

# The Identification of *Borrelia* Strains Carrying the BstBI Mutation in the *fur* Gene Homologue

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## Social Impact

Lyme disease affects thousands of people in the United States with symptoms ranging from nerve paralysis to heart attacks. This research looks into studying gene mutation in *Borrelia burgdorferi*, the bacterial vector of Lyme disease. Certain gene mutations in *Borrelia* render the bacteria defenseless against the human immune system. This research focuses on one mutation site called BstBI. Although there are laboratory grown strains of *Borrelia* with this mutation, up until this point, no naturally occurring strains have been isolated carrying the mutation. By analyzing more than 40 strains of *Borrelia*, this paper describes a naturally occurring strain containing the same mutation. This unique discovery indicates that more research should be directed into developing a therapy that induces a strategic gene mutation in *Borrelia*, either through a vaccine or another mechanism.

“Our study supports the theory that the BstBI mutation exists in the wild.”



## Biography

Robert Sampson is a second-year biology major from Stony Brook, New York. At the University of Virginia, he is the Chief Information Officer of the McIntire Investment Institute, a Peer Advising Family Network Advisor, a volunteer with Madison House, a Resident Advisor, and an EMT. His research on Lyme disease was conducted during high school at State University of New York (SUNY) at Stony Brook under Dr. Jorge Benach, James Coleman, and Dr. Laura Katona. SUNY Stony Brook is one of the paramount locations in the world to study Lyme disease and is where the first one-hour assay for the disease was developed. Currently, Robert works in Dr. Tung's laboratory at the University of Virginia studying the immunology and pathology of autoimmune diseases. In the future, Robert plans to continue researching while pursuing a career in Medicine. In his free time, Robert enjoys playing cello, fencing, trading stocks, playing ping-pong, and spending time with his friends.

## Abstract

To cure Lyme disease, a better understanding of transcriptional proteins in the bacterial vector *Borrelia burgdorferi* must be gathered to learn more about how the bacterium regulates its genome. Ferric uptake regulation protein (*fur*) is a transcriptional regulator found in most bacterial species, including *Borrelia burgdorferi*. When *Borrelia* invades a human, the host immune system responds by producing Reactive Oxygen Species (ROS) to fight the infection. Properly functioning *fur* mitigates the effect of ROS with enzymes, such as superoxide dismutase B. However, improperly functioning *fur* that has a BstBI mutation site allows *Borrelia* to be highly susceptible to ROS and be easily eradicated by an immune system. In two newly studied laboratory strains of *Borrelia*, B31F and CHP100, the BstBI mutation exists, which would allow a host immune system to easily irradiate the pathogen. The validity of these strains has been called into question since they were developed in a lab and not in the wild. Scientists argue some lab strains are artifacts and are not representative of naturally occurring strains. Analysis was conducted to search for a wild strain of *Borrelia* that contains the mutation. After analyzing over 40 different strains, *Borrelia* ACA1, a wild strain, was found to also contain the BstBI mutation. This unique finding suggests there are other strains of *Borrelia* that also have this *fur* mutation. In the future, the BstBI mutation could have a role in curing Lyme disease as vaccines and other immune therapies can be developed with the *fur* protein as their target.

### Introduction

Lyme disease is an infection caused by the spirochete *Borrelia burgdorferi* (Benach et al., 1983; Steere et al., 1983). In order for *Borrelia* to infect a human, the bacterium must travel from the gut of an *Ixodes scapularis* tick, to the salivary glands, where the tick inoculates the bacteria into the skin of the host (Benach et al., 1987; Ribeiro et al., 1987; Zung et al., 1989). The spirochete's pathogenicity is altered by certain host factors, such as blood, temperature, and oxygen levels (Seshu, Gherardini, et al., 2004; Tokaraz et al., 2004).

When *Borrelia* infects a host, under normal conditions, the immune system combats the bacterium with reactive oxygen species (ROS) (Boylan et al., 2008). It has been proposed that macrophages from hosts generate ROS, such as  $H_2O_2$ , to combat pathogens via a Fenton reaction. ROS are normally hydrolyzed by superoxide dismutase B (*sodB*), an enzyme that reduces oxidative stress to protect *Borrelia* from a host immune system (Boylan et al., 2008; Thompson, et al. 2002).

