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An Analysis Of Extrachromosomal DNA Isolated From Bartonella henselae

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AN ANALYSIS OF EXTRACHROMOSOMAL DNA ISOLATED FROM Bartonella henselae.

Submitted in Partial Fulfilment of the Requirements for Graduation with Honors to the Department of Biology and Chemistry at Carroll College, Helena, Montana

Martin Janout
April 12, 1996
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ABSTRACT

An unknown sequence of DNA discovered in *Bartonella henselae* was studied in order to determine its conformation, size, and origin. The DNA was isolated from *B. henselae* cells using a standard miniprep method, commonly used to isolate small extrachromosomal DNA from cells. This DNA was subjected to a series of experiments including digestion with restriction endonucleases, S1, and Mung Bean nucleases, and hybridization to *B. henselae* genomic DNA, in order to determine whether there are any sequences in common. DNA fragments produced by digestion were separated using standard gel as well as Pulse-Field Gel Electrophoresis, and viewed after being stained with ethidium bromide. Fragments used for hybridization experiments were labeled with $^{32}$P. The results indicated that the unknown DNA contained many restriction sites for a majority of the restriction enzymes used in the experiment. Pulse Field Gel Electrophoresis of the unknown DNA digested with the S1 and Mung Bean nucleases suggested that the DNA may be linear since both enzymes, which cut strictly circular DNA, did not seem to attack the unknown DNA. The results of the hybridization experiment indicated that the possibility of the unknown DNA sharing sequences with genomic DNA is unlikely. Finally, Pulse Field Electrophoresis of undigested *B. henselae* DNA suggested that the undigested DNA itself consists of a number of fragments of various sizes which were most likely compressed into a single band on the standard electrophoresis gel. This result ultimately disproves the hypothesis that the unknown DNA was produced by a plasmid, bacteriophage, or a transposon, since such DNA would be expected to consist of fragments of uniform size.
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Bartonella henselae is a rod-shaped, piliated bacterium that stains gram negative (10). It is a parasitic species associated with the reticuloendothelia and vascular endothelial cells of vertebrates (10). This organism has been related to Cat Scratch Disease (CSD) and Bacillary Angiomatosis (BA) in humans (6). Approximately 20,000 cases of CSD are recorded in the United States each year, and more than 2,000 patients are hospitalized (3).

In order to cause disease, Bartonella henselae has to be able to adhere to and enter cells. B. henselae contains pili on the cell surface, which are thought to be a determinant of B. henselae pathogenicity (2). Indeed, B. henselae have been shown to adhere to and enter cultured cells in higher frequency than cells containing few or no pili, such as Bartonella quintana and E. coli cells (2). Further, the fact that B. henselae cultured cells which lost their pili also lost the capacity for adherence confirms the assumption that the specific attachment observed was indeed due to the presence of pili (2).

It is possible that a plasmid within a particular B. henselae strain confers to these cells the ability to adhere to and penetrate host cells, since plasmids are known to encode for properties including production of pili and other adhesins (11). Also, minor proteins located at the tip of pili, and responsible for the attachment of pili, are encoded by plasmids as well (11). Therefore, detecting any sort of extrachromosomal DNA in B. henselae cells could be of interest as it might be helpful in fully understanding the process
of adherence and penetration of this bacterium, which is currently being researched. This information could possibly be useful for regulation of the bacterium’s potency to cause disease and the development of a more efficient antimicrobial agent.

During *B. henselae* genome mapping experiments carried out previously in the lab of Dr. Ihler at Texas A&M University Health Science Center, a band migrating with 10-14 kb supercoiled markers was observed repeatedly when DNA prepared from *B. henselae* cell lysates was subjected to standard agarose gel electrophoresis. Such DNA usually falls into one of the following categories: (1) plasmid, (2) bacteriophage, or (3) transposon. Because of the size range of the unknown DNA, it was assumed that the unknown band was due to the presence of a plasmid.

Plasmids are submicroscopic agents that infect bacteria and use bacterial cell components to replicate themselves (9). Most plasmids are covalently closed, circular, double-stranded DNA molecules, although some linear plasmids also exist (1).

Plasmids can have two effects on the host cell. Some completely take over the host cell’s machinery and kill it. Others can be beneficial. Plasmids encode a variety of properties in bacteria. Such properties include resistance to antibiotics and heavy metals, virulence mediated by toxins and pili, adhesiveness, and the ability to metabolize organic compound, and to fix nitrogen (1).

