Biofilms and antibiotic resistance of *Borrelia burgdorferi*

EVA SAPI PH.D.

UNIVERSITY OF NEW HAVEN

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How long did we know about Lyme disease?

John Josselyn, who visited New England in 1638 and again from 1663–1670, wrote

"there be infinite numbers of tikes hanging upon the bushes in summer time that will cleave to man's garments and creep into his breeches eating themselves in a short time into the very flesh of a man. I have seen the stockings of those that have gone through the woods covered with them"

The examination of preserved museum specimens has found *Borrelia* DNA in an infected *Ixodes ricinus* tick from Germany that dates back to 1884, and from an infected mouse from Cape Cod that died in 1894.

The 2010 autopsy of Ötzi the Iceman, a 5,300-year-old mummy, revealed the presence of the DNA sequence of *Borrelia* making him the earliest known human with Lyme disease.

Google Image Library

Borrelia burgdorferi the spirochete that causes Lyme disease

In 1982, the etiologic agent of Lyme disease was discovered by Willy Burgdorfer who isolated spirochetes belonging to the genus *Borrelia* from the mid-guts of *Ixodes* ticks.

He showed that these spirochetes reacted with immune serum from patients that had been diagnosed with Lyme disease. Subsequently, the etiologic agent was given the name *Borrelia burgdorferi.*

Borrelia burgdorferi , FA stain (CDC)

So how can we eliminate *Borrelia*?

In vitro and *clinical* data – do they agree?

"Survival of *Borrelia burgdorferi* *in antibiotically treated patients* with Lyme borreliosis "
Preac-Mursic et al *1989*

"*In vitro* results have no proven correlation with antimicrobial clinical effectiveness *in vivo* since the relationship of MICs or MBCs of drugs against slowly dividing organisms such as B. burgdorferi" *Moody et al 1994*

"Culture positive and PCR positive blood after antibiotics therapy"
Oksi et al 1999

"Clinically treatment failures occur in 5 to 10% of EM patients (oral doxycycline or amoxicillin for 14 to 30 days)" *Smith et al 2002*

In vitro studies for different genospecies/species of *Borrelia* – Minimum bactericidal concentrations (microgram/ml)

Doxycycline:	0.25-25.0
Penicillin	0.15-6.5
Azitromycin:	0.015-2.0
Erythromycin	0.06->0.5
Clarithromycin	0.06-0.5
Telitromycin	0.002-0.03
Amoxicillin:	0.4-8.00
Ceftriaxone:	0.03-2.00*
Ciproflaxin	0.5-16.0
Tigecycline	0.05-0.19

Russel et al 1987, Agger et al 1992; Dever et al 1992, Levin at all 1993; Sicklinger et al 2003, Hunfeld et al 2004, 2005, Kim et al 2006, Yang et al 2009, Brorson et al 2009*

But how about the *in vivo* studies?

Treatment with oxytetracycline, erythromycin or doxycycline in mice failed to eradicate acute Borrelia infection or ameliorate the disease. *Moody et al 1994*

Chloramphenicol and azithromycin failed to eradicate the organism but ameliorated the disease. *Moody et al 1994*

In a dog model of infection, showed that antibiotic-treated dogs (doxycycline and amoxicilllin, 30 days) continued to have persistent Borrelia-specific DNA in their tissue albeit at lower levels than observed in untreated animals. *Straubinger et al 1997*

But how about Ceftriaxone (Rocephin)?

Bockenstedt LK, Mao J, Hodzic E et al. Detection of attenuated, noninfectious spirochetes in *Borrelia burgdorferi*-infected mice after antibiotic treatment. J Infect Dis 2002; 186: 1430–7.

Hodzic E, Feng S, Holden K et al. Persistence of *Borrelia burgdorferi* following antibiotic treatment in mice. Antimicrob Agents Chemother 2008; 52: 1728–36.

SUMMARY: **A low numbers of noncultivable spirochetes**, detected by PCR following antibiotic treatment. For at least 3 months after cessation of antibiotic, these **noncultivable forms** can be **acquired by ticks** (xenodiagnosis), transmitted by ticks, survive the molts between larvae to nymphs to adults, infect recipient mice by tissue transplant, transcribe RNA, and express antigen in ticks and tissues in the form of morphologically identifiable spirochetes

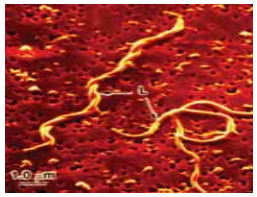
The different forms of Borrelia

Borrelia burgdorferi can convert between cyst, non-motile and normal motile spirochete forms.

The cystic forms are resistant to most antibiotic treatments and difficult to detect in the body.

- http://www.lymeinfo.net/medical/LDAdverseConditions.pdf

B. burgdorferi after exposure to penicillin concentration of 0.125 mg/L. Coiled up spirochete forming a spherical structure (spheroplast).
Schüller M; Neubert U; 1984



New York State Department of Health

The most recognized forms of *Borrelia burgdorferi*

- Spirochetes
- Round bodies (cysts, granules)

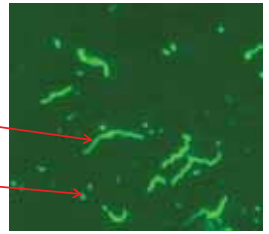


Photo by Namrata Pabbati
University of New Haven

Round bodies *in vivo*

Concurrent Neocortical Borreliosis and Alzheimer's Disease Demonstration of a Spirochetal Cyst Form

MacDonald A 1988

- An unexpected observation was the identification of cystic forms of the Borrelia spirochete in dark-field preparations of cultured hippocampus

Conversion of Borrelia garinii Cystic Forms to Motile Spirochetes *In Vivo*.

Grunter et al 2001

- *Borrelia garinii* cystic forms maintain their capability to reconvert into normal spirochetes not only *in vitro* but also *in vivo* and can therefore be considered infective, at least in BALB/c mice.

Additional Brorson et al *in vitro* studies for the antibiotic sensitivity of the cystic (round bodies) form

2001 Susceptibility of motile and cystic forms of *Borrelia burgdorferi* to **ranitidine bismuth citrate**. Int Microbiol, 4(4):209-15.

2002 An *in vitro* study of the susceptibility of mobile and cystic forms of *Borrelia burgdorferi* to **hydroxychloroquine** Int Microbiol, 5(1):25-31.

2004 An *in vitro* study of the susceptibility of mobile and cystic forms of *Borrelia burgdorferi* to **tinidazole**. Int Microbiol, 7(2):139-40.

2009 Destruction of spirochete *Borrelia burgdorferi* round-bodies by **tigecycline** PNAS 106(44):18656-61.

Agents for the cystic forms (RB)

An *In Vitro* Study of the Susceptibility of Mobile and Cystic Forms of Borrelia burgdorferi to Metronidazole *Brorson et al 1999*

"B. burgdorferi has the ability to make cystic forms both in vivo and in vitro, e.g. when exposed to antibiotics commonly used for treating Lyme borreliosis. This phenomenon, combined with the ability of the **cysts to reconvert to normal mobile spirochetes** may explain a reactivation of the disease after an illusory cure – and not a "post Lyme syndrome" as postulated by other researchers."

Ineffectiveness of Tigecycline against Persistent *Borrelia burgdorferi in vivo*

Genetically altered **non-cultivatable** *B. burgdorferi* could be isolated from mice treated with ceftriaxone and tigecycline.

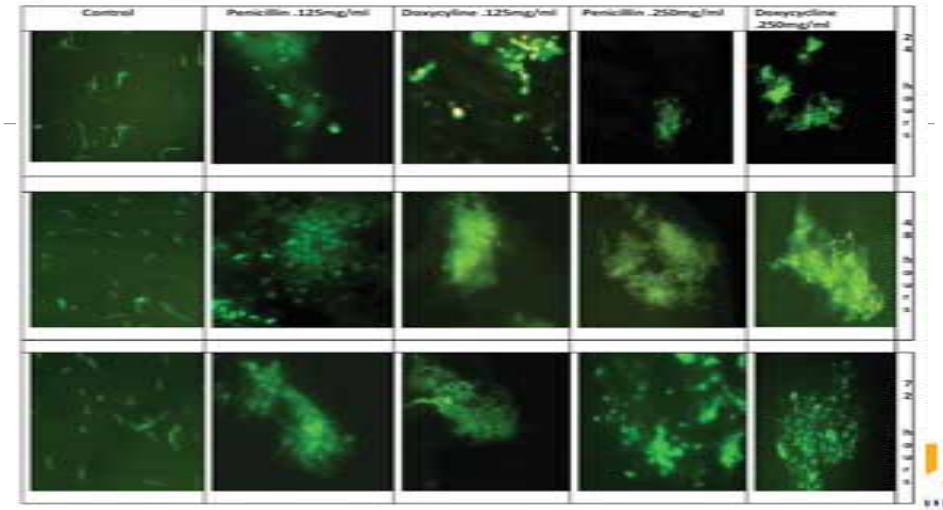
Mice remained **infected with non-dividing, but infectious spirochetes**, particularly when antibiotic treatment was commenced during the chronic stage of infection

It was resurgence of spirochete flaB DNA in multiple tissues at 12 months, with flaB DNA copy levels nearly equivalent to those found in saline-treated mice.

