Building a Better Spirochete Trap:

Using engineered proteins to create effective immunization against the unusual pathogen *Borrelia burgdorferi.*

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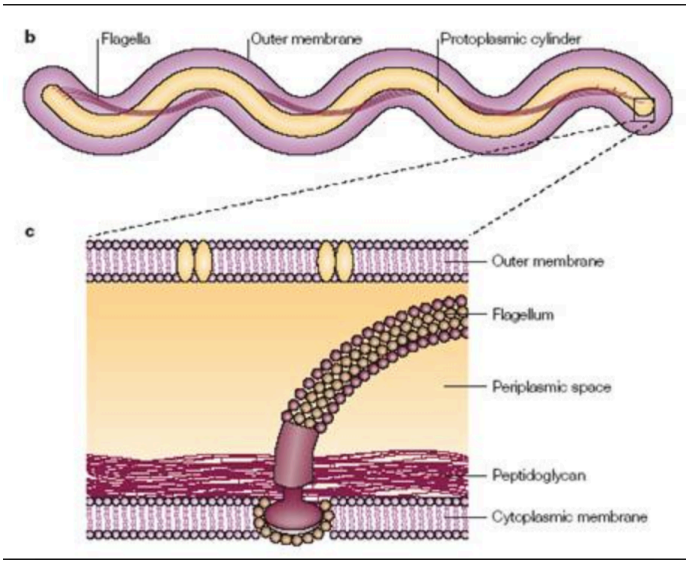
Borrelia burgdorferi, the etiologic agent of Lyme Disease, is an unusual, spirochetal, bacterium which has proven difficult to treat and immunize against. With a life-cycle that includes multiple stages and adapts to disparate arthropod and mammalian hosts, *Borrelia* exhibits adaptability due to differential expression of genes resulting in extraordinary virulence. This host-driven expression confers survival advantage through changes in morphology, behavior, and defensive capabilities. Attempts to create an effective vaccine against *Borrelia burgdorferi* have so far fallen victim to its biochemical sleight of hand, but recent advances have allowed the construction of a novel chimeric protein which may hold the answer to immunization against the pathogen. Composed of multiple epitopes of variable outer surface proteins, this vaccinogen seems to allow mammalian specific immune response to overcome *borrelial* antigen-switching evasion. This new protein forms the basis of an effective canine vaccine which is being adapted for use both in human vaccines, and immune- assays. It represents the newest weapon in the fight against Lyme disease.

I. *Borrelia burgdorferi*: an unusual bacterium.

Categorized as gram-negative-similar, due to its diderm structure but lack of gram-negative-characteristic external Liposaccharide (LPS) proteins, *Borrelia* is the only organism known to exist without a need for iron- instead replacing iron's superoxide defense role with manganese-dependent SOD. (Troxell, et al, 2012). The bacterium has 7-12 internal flagella originating from each pole, internal to the outer membrane, which extend along the periplasmic space, and wrap around the cell body to meet at the middle of the organism. **Figure 1: structure of *B.burgdorferi* (Rosa, et al. 2005)**



The counter-rotation of these flagella, driven by rotary nano-motors embedded in the inner membrane (Kudrichev, et al 2010) give *Borrelia* its notable flat-wave motion- a movement mechanism unique among bacteria (Goldstein, et al, 1994).

B.burgdorferi is microaerophilic, fastidious (using only glucose, glycerol, maltose, mannose, N-acetyl glucosamine (GlcNAc), trehalose or chitobiose as carbon sources), and slow-growing due to its sole reliance on glycolysis for ATP. (Lackham , et al, 20050, Corona, et al, 2015) These factors make *in-vitro* study difficult and time consuming and are further complicated by *Borrelia*'s tendency to lose plasmids (and thus function) rapidly under cultivation. With the low bacterial load necessary for infection (~ 0.1 bacterium per ml of blood)(Wolgemuth, 2016), and the organism's immune-evasive practice of antigen-switching *in-vivo,* detection and study can also be problematic.

The bacterium possesses a condensed genome: a small linear chromosome containing 853 genes, and 17-22 relatively large linear and circular plasmids that contain additional information essential for virulence and infectivity (Frasier, 1997 and Kenedy, et al, 2013). As an obligate parasite, *Borrelia* is entirely dependent upon its hosts for survival. The limited chromosome holds coding only for essential DNA processes, basic metabolism, and more that 50 genes coding for transport proteins specific to nutrients and cofactors. These transports are essential because *Borrelia* has no pathways for *de novo* biosynthesis and must import fatty acids, amino acids and nucleotides from external sources. (Corona, et al 2015)

Fully 56% of the genes in chromosome and plasmid have no predicted function, with 39% of the total having neither predicted function or known homologs (Groshong, et al, 2014). Amino acid comparisons between chromosome and plasmids support the experimental conclusion that the highly conserved plasmids impart significant redundancy of function via paralogous genes, meaning that, despite some correlation with plasmid degradation and reduction of infectivity (Pusere, eta al 2000), loss-of-function studies to determine genetic function have proven impracticable (Casins, et al 2000).