Ferric uptake regulation protein (*fur*) is a transcriptional protein that regulates genes in most bacterial species. It is currently unknown if the function of *fur* in *Borrelia* is an activator or a repressor of gene transcription (Boylan et al., 2003; Katona et al., 2008). Some researchers suggest *fur* is a global regulator, while others contest *fur* has no role in the regulation of *Borrelia* (Andrews et al., 2003; Boylan et al., 2003). Even though some functions for *fur* have been discovered in bacteria, we are just beginning to understand the way *Borrelia* responds to its dynamic environment.

In response to ROS, it is believed *fur* is less able to bind to target genes such as *sodB* when the *fur* homologue possesses a mutation on the BstBI restriction site (Seshu, Hyde, et al., 2004). An underexpression of *sodB* renders the bacteria unable to hydrolyze  $H_2O_2$ , thus killing the spirochete in high enough concentrations. In noninfectious *Borrelia burgdorferi* CHP100, a laboratory derivative of B31, the *fur* gene was found to have a single point mutation of an adenine to a guanine nucleic base, which ultimately caused a change in amino acid 39 from an arginine to a lysine (Hyde et al., 2006; Seshu, Gherardini, et al., 2004; Seshu, Hyde, et al., 2004). Studies have shown when CHP100 is exposed to oxidative stress, its viability decreases five orders of magnitude in the presence of 1mM of *t-butyl*hydroperoxide, another ROS similar to  $H_2O_2$ , when compared to infectious *Borrelia burgdorferi* MSK5, a clonal derivative of B31 (Seshu, Gherardini, et al., 2004). The bacteria's inability to survive with high ROS levels may be due to the change in amino acids of *fur* between lab derivative strains CHP100 and MSK5.

To date, only two strains of *Borrelia* have been discovered with a mutation on the *fur* homologue, *Borrelia burgdorferi* strain CHP100, and *Borrelia burgdorferi* strain B31F, also a lab derivative of *Borrelia burgdorferi* B31 (Katona, 2008; Seshu, Hyde, et al., 2004). New information suggests an additional strain, ACA1, also possesses the *fur* BstBI mutation. The presence of the BstBI mutation was investigated in 42 strains of *Borrelia* to compare the B31

derivative strains (CHP100 and B31F), which are known to be less virulent than wild-type strains occurring in nature. To confirm each sample's authenticity after being studied, widely studied taxonomical molecular markers, such as *groEL*, were utilized (Park et al., 2004).

## Materials and Methods

### Storage and Culturing of *Borrelia* species:

*Borrelia* species were made available from previous laboratory experiments frozen in liquid nitrogen for long-term storage. Spirochetes were grown in 50 mL tubes in Barbour-Stoenner-Kelly (BSK-H) complete liquid (6% rabbit serum) and incubated at 33°C.

### DNA Extraction

*Borrelia* cultured in BSK-H complete (6% rabbit serum) was centrifuged at 7000 g and washed in 2X PBS solution. *Borrelia* was then transferred to 50  $\mu$ L of Tris-Borate-EDTA (TBE Buffer). Samples were boiled for 5 minutes, cooled to room temperature, and centrifuged again at 7000 g for 5 minutes. The supernatant was separated from the pellet, which became the template for the Polymerase Chain Reaction (PCR) reaction.

### Primers

Primers were designed to bind efficiently and optimal annealing temperatures were calculated using MacVector version 8.1 software (Macintosh). All oligonucleotides are gel filtration grade and were synthesized at The Midland Certified Reagent Company, Inc. (Appendix, Table 1).

### Polymerase Chain Reaction and DNA Purification

Amplification of *fur* in *Borrelia* species was conducted using an Applied Biosystems thermocycler with Taq DNA polymerase (Roche Molecular Systems, Summerville, NJ). PCR conditions were as follows: 2 minutes at 95°C, 35 cycles of 1 minute at 95°C, 30 seconds at 54.5°C, and 1.5 minutes at 72°C, and a terminal extension of 15 minutes at 72°C.