Barthold SW et al 2010 and 2014

Embers ME et al:
Persistence of *Borrelia burgdorferi* in Rhesus Macaques following Antibiotic Treatment of Disseminated Infection. PLoS ONE 2012

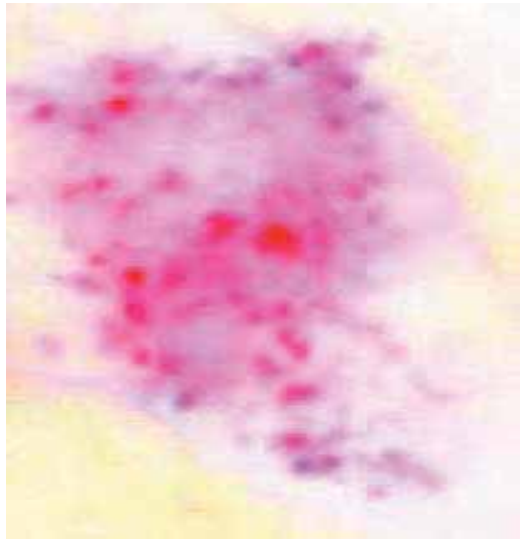
- found that Borrelia persist after 90 days in monkeys treated for chronic Lyme disease.
- further, the antibody tests used to diagnose Lyme disease fail to detect disease in late Lyme disease at least 50% of the time.



Rao P, Azano, D & Sapi E, unpublished data 2008

So what can we do now ????

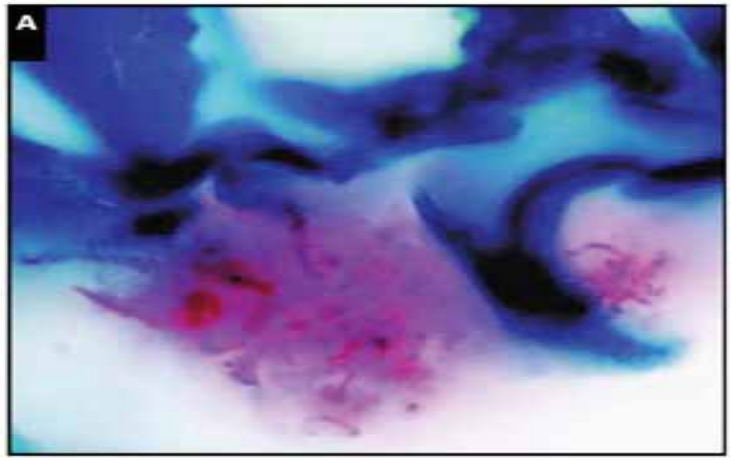
What other escape route Borrelia could have??



K. Eisendle et al. AJCP 2007,127:213-222 Acrodermatitis Chronica Atrophicans Immunohistochemistry

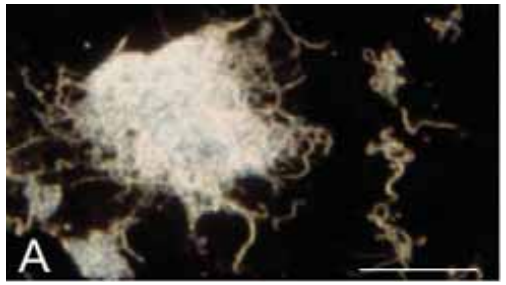
"Granular forms of *B burgdorferi* in a "colony" with a "Reddish veil"

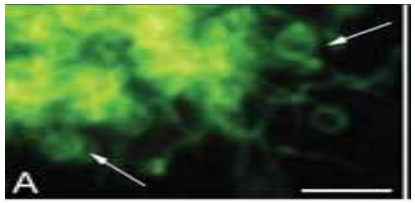
Eisendle et al 2007

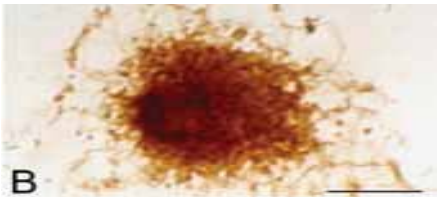


Borrelial lymphocytoma with medusa-like colony of Borrelia

Borrelia burgdorferi "colonies"

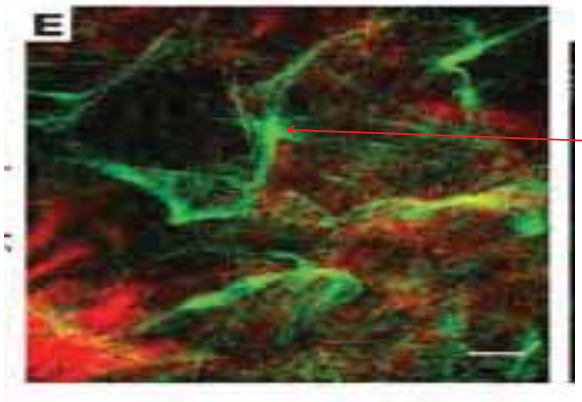






Miklossy J et al 2008

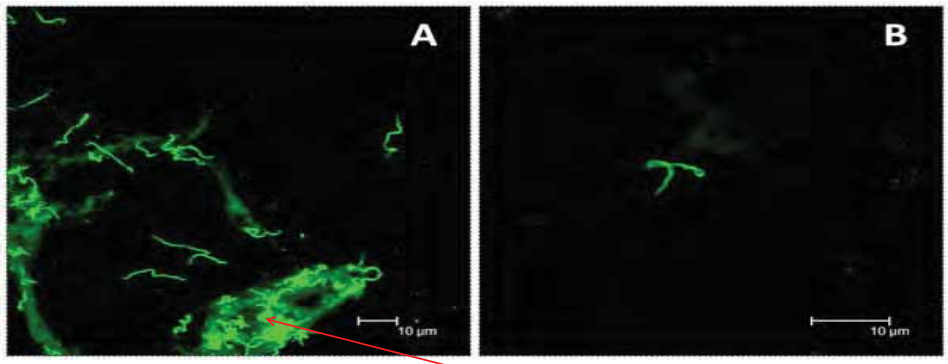
B. burgdorferi in nymphal midgut during feeding in the form of epithelial cell–associated networks



Borrelia colonies or aggregates?

Dunham-Ems et al 2009

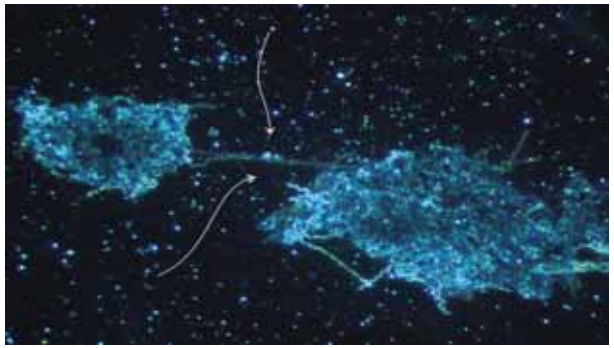
The Rhesus Macaques' study: Embers ME 2012



Borrelia colonies or aggregates?

