCTGAGATTAATAT
TGTAAGCTCTAATGAAGATCAGCCTAATAATCAAAAGTCTAATATAGTTGAAG
AGCTAACTTATCTTAGTGATTCTCAAAAAGAAGAGGCTTATAAAAATTTAGGT
ATTCTAAGGTCTGAATTTTTATTAAAAAATCTTAAGTCCAAGCTAGAGTCAAT
AATTTTTAAGCCTTATGTTACTAGTGATCCTAATTTTTCAATAATTAATCAGAT
GGGAGTTTTTACAAATTCCATTTCTTCTTCTGGTGGACTTTCTAGATATTTAAG
ACTTGATGAGAAAAAGTTTGATGAATCAATTCGTAATAATATTGATAATGTTA
GAGAGCTTTTTTTATATGATCTTAATGGTGACAGAGTGTATGATAATGGAATT
GCTAAAATGCTAGGAGATTGTCTGTCGCCTCTTGTGGCTTCCGGAGGAGTTAT
TTATAATAAAATAAAGAATTACGACTTGAAAATTTTTAATCAAAAAAATAAA
GTTGAAGATTATAAAAAGAAGTACGAAGATAGAGAGAGAAAAGTGGAAGGT
GAACTTAATACCTTGGATTTTACCGTTAAGCGCATGAAAGATCAAGAAAATA
CATTAAAGGCTTTTGATTTTAATCAAAGAAATAAATAA

**gene BB_A16**

GenBank accession number: P17739

aa sequence

MRLLIGFALALALIGCAQKGAESIGSQKENDLNLEDSSKKSHQNAKQDLPAVTED
SVSLFNGNKIFVSKEKNSSGKYDLRATIDQVELKGTSDKNNGSGTLEGSKPDKSK
VKLTVSADLNTVTLEAFDASNQKISSKVTKKQGSITEETLKANKLDSKKLTRSNG
TTLEYSQITDADNATKAVETLKNSIKLEGSLVGGKTTVEIKEGTVTLKREIEKDGK
VKVFLNDTAGSNKKTGKWEDSTSTLTISADSKKTKDLVFLTDGTITVQQYNTAGT
SLEGSASEIKNLSELKNALK.

GenBank accession number: L23136.1

Nucleotide sequence from GenBank

ATGAGATTATTAATAGGATTTGCTTTAGCGTTAGCTTTAATAGGATGTGCACA
AAAAGGTGCTGAGTCAATTGGTTCTCAAAAAGAAAATGATCTAAACCTTGAA
GACTCTAGTAAAAAATCACATCAAAACGCTAAACAAGACCTTCCTGCGGTGA
CAGAAGACTCAGTGTCTTTGTTTAATGGTAATAAAATTTTTGTAAGCAAAGAA
AAAAATAGCTCCGGCAAATATGATTTAAGAGCAACAATTGATCAGGTCGAAC
TTAAAGGAACTTCCGATAAAAACAATGGTTCTGGAACCCTTGAAGGTTCAAA
GCCTGACAAGAGTAAAGTAAAATTAACAGTTTCTGCTGATTTAAACACAGTAA
CCTTAGAAACATTTAATGCCAGCAACCAAAAAATTTCAAGTAAAGTTACTAA
AAAACAGGGGTCAATAACAGAGGAAACTCTCAAAGCTAATAAATTAGACTCA
AAGAAATTAACAAGATCAAACGGAACTACACTTGAATACTCACAAATAACAG
ATGCTGACAATGCTACAAAAGCAGTAGAAACTCTAAAAAATAGCATTAAGCT
TGAAGGAAGTCTTGTAGGCGGAAAAACAACAGTGGAAATTAAAGAAGGTACT
GTTACTCTAAAAAGAGAAATTGAAAAAGATGGAAAAGTAAAAGTCTTTTTGA
ATGACACTGCAGGTTCTAACAAAAAAACAGGTAAATGGGAAGACAGTACTAG
CACTTTAACAATTAGTGCTGACAGCACAAAAACTAAAGATTTGGTGTTCTTAA
CAGATGGTACAATTACAGTACAACAATACAACACAGCTGGAACCAGCCTAGA
AGGATCAGCAAGTGAAATTAAAAATCTTTCAGAGCTTAAAAACGCTTTAAAA
TAA