To purify the PCR products, The Promega Wizard PCR Preps DNA Purification System was used and the manufacturer's instructions for direct purification of DNA from PCR amplifications were followed. DNA presence was verified using spectrophotometry with a wavelength of 260 nm via NanoDrop apparatus.

### Restriction Enzyme Digestion and Gel Electrophoresis

300-400 ng of purified DNA from PCR reactions were digested with 1  $\mu$ L of restriction enzyme BstBI (New England BioLabs) for a final reaction volume of 30-40  $\mu$ L. Samples were incubated for two hours at 65°C. If restriction digestions were effective, DNA was cut, creating 112 and 419 base pair sequences.

Digested purified PCR products were separated by gel electrophoresis on 2.0% agarose gels for 1.5 hours and stained with ethidium bromide. A 10-kb DNA Molecular Ladder (manufactured by Fermentas Life Sciences) was used to verify DNA size.

### The use of B31F as a positive control

Preliminary studies in the lab suggested B31F would be a suitable "positive control" based on positive restriction enzyme digestions and positive bioinformatics results indicating the presence of the BstBI mutation.

### DNA Sequencing

*Borrelia* DNA samples that demonstrated a successful restriction enzyme reaction as well as products that were amplified via *groEL* specific primers were processed with an Applied Biosystems 3730 DNA Sequencer. For each sequencing reaction, 5-20 ng of PCR product template was used along with 3.2 pM of primer in a total sample volume of 8.0  $\mu$ L.

## Results and Discussion

Forty-two strains of *Borrelia* were analyzed. Twenty-four of these strains were tested via restriction enzyme assay, and eighteen were tested via bioinformatics approach using MacVector version 8.1 (Macintosh). The bioinformatics approach entails analyzing genetic code downloaded from



**Figure 1. Restriction Digestion Assay.** Molecular Standard (gel well 1), *Borrelia burgdorferi* B31F (gel well 2), Empty (gel well 3), Unknown *Borrelia* species (gel well 4), *Borrelia garinii* 20001 (gel well 5), Molecular Standard (gel well 6). The red circle isolates the successful cutting of B31F *fur*, indicating a mutation exists.

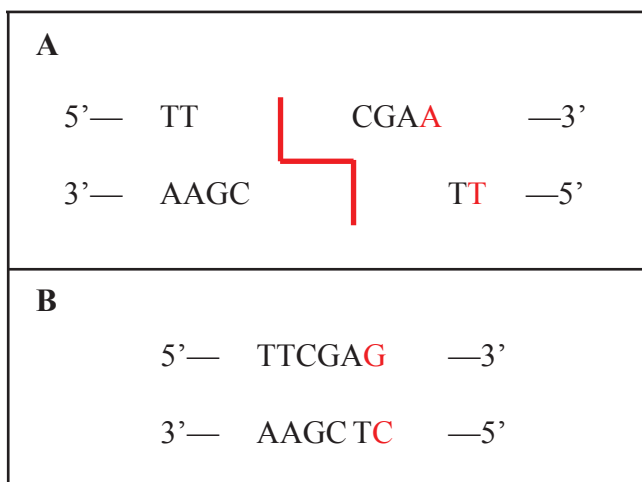
a national *Borrelia* database and scanning for the mutation site via MacVector. As we began testing the *Borrelia* strains, the B31F strain was studied and identified to possess the BstBI mutation. The restriction enzyme assay revealed that a cut was made on B31F *fur*, and therefore a mutation was present (Figure 1).

In Figure 1, Well 2 shows a successful restriction enzyme cut

that suggests a mutation is present. In Well 4, the *Borrelia afzelii* ACA1 strain also shows a successful restriction enzyme cut suggesting it too possesses the mutation. Normally there should be two distinct bands in a digest for a successful cut of *fur* DNA, a 112 base pair strand and a 419 base pair strand. However, the presence of a third band suggests the restriction digestion did not go to completion, which is irrelevant since it is most likely due to the limiting reactant of the quantity of the BstBI enzyme used. However, in Well 5, the restriction enzyme digest did not yield a cut. Therefore, we concluded there was no mutation present and as a result, only one band is shown, one piece of uncut DNA.