Fluorescent staining of B. burgdorferi spirochetes found in xenodiagnostic tick midgut culture (A)
or tick midgut preparation (B) from treated animals

Borrelia burgdorferi "Photo 51"



Alan MacDonald 2008

What is Biofilm?

collections of microorganisms (bacteria, yeasts, and protozoa) that form on a hard surface (exception floating biofilms)

examples: plaque that forms on teeth and the slime that forms on surfaces in watery areas (shower)

surrounded by slimy secretions:mucoid polysaccharide structure which attaches the community to a surface

estimated that over 90% of bacteria live in biofilm (Costerton WJ)

MacDonald A, & Sapi E: Biofilms of *Borrelia burgdorferi* in chronic cutaneous borreliosis AJCP 2008 June

- We propose the hypothesis that that *Borrelia burgdorferi* can form biofilm structures in lymphocytomas and acrodermatitis chronica atrophicans.
- Our close examination of these pictures revealed striking similarity to previously published biofilm pictures and our preliminary findings on specific biofilm-like colony formation of *Borrelia burgdorferi* when cultured in the presence of human plasma

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Microorganisms found in medical devices

Staphylococcus

Streptococcus

Enterococcus

E. coli

Klebsiella

Pseudomonas

Bacteria may originate from the skin of the patient, or a healthcare worker and tap water

Common biofilms

Dental plaque

Bacterial endocarditis

Urinary tract infections

Cystic fibrosis

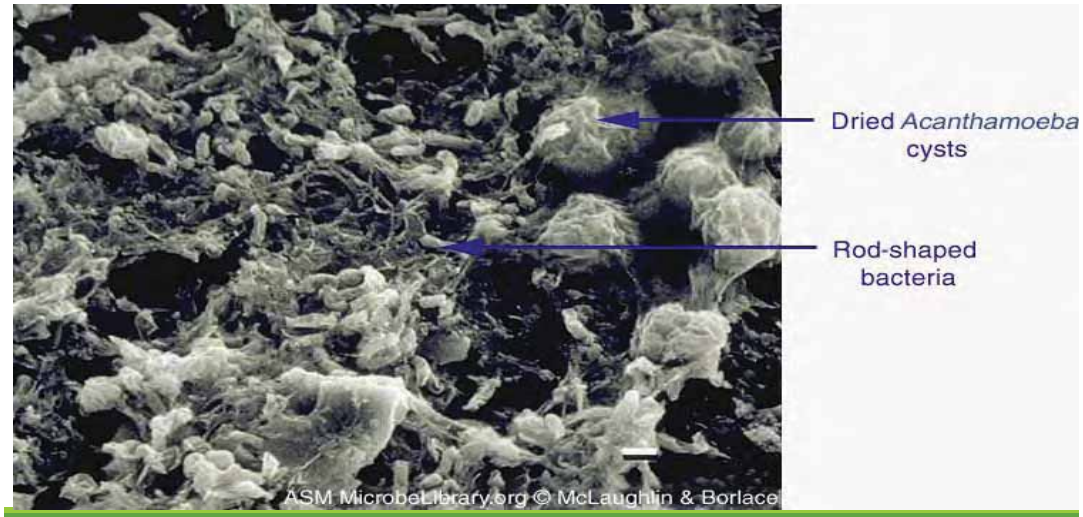
Staphylococcus osteomyelitis

Middle ear infection

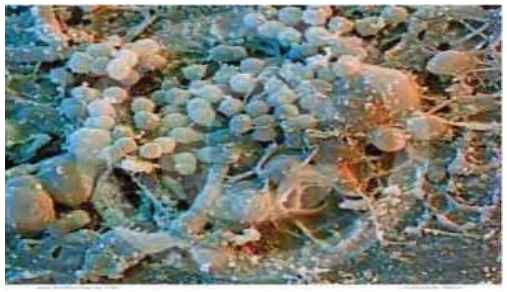
Chronic prostatitis

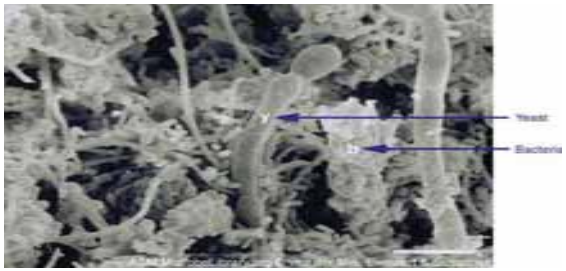
Infectious kidney stones

Contact lens



Dental Plaque: Complex community





Biofilm is like a city

Careful selection of location

Limit settlement of too many organisms

Division of labor (planktonic and sessile cells)

Storage of energy (exopolysaccharides)

Transfer of information (genetic transfer)

Intercellular communication

Emigration when population gets too large for resources

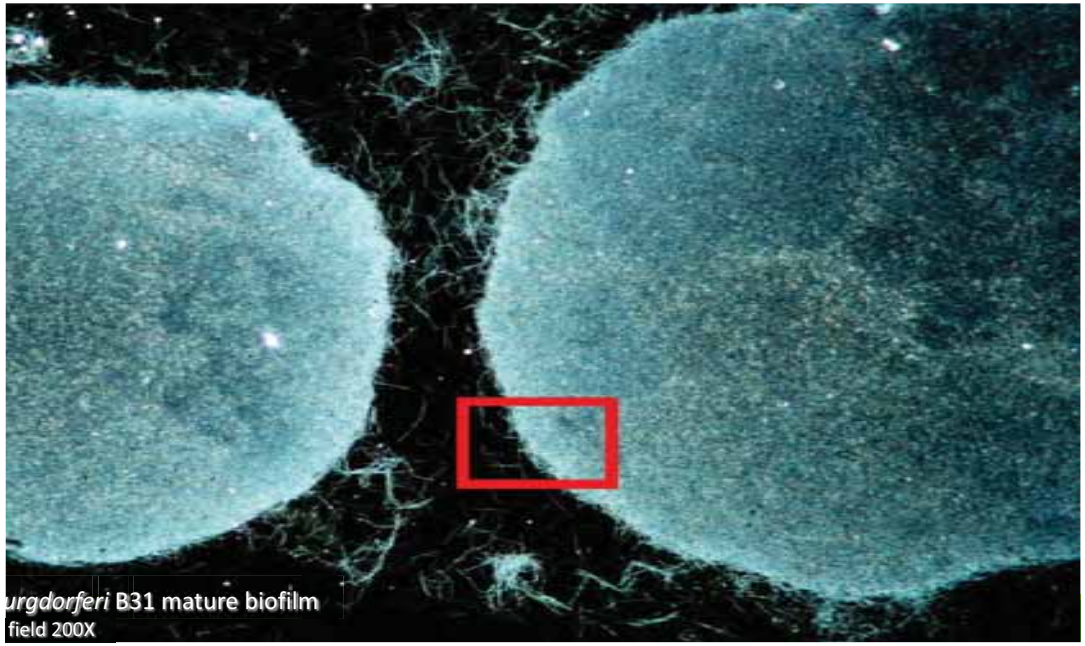
What makes a bacterial colony to a true biofilm?

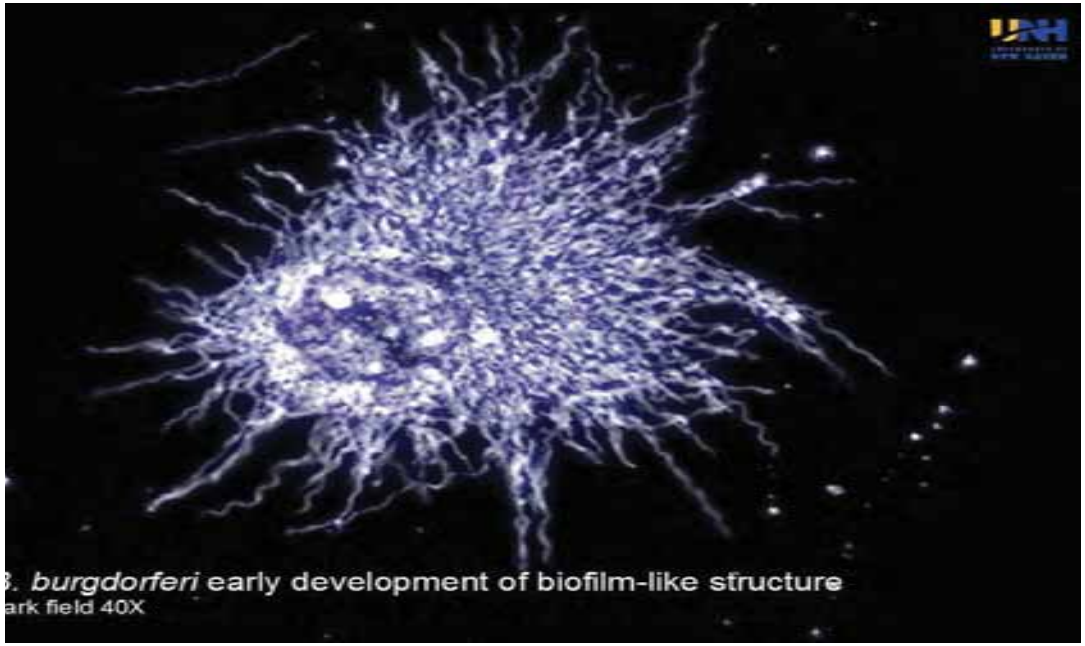
Loss of motility?