**gene BB_0115**

GenBank accession number: O51142

aa sequence

MIKRYEACFLFKSEEIEYKGSLEEVKKSLEFFGATDVVSNFIGERALEYPIKKQAR
GRYEIIEFSMEGNNLKEFESRLKLIRNLLRYMILVKIVRKINTKKIKRRNFREFRDNI
DKDSLK GASKVETPTGPESTDIQEK.

NCBI Reference Sequence: NZ_CP019767.1

Nucleotide sequence from GenBank

ATGATTAAAAGATATGAGGCATGTTTTTGTTTAAAAGTGAAGAAATTGAATA
TAAGGGTTCTTTAGAAGAGGTTAAAAAATCTTTAGAGTTTTTGGTGCAACTG

ATGTTGTTAGCAATTTTATTGGAGAGAGAGCCTTAGAATATCCTATTAAAAAG
CAGGCTAGAGGTCGTTATGAAATAATAGAGTTTAGTATGGAAGGCAATAAT
TTAAAAGAATTTGAATCAAGGCTTAAGTTAATTAGAAACTTGCTTAGGTATAT
GATTTTGGTGAAAATAGTTAGAAAGATCAATACTAAAAAAATCAAAAGAAGA
AATTTTAGAGAATTTAGAGACAATATTGACAAAGACAGTCTTAAAGGTGCCTC
TAAAGTTGAAACACCAACAGGTCCTGAAAGCACAGATATTCAGGAAAAATAA

**gene BB_0780**

GenBank accession number: O51721
aa sequence
MATSKSGGSSKNGRDSISKRLGVKRSGGQFVKAGEIIVRQRGTKFHKGKNVGLG
RDYTIF ALSSGKVEFKTLKGRKYVSIV.

NCBI Reference Sequence: NZ_CP019767.1
Nucleotide sequence from GenBank
ATGGCAACAAGTAAAAGTGGTGGTAGTTCAAAAAATGGACGAGATTCTATAT
CCAAGCGACTTGGAGTTAAAAGAAGTGGTGGTCAGTTTGTTAAAGCTGGAGA
GATAATTGTTAGACAAAGAGGTACAAAGTTTCATAAAGGTAAAAACGTTGGT
CTTGGAAGAGATTATACAATATTTGCGCTTTCATCTGGTAAGGTAGAGTTTAA
AACTTTAAAGGGGCGAAAATACGTAAGTATTGTTTAG

**gene BB_0614**

GenBank accession number: O51559
aa sequence
MKDGVRKPSGNRASFSAQPNVKGLRKNNSFSCFAKNNLGKSFSKGKKRGK.

NCBI Reference Sequence: NZ_CP019767.1
Nucleotide sequence from GenBank
ATGAAAGATGGAGTTAGAAAGCCTTCGGGCAATAGAGCGTCTTTTAGCGCTC
AGCCTAATGTTAAGGGCTTGAGAAAGAATAATAGTTTTTCTTGTTTTGCTAAG
AATAATTTGGGTAAAAGTTTTTCAAAGGGTAAAAA AAGGGGTAAATAA