II. *Borrelia*'s Multi-stage life-cycle

As a highly virulent zoonotic pathogen, *Borrelia*'s complicated life cycle is comprised of several morphologies in several hosts, upon whom it depends for resources due to its condensed genome. It is adapted to use tick antifreeze (glycerol), and chitobiose derived from the tick cuticle as carbon sources in addition to the more common glucose and mannose available in mammalian blood (Pappas, et al, 2011). In its arthropod vector (*Ixodes* ticks), the spirochete lives in the oxygen-poor, alkaline midgut. However, once a blood meal is taken, chemotaxis and differential expression of external proteins is activated by the concomitant increase in temperature, acidity, and oxygenation (Caimano 2016). *Borrelia* moves to the salivary glands and is thereby injected into the mammal host during feeding (Carroll, et al, 1999). In the mammal, *Borrelia* is virulent and highly invasive, eluding innate and acquired immune responses and disseminating beyond the initial infection site quickly to colonize secondary immune-secluded sites such as joints, central nervous system, and vascular passageways- including the heart.

Small rodents, whose habitats often coincide with that of humans, offer a target-rich environment for ticks and act as *Borrelia*'s primary biological reservoir. (Radolf, et al, 2012). Were they to have the same morbidity and mortality rates as human hosts, Lyme disease would be a self-contained mouse epidemic- limited by rodent ability to disseminate ticks into the environment. However, rodents respond differently to the infection than humans, being seemingly immune to the debilitating and painful symptoms of bacterial infection suffered by larger hosts (Tracy, et al 2017). This lack of symptom is of evolutionary benefit to *Borrelia*, allowing it to spread more widely via asymptomatic carriers: a cooperative evolution which contributes to the survival and proliferation of the bacteria (Pappas, et al, 2011).

III. Outer Surface Proteins

Differential expression of outer surface proteins is one of many survival strategies employed by *Borrelia*. As an extracellular pathogen, the spirochete's outer membrane is the point of contact between bacterium and host: making its constituents hugely important for survival, and therefore virulence. (Groshong, et al, 2014). External protein expression is controlled by the Rrp2-RpoN-RpoS regulatory pathway, and triggered by environment, specifically a blood meal which alters temperature, pH, oxygenation and cell density near the bacteria. (Seshu, et al 2004)

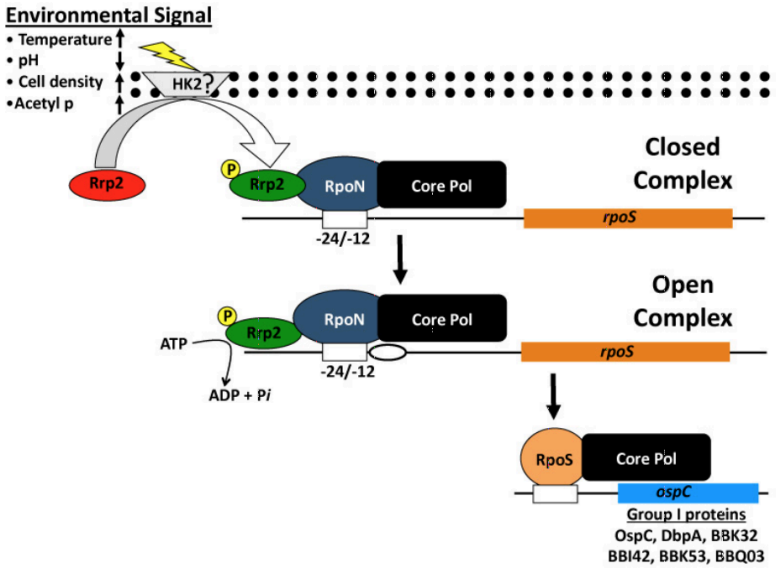


Figure 2: RpoS signaling pathway. Groshong, et al, 2014)

RpoS is the central regulator of this pathway, and hence of differential protein expression necessary for transfer and survival between arthropod and mammalian hosts. Regulation occurs via induction of the alternative sigma factor cascade by phosphorylation of alternative sigma factor Rrp2 . Rrp2, in turn, donates activation energy to regulatory holoenzyme RpoN which binds upstream of the RpoS promotor and initiates its transcription. Once

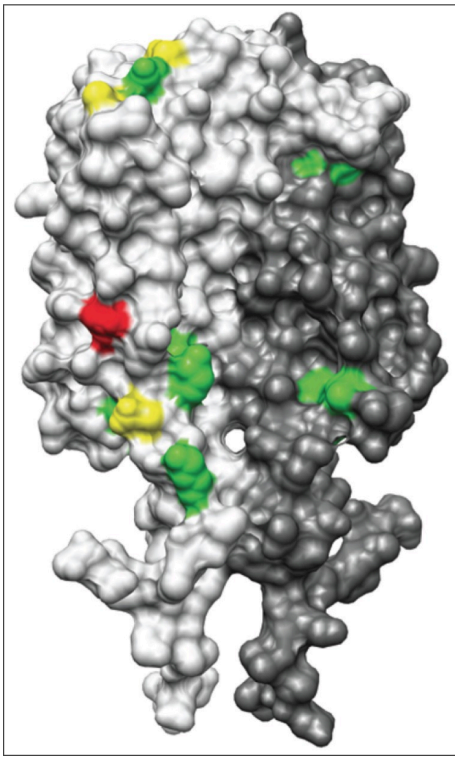
transcribed, RpoS mediates the OspA/OspC balance to optimize infection, dissemination and bacterial survival. (Yang , 2003)