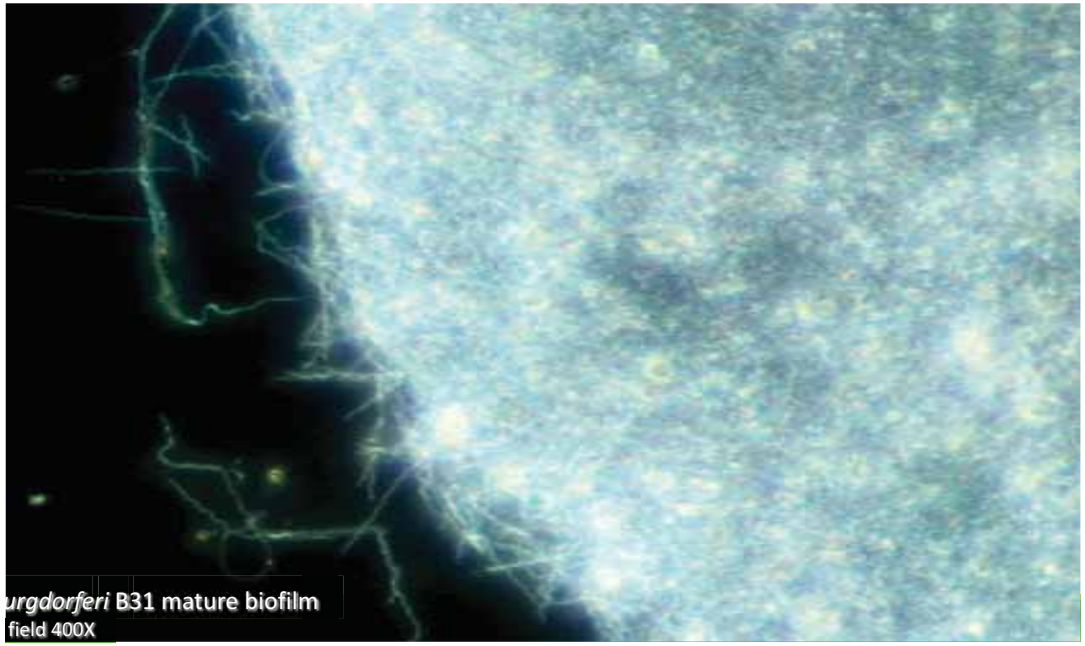
Internal morphological rearrangement

A colony embedded in a matrix of extracellular polymeric substance (EPS) separated by a network of open channels

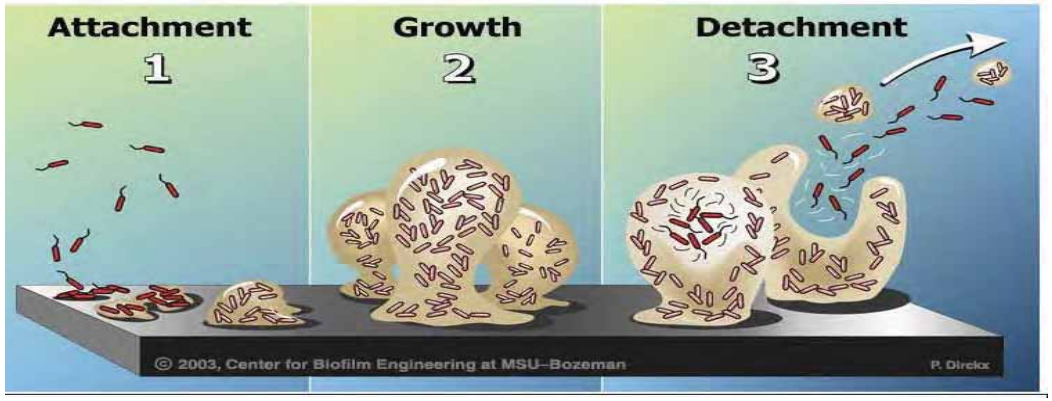
Communication network- quorum sensing



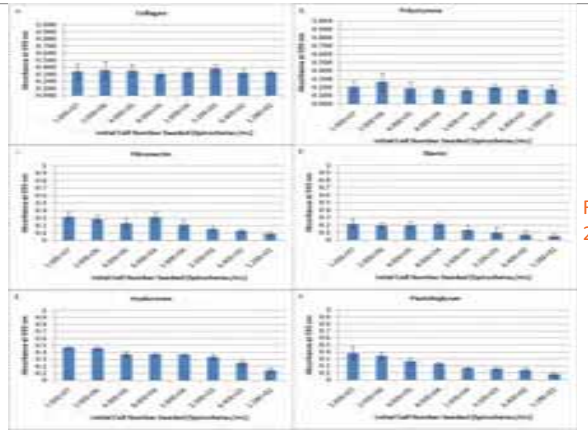




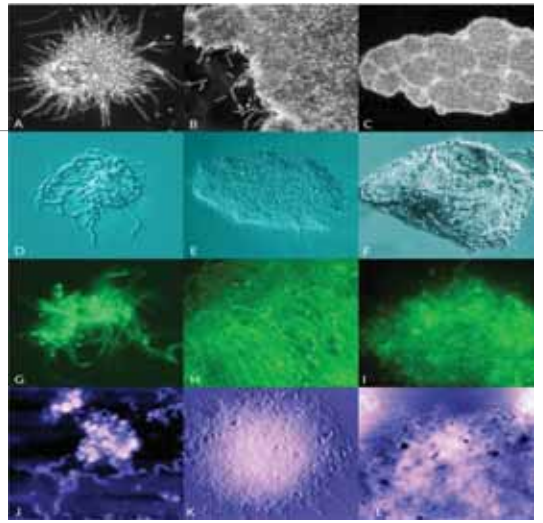
Stages of biofilm development



Matrix requirement for *Borrelia* biofilm



Rattelle A, Sapi E 2011



Dark-field microscope

DIC

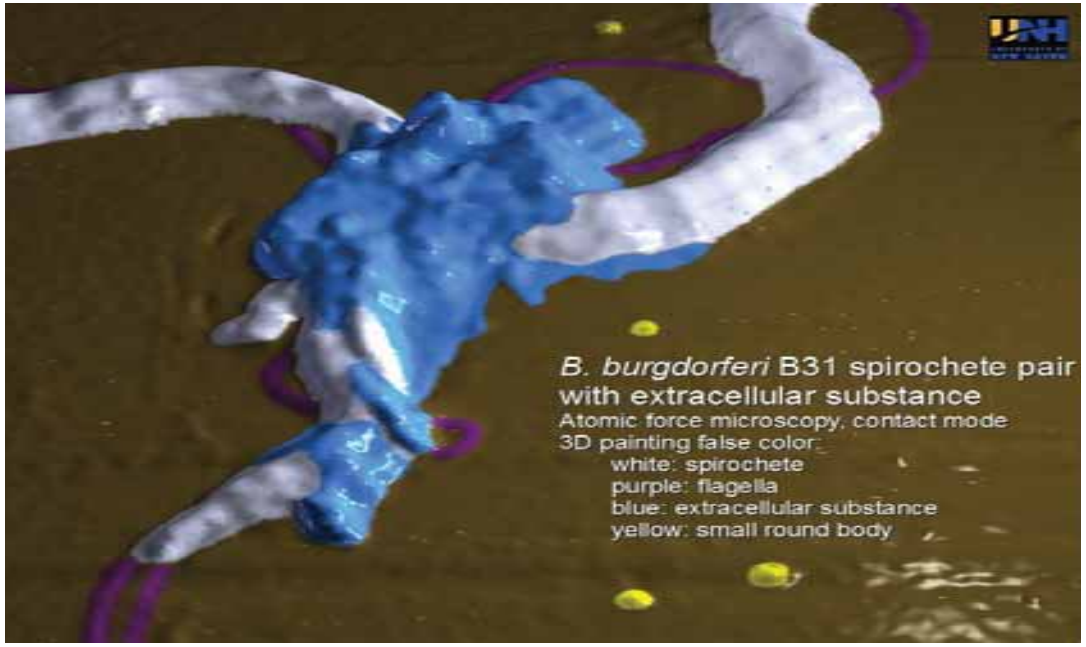
Fluorescent microscope

Atomic Force Microscope

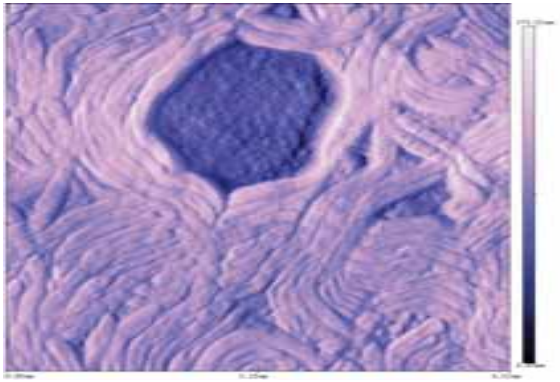
Borrelia burgdorferi biofilm Sapi E at al: PLoS One 2012