**gene BB_0337**

GenBank accession number: O51312

aa sequence

MGFHIYEIKARQIIDSRGNPTVEADVILEDGTYGRAAVPSGASTGINEAVELRDGD
KSVYMGKGVLKAIENIKNIIAPELEGMSALNQVAIDRKMLELDGTPTKEKLGANA
ILAVSMATAKAAAKYLGLRPYQYLGAYKANILPTPMCNIINGGAHSDNSVDFQEF
MIMPIGAKTFSEAIRMAAEVFHTLKGILSGKGYATSVGDEGGFAPNLKSNEEACE
VIIEAIKKAGYEPGKDIAIALDPATSELYDPKTKKYVLKWSTKEKLTSEQMVEYW
AKWVEKYPIISIEDGMAEEDWDGWKKLTDKIGNKIQLVGDDLFVTNTSFLKKGIE
MGVANSILIKVNQIGTLTETFEAVEMAKKAGYTAIVSHRSGETEDTTIADLVVAL
GTGQIKTGSLSRTDRIAKYNQLIRIEEELETTAE YHGKSVFYSIKQK.

NCBI Reference Sequence: NZ_CP019767.1

Nucleotide sequence from GenBank

ATGGGTTTTCACATTTATGAAATCAAAGCCAGACAAATCATTGATTCTAGAGG
GAATCCAACAGTTGAAGCTGATGTCATTTTAGAAGATGGAACTTACGGAAGA
GCTGCCGTACCATCAGGTGCATCAACAGGAATTAACGAGGCTGTTGAGCTTA
GAGATGGTGATAAGTCTGTATATATGGGAAAAGGGGTTTTAAAGGCAATTGA
AAATATAAAAAACATAATTGCCCCAGAACTTGAAGGTATGAGTGCCTTAAAT
CAGGTTGCAATCGACAGAAAAATGCTTGAACTTGATGGCACCCCTACAAAAG
AAAAGCTTGGTGCTAATGCAATTTTAGCAGTTTCAATGGCTACAGCTAAAGCT
GCTGCAAAGTACCTTGGACTTAGGCCTTATCAATATCTTGGAGCGTACAAAGC
CAACATTTTGCCTACACCTATGTGTAATATTATTAATGGCGGTGCACACTCTG
ACAACTCTGTTGACTTTCAGGAGTTCATGATAATGCCAATAGGAGCAAAAAC
ATTCAGTGAAGCAATAAGAATGGCAGCAGAGGTTTTTCATACGCTAAAGGGC
ATTCTAAGTGGCAAAGGGTATGCAACTTCTGTTGGAGATGAAGGGGGATTTGC
TCCAAATTTGAAATCAAATGAAGAAGCTTGTGAAGTGATTATAGAGGCAATA
AAGAAGGCAGGATATGAACCTGGAAAAGACATTGCAATAGCTCTTGATCCCG
CAACATCTGAGCTTTATGATCCAAAAACAAAAAAATACGTACTTAAATGGTC
AACAAAAGAAAAACTTACTTCCGAACAAATGGTTGAATATTGGGCAAAGTGG
GTAGAAAAATATCCAATCATTTCAATTGAAGATGGAATGGCTGAAGAAGATT
GGGATGGATGGAAAAAACTTACAGACAAAATTGGAAACAAAATACAACTTGT
TGGAGATGATTTATTTGTAACAAATACCTCGTTTCTTAAAAAAGGAATTGAAA

TGGGAGTTGCCAATTCAATCCTTATTAAGGTCAATCAAATTGGAACACTAACA
GAAACATTTGAGGCTGTAGAAATGGCTAAAAAAGCGGGTTACACAGCAATAG
TCTCTCACAGATCGGGAGAAACAGAAGATACAACAATAGCTGATCTTGTAGT
AGCTCTTGGAACAGGACAAATCAAAACTGGTTCACTCTCAAGAACAGATAGA
ATAGCAAAATACAATCAACTCATAAGAATAGAGGAAGAATTGGAAACAACTG
CTGAA TACCACGGTAAGAGCGTCTTTTATTCTATTAAACAAAAATAA

**gene BB_A40**

GenBank accession number: O50933
aa sequence
MSDSIDFQKEIEKLKASKVELESQLESLKKNQAQKIVLDKLQSVNASSYPVFESHK
FQDEGLYFAQKGGLKSSSADKFENYQALDFCYKCGVKLIVNGSNLQIAKGGGSD
LYGVCVDFDDFSRTGTVVPITCSFECVLITKDKTIKAEDKLIINSEGVLEKSSKNAS
VIHALALGPALEFKDRRDVYGVRVLFLVKQIKDSI.