By using bioinformatics, we also confirmed that B31F possessed the mutation and could be used as a positive control. We compared all other strains to this positive control in the experiment. In Figure 2, the BstBI restriction enzyme cut site of B31F is circled in red (see appendix).

To further understand this important cut site, Figure 3A shows the recognition site of the BstBI restriction enzyme. *Borrelia burgdorferi* B31F is thus able to be cut by the BstBI restriction enzyme because it possesses a mutation on the 116<sup>th</sup> base pair, which changes a guanine base pair to an adenine base pair. In Figure 3B, no mutation exists on the 116<sup>th</sup> base pair, so it is impossible for restriction enzyme BstBI to make a cut.



**Figure 3. Cut sites.** A. BstBI cut site shown. 116<sup>th</sup> base pair highlighted. B. No BstBI cut site exists. *Borrelia* species without BstBI mutation shown. The highlighted base pairs do not permit cutting by restriction enzyme digestion.

After successfully cutting B31F in a restriction enzyme digest, we found *Borrelia afzelii* ACA1 also cut, as seen in Figure 1. To confirm this, we sequenced *fur* in ACA1 and found it possessed the same sequence as B31F. In *Borrelia*, identical sequences of genes are common among different strains when small samples of the genome are used.

Since both strains possessed the same sequence of *fur*, we sequenced the *groEL* gene in ACA1 to verify that B31F and ACA1 were different (Park et al., 2004). According to Park et al., the *groEL* gene is one of the least conserved identification genes, making it a valuable tool in identifying *Borrelia* strains.

Our ACA1 sequence of *groEL* when put through a BLAST analysis revealed 10 strains of *Borrelia* that had a 100% genetic match to ACA1. These findings indicated that *groEL* did not provide us with a unique signature for ACA1, and that among *Borrelia*, there are many identical sequences of genes.

## Conclusion

The BstBI mutation is an important aspect of a spirochete to study since mutated strains are five magnitudes more susceptible to reactive oxygen species (Seshu, Gherardini, et al., 2004).

Future research will be directed into verifying *Borrelia burgdorferi* ACA1 since it has been found to possess a mutation on the *fur* homologue. Our study supports the theory that the BstBI mutation exists in the wild. As a result, it would be reasonable to deduce this mutation may be prevalent in many strains throughout the world since the mutation has occurred in three out of 42 investigated strains. This is a high ratio when considering the existence of millions of strains of *Borrelia*.

Current research suggests *fur* reduces oxidative stress in *Borrelia* by binding to *sodB*, which protects *Borrelia* from a host immune Fenton reaction (Boylan et al., 2008; Thompson et al., 2002). When there is a BstBI mutation, it is believed *fur* loses the ability to bind to *sodB*. Thus, wild-type strains of *Borrelia* that possess this mutation will naturally be attenuated when they infect a host. The recognition and awareness of the BstBI mutation may help predict the pathogenicity of infectious *Borrelia* species, and may give certain Lyme disease patients a better prognosis (Litwin & Calderwood, 1993).