Atomic force microscopy images of live *Borrelia* colony growing on agarose – early development

Luecke D, Datar A, and Sapi E 2009



Atomic force microscopy images of live *Borrelia* colony growing on agarose – mid-phase development

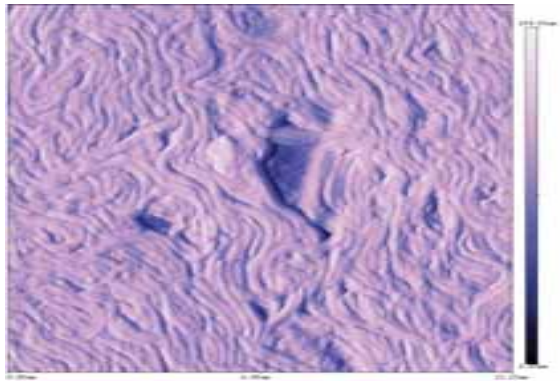


Bastian S, Luecke D, Datar A, Sinha S and Sapi E 2011

Atomic force microscopy images of live *Borrelia* colony growing on agarose – early development

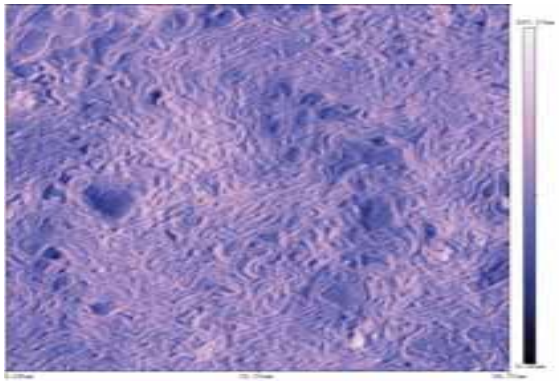
Bastian S, Luecke D, Datar A, Sinha S and Sapi E 2011

Atomic force microscopy images of live *Borrelia* colony growing on agarose – midphase development – 2 days later



Bastian S, Luecke D, Datar A, Sinha S and Sapi E 2011

Atomic force microscopy images of live *Borrelia* colony growing on agarose –late phase



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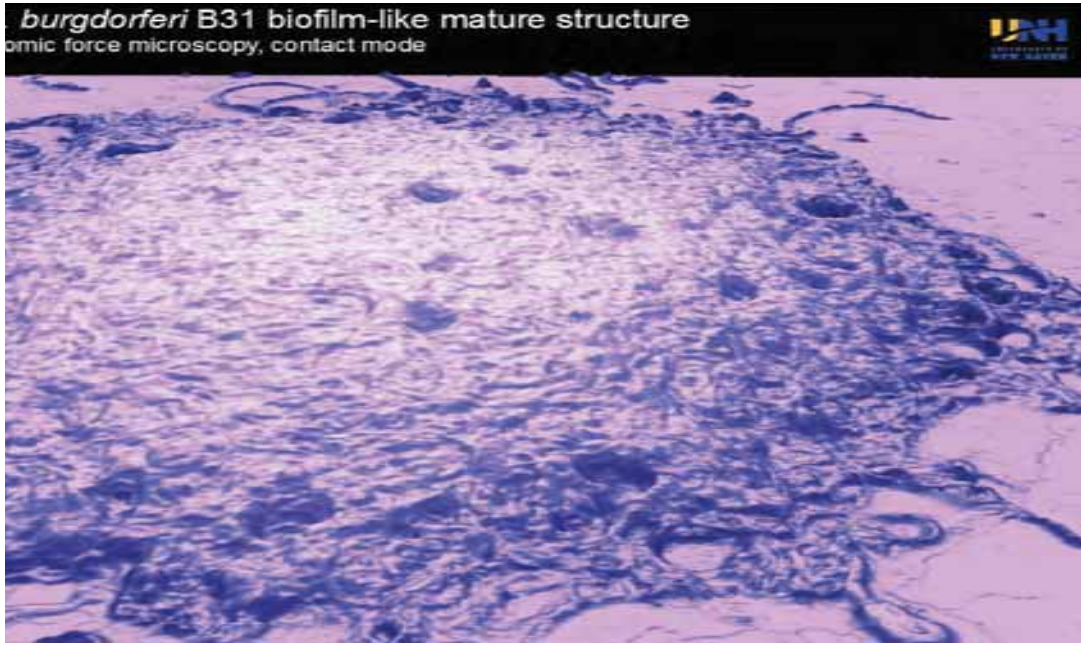

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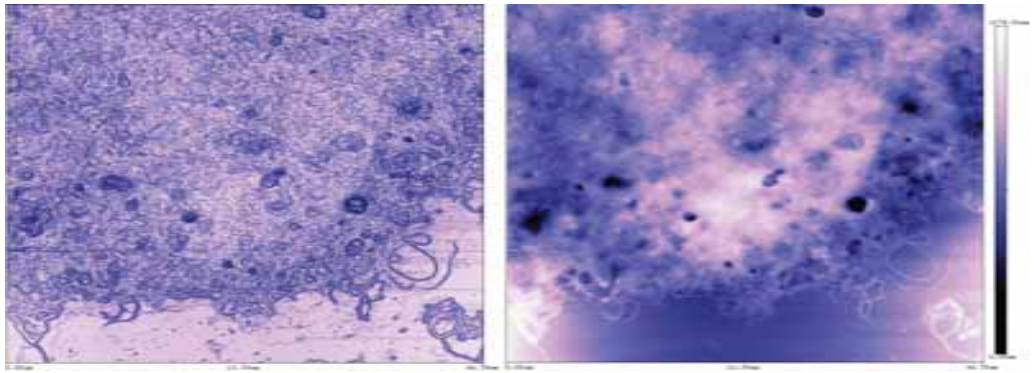