Nucleotide sequence from GenBank
TATTTTGCTCAAAAAGGCGGACTTAAAAGCTCTTCTGCTGATAAGTTTGAAAA
TTATCAAGCTttaGATTTTTGCTACAAATGCGGGGTTAAGCTTATTGTTAACGGT
TCAAATTTGCAAATAGCAAAAGGCGGTGGTAGTGATCTTTATGGGGTTTGTGT
AGACTTTGACGATTTTTCAAGAACCGGCACGGTTGTTCCAATCACTTGTAGTTT
TGAATGTGTTTTGATTACTAAAGACAAAACCATCAAAGCAGAAGACAAATTA
ATAATAAACAGCGAAGGGGTTTTAGAAAAATCTAGCAAAAATGCATCTGTTA
TTCACGCTTTAGCATTAGGTCCTGCA

**gene BB_0658**

GenBank accession number: O51602
aa sequence
MYKLVLVRHGESEWNKENLFTGWTDVKLSDKGIDEAVEAGLLLKQEGYSFDIAF
SSLLSRANDTLNIILRELGQSYISVKKTWRLNERHYGALQGLNKSETAAKYGEDK
VLIWRRSYDVPPMSLDESDDRHPIKDPRYKHIPKRELPSTECLKDTVARVIPYWTD
EIAKEVLEGKKVIVAAHGNSLRALVKYFDNLSEEDVLKLNIPTGIPLVYELDKDLN
PIKHYYLGDESKIKKAMESVASQGKLK.

NCBI Reference Sequence: NZ_CP019767.1

Nucleotide sequence from GenBank

ATGTATAAATTAGTTTTAGTAAGACACGGAGAGAGTGAGTGGAATAAAGAAA
ATCTTTTTACTGGTTGGACAGATGTTAAACTTTCTGACAAGGGTATCGATGAG
GCTGTTGAGGCGGGTTTGCTTCTCAAACAAGAAGGCTATTCTTTTGATATTGCT
TTTAGTTCTTTGTTGTCAAGAGCTAATGACACTTTAAATATTATTTTGCGAGAA
TTAGGGCAATCTTATATTAGTGTAAAAAAAACCTGGAGATTAAATGAAAGGC
ACTATGGAGCTTTGCAAGGTTTAAATAAGTCAGAAACAGCTGCAAAATATGG
GGAAGATAAGGTTTTAATTTGGAGACGTAGTTATGATGTGCCCCCAATGTCTT
TGGATGAGTCTGATGATCGTCATCCCATAAAAGATCCAAGATATAAACATATC
CCCAAAAGGGAACTTCCTTCAACAGAGTGCCTTAAAGATACTGTTGCAAGAG
TTATTCCTTATTGGACTGATGAGATTGCAAAAGAAGTTCTTGAAGGTAAAAAA
GTTATTGTTGCTGCTCACGGTAATTCTTTAAGAGCGCTTGTTAAATATTTTGAC
AATTTAAGTGAAGAAGATGTTTTAAAGCTTAACATTCCCACAGGCATTCCTTT
AGTTTACGAATTAGATAAAGATTTAAATCCCATTAAACATTACTATCTAGGTG
ATGAGAGCAAAATTAAAAAGGCAATGGAATCTGTTGCTAGTCAAGGAAAGTT
AAAGTAA