The size range of the unknown DNA would not, however, exclude bacteriophages, which are viruses infecting bacteria. Bacteriophages are similar to plasmids in that they lack the organelles necessary for replication and also reproduce only inside living cells. Most bacteriophages consist of a head containing genetic material and a tail, which
adheres to the bacterial surface and serves as a passageway for DNA/RNA entering the host cell (9). Unlike plasmids, however, bacteriophages contain genes that code for proteins that form a protective shell encasing their genetic material (9). Unlike plasmids, bacteriophages have a narrow host range because a particular phage would have a tendency to infect only a specific type of host cell. Also, the process of adsorption of a bacteriophage to the host cell membrane is highly specific.

Transposons are small, discrete sequences of nucleotides scattered among the genes of living cells that can duplicate themselves, with the new copy being able to “hop” to another DNA molecule (9). Their movement between molecules of DNA is called “transposition.” Consequences of transposition depend on where the new copy becomes inserted (9). Gene information can be interrupted by a transposon, resulting in the functional protein’s no longer being produced (9). Cases have also been reported in which a transposon activated a gene by inserting near the gene (9). Transposons can also carry genes for drug resistance or other genetic abilities (12).

The purpose of the present study was to obtain information about this DNA that could lead to determining its nature and origin, and result in classification of the unknown DNA.

In order to determine its size and conformation, the unknown DNA was digested with a variety of restriction endonucleases, as well as S1 and Mung Bean nucleases in an attempt to cut the DNA only once. The size of the DNA could then be determined when it was subjected to gel electrophoresis. If a single band traveling slower than undigested DNA resulted from the digestion, then linearization of supercoiled DNA could be inferred.
Two bands adding up to the size of the undigested DNA, on the other hand, would lead to the conclusion that it was linear to begin with.

Linear DNA could be radioactively labeled and hybridized to *B. henselae* genomic DNA to see if there are any sequences in common. Further, it would be possible to sequence the DNA once it was rendered linear.
MATERIALS AND METHODS

DNA Extraction

A standard mini-prep method was used to isolate DNA from *Bartonella henselae* cells. A single bacterial colony was transferred into 5 ml of LB medium (10 g tryptone/ 5 g NaCl/ 1 ml 1N NaOH/ 1 l final volume) and incubated at 37°C overnight with vigorous shaking. After a 24-hr incubation, 1.5 ml of the culture were poured into a microfuge tube and centrifuged at 12,000g for 1 min at room temperature. The medium was removed by aspiration and the bacterial pellet was allowed to air dry.

The bacterial pellet was resuspended in 100 µl of ice-cold Solution I (50 mM glucose/ 25 mM Tris-HCl, pH 8.0/ 10 mM EDTA, pH 8.0) by vigorous vortexing. Solution II (200 µl of 0.2N NaOH/ 1% SDS) was added to the suspension. The contents were mixed by inverting the microfuge tube rapidly five times and stored on ice for five min. Solution III (150 µl of 5M potassium acetate/ glacial acetic acid) was added to this mixture and the contents mixed by vortexing. The microfuge tube was then stored on ice for five min.

After centrifuging at 12,000g for 10 min, the supernatant was transferred to a fresh microfuge tube while large genomic DNA remained trapped at the bottom. To this mixture, an equal volume of phenol was added in order to precipitate any protein or lipid
present. Centrifuging at 12,000g and subsequent precipitation with 1/4 volume 1M NaCl yielded a pellet which was then washed with 70% ethanol. This step removes excess salt while keeping the DNA precipitated. The resulting nucleic acid was redissolved in TE buffer (10mM Tris-HCl, pH 8.0/ 1 mM EDTA) and stored at -20°C.

DNA Digestion with Standard Restriction Enzymes

Two successive digestions of *B. henselae* DNA were carried out with two different sets of restriction enzymes. Each digestion tube contained 5 \mu l of bacterial DNA/ 1 \mu l of 10X buffer for the corresponding endonuclease diluted in 1 \mu l of 10X BSA buffer/ 2 \mu l of double distilled water/ 1 \mu l of stock enzyme.

Restriction enzymes used in this digestion were Pst I, Hind III, BamHI, EcoRI, MluI, KpnI, DraIII, XmnI, EcoRV, NotI, SfiI, XbaI, NarI, SmaI, and SphI. The digested fragments were compared to a standard \lambda-Hind III ladder, which is suitable for sizing linear double stranded DNA from 125 bp to 23.1 kb. All bands were visualized using ethidium bromide staining.

*Bartonella henselae* DNA Labeling and Hybridization

DNA transfer from gel onto a nylon membrane took place by using the Southern blotting procedure. The gel was fixed on a steel screen, covered with a nylon membrane,
and flooded with 0.4N NaOH. Then a vacuum was applied and maintained at a constant suction (2-5 in of mercury).