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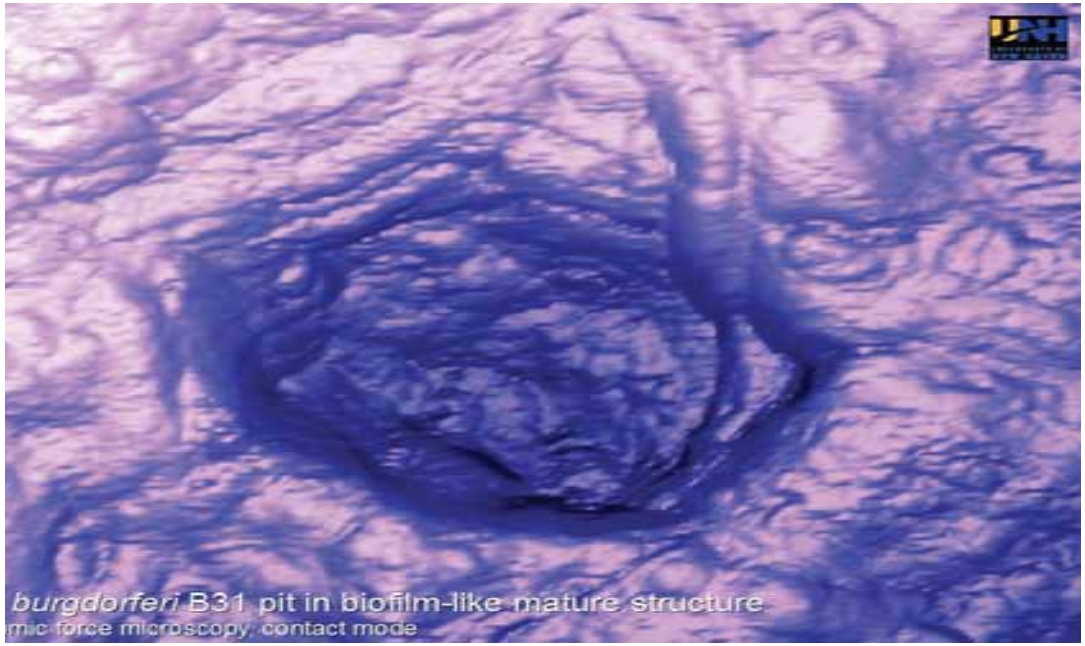
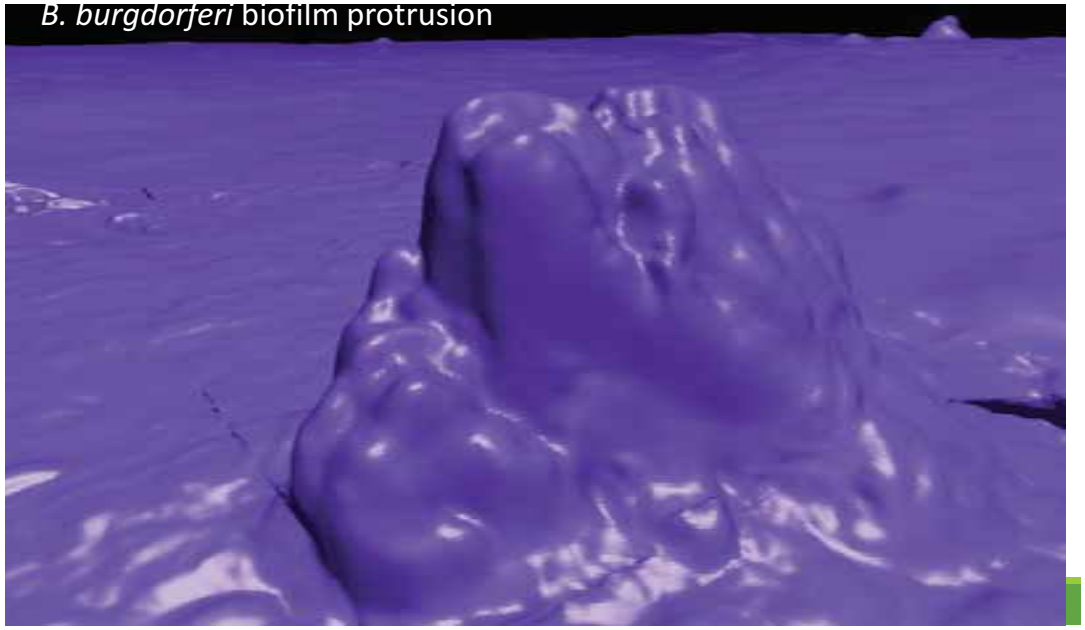
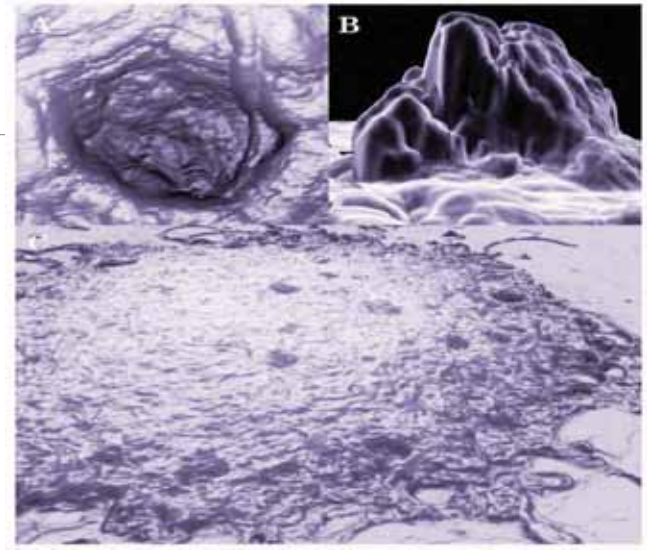
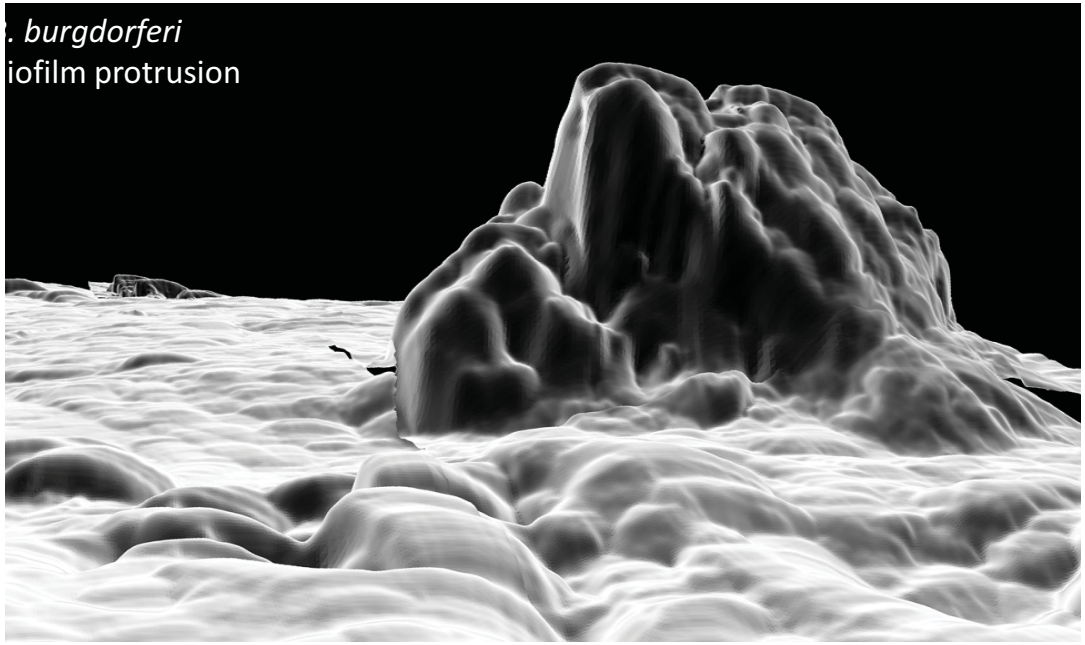






Sapi E et al 2012 PLoS ONE



Extracellular Polymeric Substances

The EPS matrix: The "house of biofilm cells"

Composed of mucopolysaccharides (slime), proteins (enzymes) glycoproteins, glycolipids, extracelullar DNA

Some of these polysaccharides are polyanionic (like uronic acid) – can bind to calcium and magnesium

Costerton JW and Irvin RT 1984, Flemming HC 2007

Spicer Meyer aldehyde fuchsine – alcian blue stain sequence for mucopolysaccharides

Aldehyde fuchsine:

Stains acidic sulfated mucins

Alcian blue:

Stains remaining non-sulfated mucins, carboxylated mucopolysaccharides

Spicer & Meyer, 1960

Borrelia EPS component: alginate?

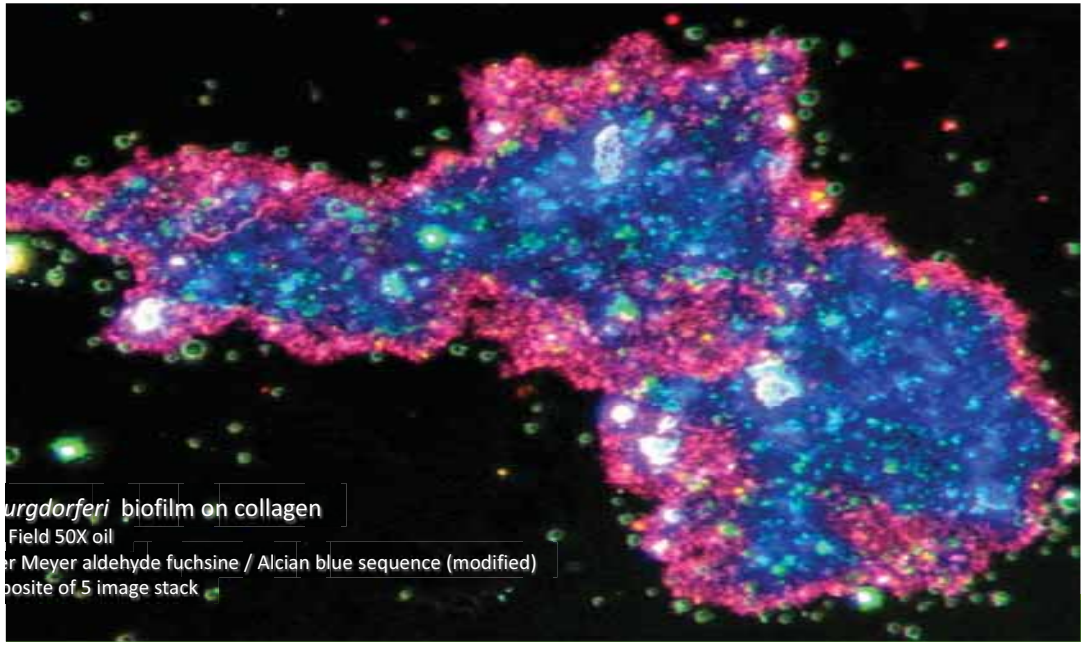
Alginate is well established as a viable primary EPS compound

Composed primarily of **polyuronic acid polymer – alginate** (Kjelleberg 2007)

Chemical analysis of two varieties of slime produced by *Pseudomonas aeruginosa* (Murakama 1973)