## 7.2 Primer pairs

*Table 15. Primer combinations designed for Bb replicating spirochetes and biofilms over the course of this study. Highlighted in yellow are the primer pairs that showed promising results.*

| Forward primer | Sequence (5’- 3’) | Reverse Primer | Sequence (5’- 3’) | Size of Primer [bp] | Annealing temperature [°C] |
|---|---|---|---|---|---|
| **Gene BB_0383** Basic Membrane protein A | | | | | |
| F1 | AAGGATGCGGGCTCAGATTT | R1 | GCAACGCTTCTACCAGCTTC | 368 | 56.6 |
| F2 | AAGGTAGTCTTGGGAGCGAA | R2 | AAATCTGAGCCCGCATCCTT | 199 | 55.3 |
| F5 | AGAGCTCAAGAGGGTGCATT | R5 | TTGCAACCTCAATAGCCCCAA | 292 | 56.0 |
| F1 | AAGGATGCGGGCTCAGATTT | R5 | TTGCAACCTCAATAGCCCCAA | 427 | 56.6 |

| | | | | | |
|---|---|---|---|---|---|
| F2 | AAGGTAGTCTTGGG AGCGAA | R1 | GCAACGCTTCTACCA GCTTC | 527 | 55.3 |
| **Gene BB_0389** DNA-directed RNA polymerase Subunit beta | | | | | |
| F1 | GACGTGTGGGGCGG TATAAA | R1 | AATTGGTCCGTCACC ACTCC | 613 | 57.4 |
| F3 | TAGCTGACGGTCCT GCTACT | R3 | CGTCCAGCCATTTTA TCGCC | 559 | 56.7 |
| F5 | AAACCCCTGAAGGG CCAAAT | R5 | ACGCTTTCCATACCC GTACC | 436 | 57.0 |
| F9 | GGTACGGGTATGGA AAGCGT | R9 | TCCAGCCATTTTATC GCCCT | 795 | 56.4 |
| F1 | GACGTGTGGGGCGG TATAAA | R5 | ACGCTTTCCATACCC GTACC | 905 | 57.1 |
| F5 | AAACCCCTGAAGGG CCAAAT | R9 | TCCAGCCATTTTATC GCCCT | 1191 | 56.4 |
| **Gene BB_0123** 30S ribosomal protein S2 | | | | | |
| F1 | CCGGAGTTCATTTTG GCCATC | R1 | ATGCCCCCAAGCCAT CTATT | 265 | 56.3 |
| F12 | ACAGGCATCAAGGA CATGGAA | R2 | TCGCATCATCATTGC CAGGAA | 175 | 56.2 |
| F10 | GAGCCTGTTAGAGG CCGGAG | R10 | GTTTCCATGTCCTTG ATGCCTG | 447 | 58.5 |
| F10 | GAGCCTGTTAGAGG CCGGAG | R2 | TCGCATCATCATTGC CAGGAA | 578 | 58.7 |
| **Gene BB_0149** Flagellar hook-associated protein 2 | | | | | |
| F1 | TTAATCCCGGAGGG GCTACA | R1 | CCACAAGAGGCGAC AGACAA | 951 | 57.5 |
| F5 | AGGGGAGGGATTGT CTTTTGC | R5 | AAATGTAGCCCCTCC GGGAT | 289 | 57.9 |
| F9 | GAGCAAGAGAAAA GTGCCTGG | R9 | TTAAATGTAGCCCCT CCGGG | 740 | 56.2 |
| F5 | AGGGGAGGGATTGT CTTTTGC | R1 | CCACAAGAGGCGAC AGACAA | 1197 | 57.4 |
| F9 | GAGCAAGAGAAAA GTGCCTGG | R1 | CCACAAGAGGCGAC AGACAA | 1646 | 56.9 |
| **Gene BB_A16** Outer surface protein B (OspB) | | | | | |
| F1 | AAACAAGACCTTCC TGCGGT | R1 | CCACTGTTGTTTTC CGCCT | 475 | 55.9 |