Prehybridization

Powdered milk solution (1.5 ml consisting of 0.5 g powdered milk/ 10 ml 20X SSPE (3.6M NaCl, 0.2M NaH₂PO₄, 0.02M EDTA, pH 7.7)/ 1.5 10 % SDS/ 50 ml double distilled H₂O) was added to a plastic bag containing the nylon membrane. During this prehybridization, casein occupied all of the sites on the membrane which did not contain DNA so that the radioactively labeled probe could not bind to anything except the digested \textit{B. henselae} DNA.

Labeling of \textit{B. henselae} DNA

\textit{B. henselae} DNA was labeled with a “Ready-To-Go” kit in order to make a radioactive probe, which would then be mixed with more \textit{B. henselae} DNA to see if hybridization would take place.

Digested \textit{B. henselae} DNA was dissolved in TE buffer at a concentration such that 25-50 ng could be added to the reaction in a volume of no more than 25 μl. The pellet in the Reaction Mix tube was resuspended in distilled water and stored on ice. \textit{B. henselae} DNA was denatured using the Bio-Rad Gene Linker at exactly 60 Joules of UV energy. The nicked/denatured DNA was added into the reaction tube. The reaction tube then
contained the reconstituted reaction mix, denatured bacterial DNA, \( \alpha^{-32}\text{P} \) dCTP (radioactively labeled nucleotides), and distilled water. The final mixture was incubated at 37°C for 15 min. Then, it was added to the blocking solution and nylon filter and mixed thoroughly.

Autoradiography

All procedures were done under a darkroom safelight. The dried nylon membrane was placed on the bottom of an open X-ray film holder cassette. A piece of Kodak XOMAT XAR-5 X-ray film was placed on top of the nylon filter and incubated at room temperature overnight without an intensifying screen. The exposed X-ray film was developed, fixed, and washed via the X-ray film developer machine in the dark room.

S1 Nuclease and Mung Bean Nuclease Digestion of \( B. henselae \) DNA

A dilution using the stock S1/Mung Bean nuclease was made using 1000 µl of ice-cold 1X S1 buffer and 0.5 µl of stock enzyme (1058 Units/µl). The final concentration of enzyme dilution was 0.5 Units (U) per µl of dilution. The digestion tube contained \( B. henselae \) DNA, the desired amount of enzyme, 2X S1 nuclease buffer, and distilled water, and was incubated at 37°C for a specific period of time.
Pulsed Field Gel Electrophoresis (PFGE)

Pulsed Field Gel Electrophoresis was used to separate the larger DNA fragments into distinct bands as these fragments can be compressed into one thick band if run on a standard 0.8% agar minigel. A Dr II unit used to analyze *B. henselae* DNA fragments operates on a principle of alternating electric current that tends to drag DNA through the gel in a "snake-like" manner which results in a more complete separation of fragments of size above approximately 30 kb. One disadvantage of this method was the amount of time (approx. 20 hrs) it took for DNA to travel from one end of the gel to the other.

Preparation of the 1% Pulse Field Agarose (PFA)

Overall, 100 ml of the Pulse Field Gel were made per experiment. Pulse Field Agarose (1g) was weighed and mixed with 100 ml of 0.5X TBE buffer (445mM Tris-HCl/445mM Boric acid/10mM EDTA) in a flask. After boiling for 5 min, double distilled water was used to return the flask to its original, pre-boil weight before pouring the gel into a casting tray.
RESULTS AND DISCUSSION

Most non-linear DNA species exist as supercoiled circles because closed circles are only produced if some outside agent, such as an endonuclease, disturbs the supercoiled conformation. Therefore, it was assumed that the unknown DNA was a supercoiled circle. The size of such DNA would be best estimated by comparing it to a standard supercoiled ladder.

During standard gel electrophoresis the undigested unknown DNA traveled at the same distance as the 10-14 kb fragment of the supercoiled DNA ladder (Figure 1). The PFGE "plugs" in Figure 1 consisted of *B. henselae* genomic and extrachromosomal DNA imbedded in Pulse Field Electrophoresis Gel. The genomic DNA remained in the wells as it was too large to enter the gel. The 10-14 kb fragment represents the extrachromosomal DNA, and can be seen approximately half way down the gel.