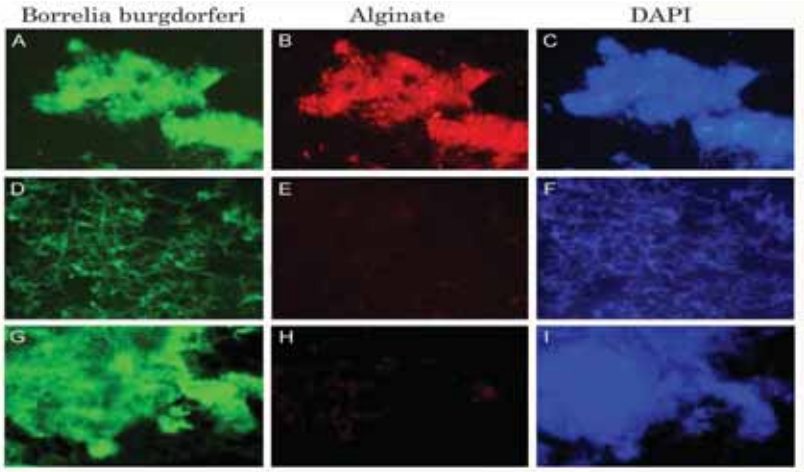
"The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN-gamma-mediated macrophage killing." (Leid 2005)

"Alginate is not a significant component of the extracellular polysaccharide matrix" (Wozniak 2003)



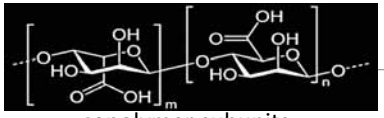
urgdorferi biofilm on collagen
Field 50X oil
r Meyer aldehyde fuchsine / Alcian blue sequence (modified)
posite of 5 image stack

Alginate on the surfaces of *Borrelia* aggregates



Sapi E et al 2012 PLoS ONE

EPS: alginate and calcium



copolymer subunits

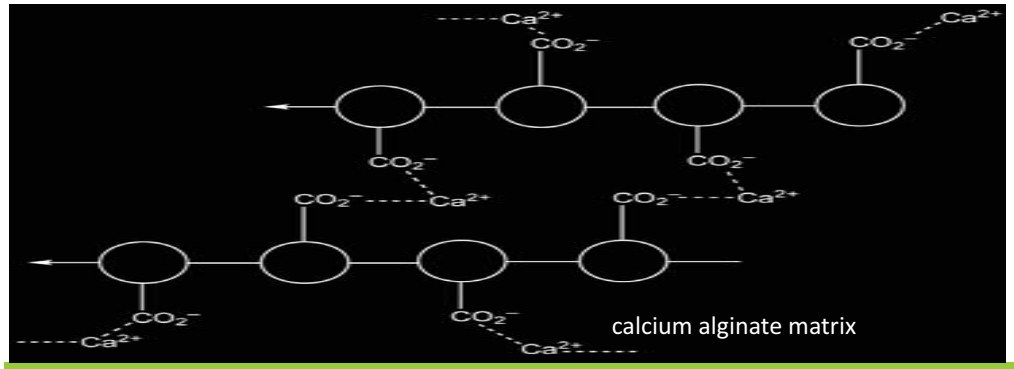
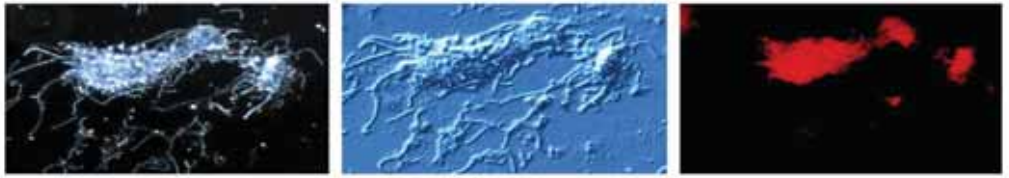


image: RSC.org

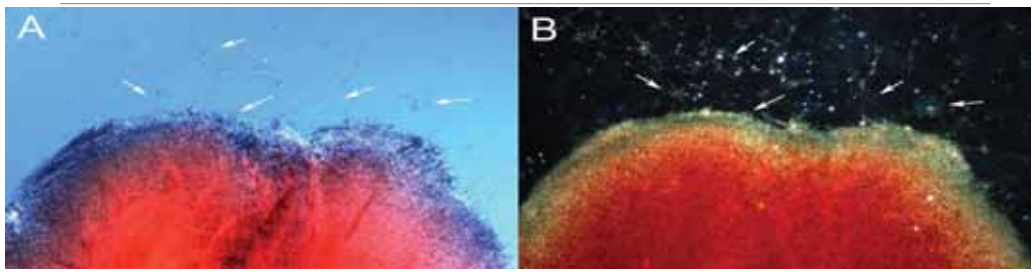
Extracellular DNA on the surface of *Borrelia biofilms*



Borrelia burgdorferi B31 aggregates surrounded by individual spirochetes (marked with white arrows) stained with the DDAO [7-hydroxy-9H-(1, 3-dichloro-9, 9 dimethylacridin-2-one DNA binding fluorescent dye. A: Dark field image B: Differential interference contrast image, and C: DDAO red stained fluorescent image of the same cellular structures. 400x magnification.

Sapi et al PLoS ONE 2012

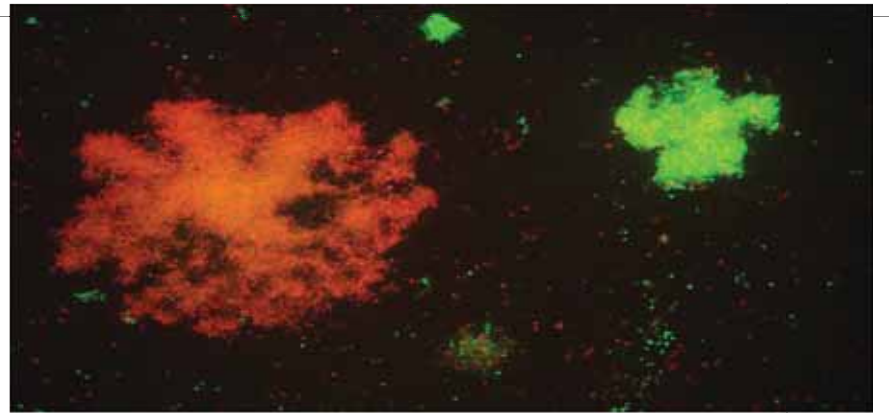
Calcium on the surface of *Borrelia* aggregates



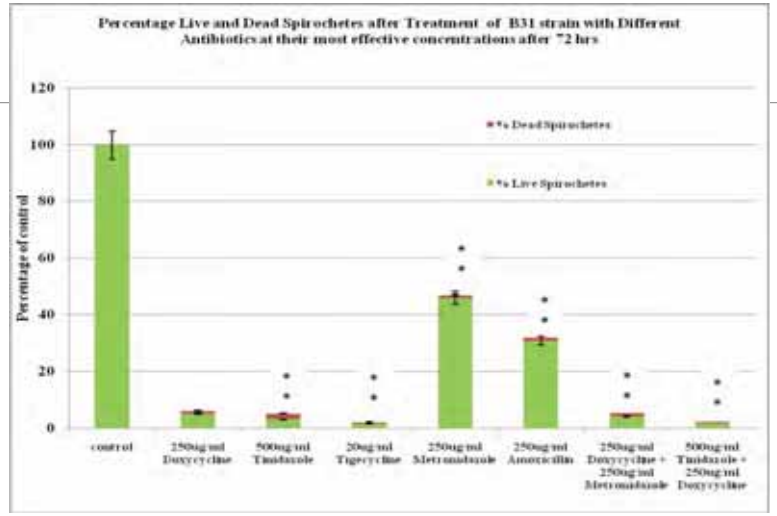
Borrelia burgdorferi B31 strain large aggregate surrounded by individual spirochetes and several small aggregates stained with the calcium-detecting stain Alizarin. Red coloration indicates presence of calcium, by differential interference contrast (Panel A) and dark field microscopy (Panel B).