| F9 | CCCTTGAAGGTTCAAAGCCTG | R9 | TTCCGCCTACAAGACTTCCTTC | 278 | 56.3 |
|---|---|---|---|---|---|
| F10 | GAAGGAAGTCTTGTAGGCGGA | R10 | CTGATCCTTCTAGGCTGGTTCC | 246 | 56.4 |
| F1 | AAACAAGACCTTCCTGCGGT | R10 | CTGATCCTTCTAGGCTGGTTCC | 687 | 56.6 |
| F9 | CCCTTGAAGGTTCAAAGCCTG | R10 | CTGATCCTTCTAGGCTGGTTCC | 524 | 56.7 |
| **Gene BB_0115** 30S ribosomal protein S6 | | | | | |
| F1 | AAAGCAGGCTAGAGGTCGTT | R1 | TGCTTTCAGGACCTGTTGGT | 245 | 56.4 |
| F3 | TGGTGCAACTGATGTTGTTAGC | R1 | TGCTTTCAGGACCTGTTGGT | 285 | 55.9 |
| **Gene BB_0658** gmpA | | | | | |
| F1 | GTTGAGGCGGGTTTGCTTCT | R1 | TGGGGGCACATCATAACTACG | 250 | 57.6 |
| F4 | ATGATGTGCCCCCAATGTCTT | R4 | ACCGTGAGCAGCAACAATAAC | 200 | 56.1 |
| F10 | AAGGCACTATGGAGCTTTGC | R10 | AAGGAAGTTCCCTTTTGGGGA | 180 | 55.6 |
| F1 | GTTGAGGCGGGTTTGCTTCT | R4 | ACCGTGAGCAGCAACAATAAC | 440 | 52.0 |
| F10 | AAGGCACTATGGAGCTTTGC | R4 | ACCGTGAGCAGCAACAATAAC | 290 | 55.4 |
| F10 | AAGGCACTATGGAGCTTTGC | R1 | TGGGGGCACATCATAACTACG | 100 | 56.1 |
| **Gene BB_0337** Enolase | | | | | |
| F2 | GAAGAGCTGCCGTACCATCA | R2 | TGTTGGCTTTGTACGCTCCA | 324 | 56.8 |
| F3 | TCCCGCAACATCTGAGCTTT | R3 | ACCGTGGTATTCAGCAGTTGT | 541 | 56.2 |
| F4 | TTGAACTTGATGGCACCCCT | R4 | GATGTTGCGGGATCAAGAGC | 454 | 56.5 |
| F2 | GAAGAGCTGCCGTACCATCA | R4 | GATGTTGCGGGATCAAGAGC | 620 | 56.5 |
| F4 | TTGAACTTGATGGCACCCCT | R3 | ACCGTGGTATTCAGCAGTTGT | 962 | 56.2 |
| **Gene BB_0780** 50S ribosomal protein L27 | | | | | |

| F1 | TGGCAACAAGTAAAAGTGGTGG | R1 | ACCTTACCAGATGAAAGCGCA | 199 | 55.5 |
|---|---|---|---|---|---|
| F9 | GTGGTCAGTTTGTTAAAGCTGGA | R9 | ACTTACGTATTTTCGCCCCTTT | 158 | 53.7 |
| F1 | TGGCAACAAGTAAAAGTGGTGG | R9 | ACTTACGTATTTTCGCCCCTTT | 214 | 54.6 |
| **Gene BB_0614** Uncharacterized protein | | | | | |
| F5 | TTCGGGCAATAGAGCGTCTT | R5 | CCCTTTGAAAAACTTTTACCCAAAT | 111 | 53.6 |
| F8 | ATGAAAGATGGAGTTAGAAAGCCT | R5 | CCCTTTGAAAAACTTTTACCCAAAT | 109 | 51.2 |
| **Gene BB_A40** Uncharacterized protein | | | | | |
| F3 | GGCACGGTTGTTCCAATCAC | R3 | GCAGGACCTAATGCTAAAGCG | 190 | 56.6 |
| F5 | TACCCGGTATTTGAGAGCCAC | R5 | TCACTACCACCGCCTTTTGC | 190 | 57.3 |
| F5 | TACCCGGTATTTGAGAGCCAC | R3 | GCAGGACCTAATGCTAAAGCG | 380 | 56.1 |