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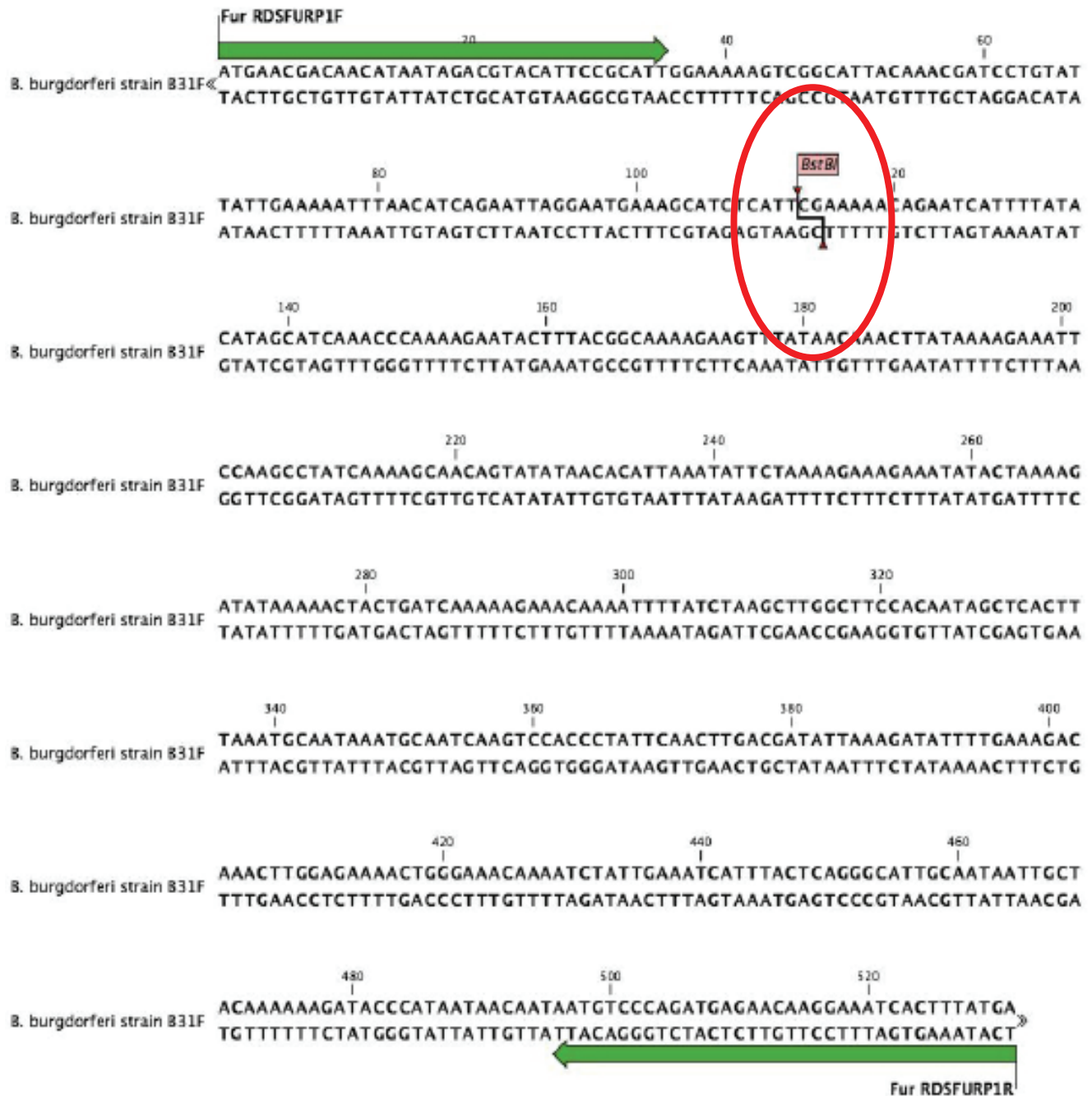
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**Table 1. Oligonucleotides used in this study.**

Purpose	Oligonucleotide	Sequence (5' to 3')
<i>fur</i> PCR Primers	RDSFURP1F	ATGAACGACAACATAATAGACGTACATTCCGCATT
	RDSFURP1R	TCATAAAGTGATTTCCCTTGTTCTCATCTGGGACATT
<i>groEL</i> PCR Primers	GF	TACGATTTCTTATGTTGAGGG
	GR	CATTGCTTTTCGTCTATCACC

**Borrelia burgdorferi B31F fur Genome**



**Figure 2. Borrelia burgdorferi B31F fur Genome.** Bioinformatics depiction of the fur gene in *B. burgdorferi* strain B31F. The BstBI mutation is identified in the red circle. The binding sites for forward and reverse fur primers (Table 2) are shown.