Sapi E et al PLoS ONE 2012

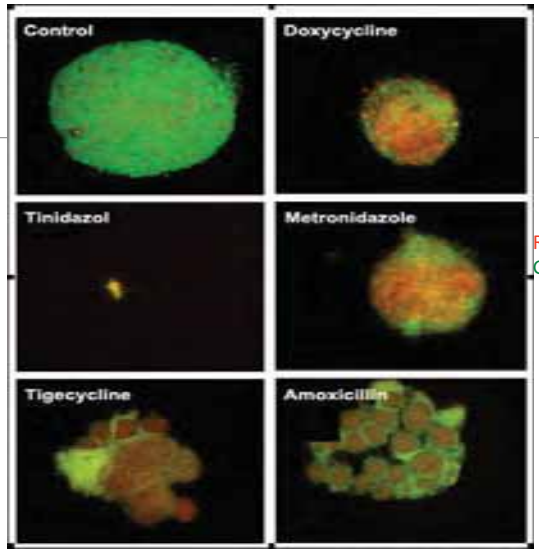
Borrelia burgdorferi treated with 25 microgram/ml of doxycyline for 3 weeks



DF Luecke, Kaur N, Datar A and E Sapi unpublished data 2012

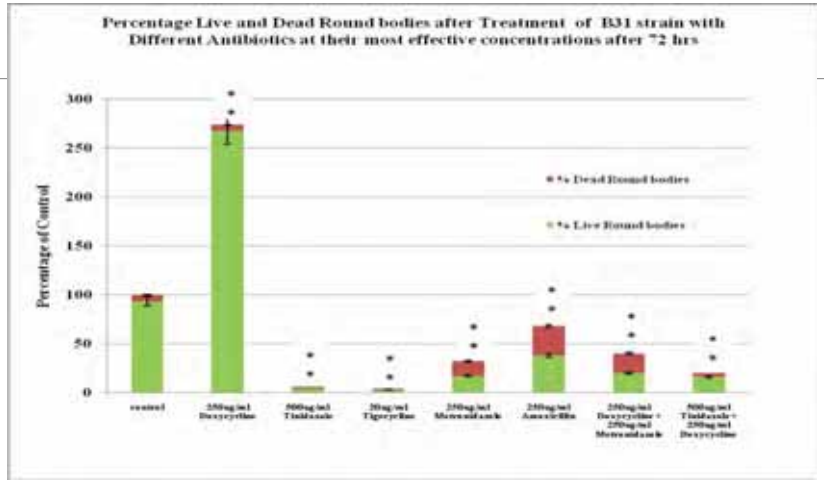


Kaur N, Sapi E 2012 unpublished data



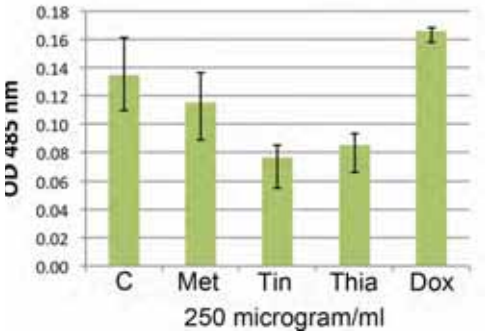
Red stain: Dead
Green stain: Viable

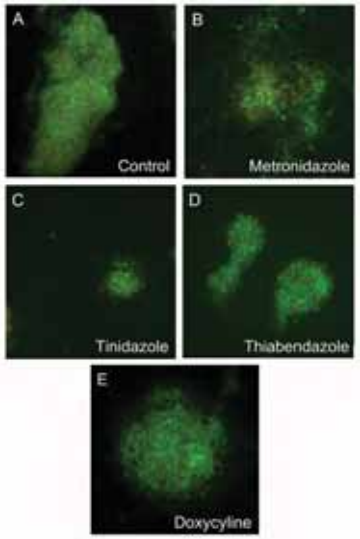
Effect of antibiotics on the aggregates of Borrelia measured BacLight staining Sapi E at al 2011



Kaur N, Sapi E 2012 unpublished data

Effects of different *nitroimidazole* antibiotics on *Borrelia* biofilm (72h)

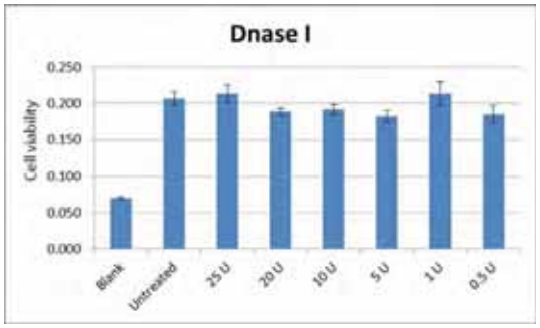




PAS Theophilus, Balu K Sapi E unpublished data 2013

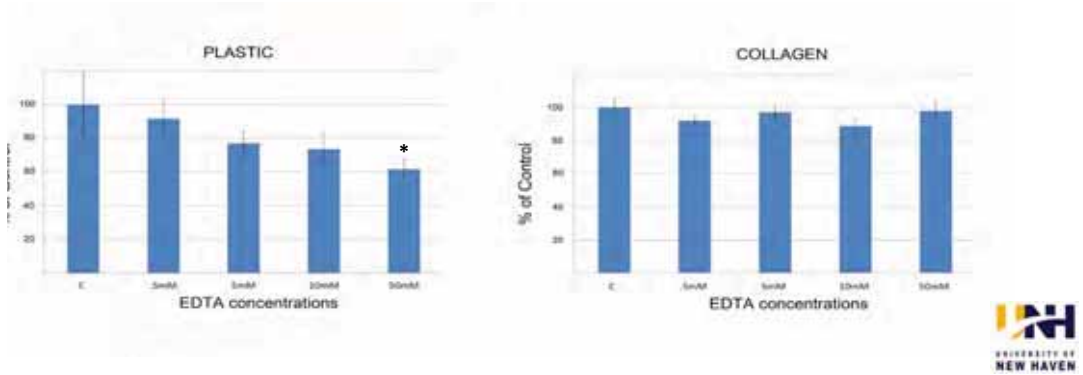
Do we need to treat the EPS of the biofilm layer with different agents?

Degrading the extracellular DNA layer?



Victoria JM, Sapi E unpublished data 2013

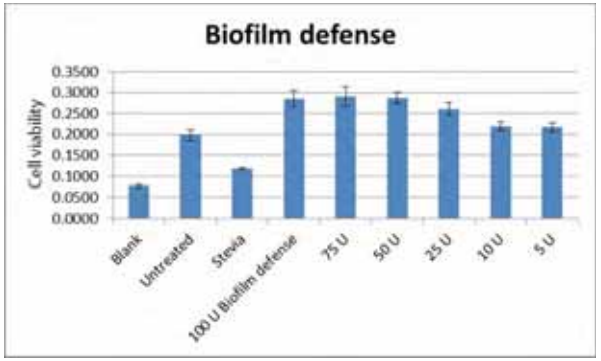
EDTA effect on *Borrelia* biofilm on plastic and on collagen - 48h



Phillips DS, Burugu D, Sapi E 2013 unpublished data

Mixture of Enzymes?

film Defense



Victoria JM, Sapi E unpublished data 2013

Do we need a Trojan Horse?

Additional work before publishing

- in situ hybridization
- AFM studies
- monoclonal staining
- additional biofilm markers (lectin binding studies)
- animal studies

Summary

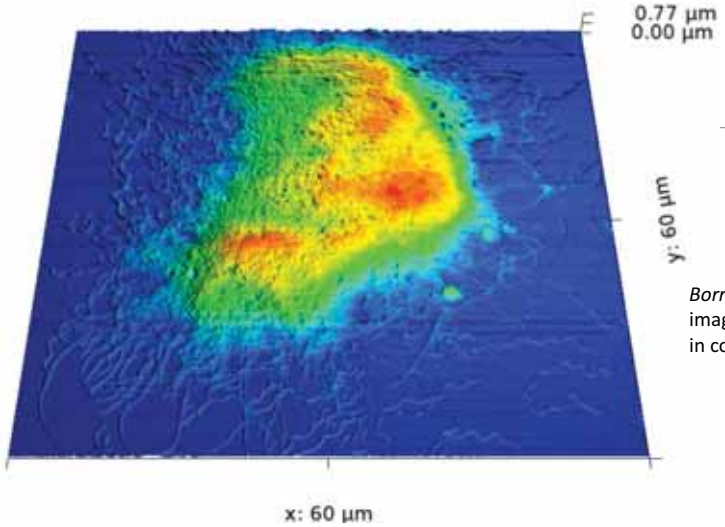
Borrelia burgdorferi does form a very organized biofilm *in vitro*

Major component is probably calcium alginate, but polymer subunits remain to be identified

Imination detected by RAMAN spectroscopy possible cross-linking for increased strength may be mechanism of maturation

Preliminary results show potential *in vivo* Borrelia biofilm in infected skin tissues

The new atomic force microscope 2013 October
UNH Lyme disease research group



Borrelia burgdorferi B31 biofilm image using AFM microscope in contact mode

Luecke DF, Sapi E 2013 unpublished image

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Question?

Please call me or send an email:

esapi@newhaven.edu

203-479-4552