*B. henselae* DNA was then digested with a wide spectrum of restriction endonucleases in order to see if any of these enzymes would cut the DNA only once (Figure 2). Circular DNA cut at a single restriction site would produce a single linear strand. Such linear DNA fragment would be expected to travel considerably slower than the original circular DNA, and its size could be determined by comparison with linear markers, such as 1 kb or 5 kb linear ladders. Linear DNA cut at a single site would produce two fragments. The size of these fragments could also be found using linear markers, and the sum of the two fragments' sizes would equal the size of the undigested DNA.
Figure 1. Agarose gel electrophoresis of undigested *B. henselae* DNA. Undigested *B. henselae* minipreps (lanes labeled 1-4 on the right side of the gel). PFGE plug on the left side of the gel contains *B. henselae* chromosomal DNA. Supercoiled DNA ladder occupies lane 7 in the middle of the gel. Nucleic acid was extracted from whole cells and resolved on 0.8% agarose gel.
Figure 2. Restriction enzyme digestion of *B. henselae* unknown DNA. The right side of the gel (Kph I-Sph I) contains DNA digested with restriction enzymes. Undigested *B. henselae* DNA occupies lane 4. DNA-size ladders are seen on the left side of the gel.
The digestion of *B. henselae* DNA with some restriction enzymes, such as Hind III, produced a smear on the gel. The Hind III enzyme seemed to digest the unknown DNA more successfully than any other enzyme since there was no DNA left in its lane at the level where the undigested DNA stopped. Some enzymes, such as Not I, Sfi I, Nar I, and Sma I, on the other hand, did not seem to digest the unknown DNA at all. Sph I and Xba I enzymes digested the unknown DNA partially.

Hybridization of $^{32}$P labeled unknown, undigested *B. henselae* DNA to the *B. henselae* genomic DNA yielded negative results, suggesting one of the following: (1) There were no sequences in the genomic DNA complementary to the unknown DNA sequence; (2) There was a malfunction during the hybridization procedure or in development of the X-ray film; (3) The undigested DNA failed to be labeled with radioactive $^{32}$P, even though hybridization might have taken place. The third possibility would indicate that the unknown DNA probe is not linear, and is therefore likely to be circular, since the Ready-To-Go kit only labels DNA that has been linearized or is linear to begin with.

Because digestion of *B. henselae* DNA with standard restriction endonucleases produced a smear on a gel, it was assumed that these enzymes cut the DNA into a large number of small fragments. To prevent such excessive digestion, two enzymes were tested that would be likely to cut *B. henselae* DNA only once or a few times. SI and Mung Bean nucleases are known to be "rare cutters" of single-stranded DNA. Such enzymes will "nick" or separate two strands of supercoiled DNA at a single site and then cut the remaining strand. Once DNA is linearized it will not be attacked by either enzyme. Since
S1 and Mung Bean nucleases only attack supercoiled DNA, it could be deduced that *B. henselae* is supercoiled if a pattern of bands different from the undigested lane were to be obtained.

The ability of S1 and Mung Bean nuclease to digest circular DNA was tested using the pSPORT plasmid, a DNA of known size and sequence (Figure 3). Prior to the Pulse Field Electrophoresis, the fragments resulting from S1 and Mung Bean nuclease digestion of *B. henselae* unknown DNA were analyzed using the standard 0.8% electrophoresis gel. When the standard 0.8% minigel was used, the digestion lanes for both enzymes produced a single band at the level of undigested DNA, suggesting that, perhaps, insufficient enzyme concentration may have been used. However, the digestion of *B. henselae* unknown DNA with S1 and Mung Bean nuclease produced surprising results after Pulse Field Gel Electrophoresis (Figure 3). When the DNA fragments resulting from S1/Mung Bean nuclease digestion were run on the Pulsed Field Electrophoresis Gel, a smear appeared in all lanes where *B. henselae* DNA was loaded, regardless of digestion with S1 and Mung Bean nucleases (see lanes 6-9 labeled S1, Mung bean, undig, and undig/treated), including the undigested lane.

Pulse Field Electrophoresis is known for its higher degree of separation of DNA fragments compared to ordinary electrophoresis due to the longer path DNA has to travel in order to cross the gel. The fact that a single band appeared on the standard gel was probably due to the lower separation ability of the 0.8% minigel and the subsequent compression of multiple bands into a single one.
These results disprove previous speculations about the nature and origin of the unknown DNA isolated from *B. henselae*. Both a plasmid and a phage DNA would be expected to consist of fragments of unique size that would in turn produce a single band when analyzed on electrophoresis gel. The smear in lanes 6-9 suggests that a spectrum of DNA fragments of various sizes is present as opposed to a unique species of DNA.

Even though the above experiments did not unveil the origin of the unknown DNA discovered in *Bartonella henselae*, several hypotheses were disproved. The question of its origin is still one to be answered.
Figure 3. S1 and Mung Bean nuclease digestion of *B. henselae* DNA (lanes 6-7). Lane 8 contains undigested unknown DNA. Lane 9 shows *B. henselae* DNA left undigested. Lane 10 contains such "undigested/treated" pSPORT plasmid DNA. Fragments were compared to 5 kb ladder in Lane 1. pSPORT DNA digested with Hind III, S1, and Mung Bean nucleases occupies lanes 3-5 respectively. Lane 2 shows undigested pSPORT DNA.


4. 1994. Epidemiologic Notes and Reports, Encephalitis Associated With Cat Scratch Disease - Broward and Palm Beach Counties, Florida. MMWR 43:909-916