Some outer surface proteins are modified to contain anchoring lipid moieties which they use to attach to the external membrane. Examples of these are OspA, OspB, OspC, OspD, OspE, OspF, (Kenedy, et al, 2012). In addition to differential expression, several of these surface-exposed lipoproteins, which are targeted to the external leaflet by default after translation, employ the protective mechanism of hiding within the bacterial periplasm (Hefty *et al.*, 2002b) – transported to this secondary location based on sequence-specific signals (Schulze and Zuckert, 2006).

IV. Vaccine Targeting

In order to be useful in imparting immunity, a vaccine must have targets that are solvent-exposed, present in many or all strains of the target species, and evident throughout the relevant portions of an organism's life cycle. Outer membrane proteins were implicated early as good vaccine target prospects in *Borrelia* (Luft, et al. 2002, and Melisha, et al 2012). And original LymeRix vaccine was targeted specifically against OspA, a membrane protein upregulated in the arthropod host. Unfortunately, *Borrelia*'s speed exceeds that of a human neutrophil (the fastest cell in the body) by over two orders of magnitude. When added to the sophisticated directional control provided by flaggelar nano-motors spirochetes are able to move into immune-deficient compartments such as joints more quickly than immune cells could capture them, (Malwesta, et al 2008) and, in some genetically predisposed cases, the patient's own immune system joined *Borrelia's* extracellular matrix degradation in destroying host tissue. The vaccine was scrapped due public outcry over autoimmune side-effects (NIgrovi 2007).

Because outer surface proteins still filled all the vaccine-target requirements, alternatives to OspA were sought. OspC, a 22kDa immunodominant lipoprotein coded on circular plasmid 26 and controlled by the RoS cascade (Fraser et al., 1997) is essential for bacterial survival due to the lytic complement complex which aids in immune evasion by extracellular matrix degradation (Kraiczy et al., 2002). It was found to be a likely alternative target. OspC binds tick saliva and is present only during initial stages of mammalian infection. It is not required for persistence and disappears two weeks after infection (Pulsova, et al. 2014).



OspC proved useful in detection of the very earliest spirochete invaders, which gave antibodies the ability to strike at the start of the infection cycle. Additionally, the disappearance of OspC after initial infection removed the possibility of autoimmune attack which had waylaid the OspA vaccine (Pulsova, et al. 2014). However, the variance in Osp C epitope presentation was a challenge, as antibody to one OspC did not necessarily work against others. Upon study, the protein was found to have a conserved core with several stable regions of difference.

Figure 3: OspC protein surface: 1 dark and 1 light grey monomer, epitopes in color (Baum, et al, 2015)

When addressing OspC s, antibody response during infection is OspC type-specific, with minor cross-reactivity-sa indicating variance in epitope rather than overall structure. Based on a 5% difference threshold for designating a new OspC type, over 30 OspC types have been defined so far. (Earnhart and Marconi, 2007c). Additionally, scientists found a relationship between OspC type and preferred host, with some genotypes associated specifically with rodent, canine or human infection. (Lovrich, et al, 2005) and (Rhodes, et al, 2013) and some types associated with different dissemination profiles and thus different disease manifestations per genotype (Norek, et al, 2017).

Shotgun vaccines containing full specimens of multiple OspC types together proved ineffective,

seemingly due to mutual inactivation, so focus moved to creating chimeric proteins containing only the epitopes from several OspC types. 21 OspC types were identified and their epitopes mapped. Cross-immunoreactivity studies were performed to locate multiple strain-relevant epitopes, and reduce redundancy. First four, then eight epitopes were chosen to created (respectively) tetravalent and octovalent chimeric immunogenic proteins for use in vaccination. (Earnhart, et al. 2007)

In order to create Immunogenic chimeras, targeted primers were used to amplify portions of the relevant OspC-coding DNA, and PCR amplification was performed with 5'overhangs to allow addition of the copies to vector plasmids. The epitope-loaded plasmids were then transfected into *E.coli* to test efficacy and purity of the inserted genetic information. These initial amplicons, once pure, were joined in sets of two, using the same PCR method, until reaching the full chimeric construct which was evaluated via DNA sequencing of the purified plasmid. (Earnhart, et al. 2007)

Once created, tests of the chimera's immunogenic activity were also completed stepwise, and showed consistent production of a high titered IgG antibody response in mice. It was additionally found that the vaccinogen elicited antibodies to each of the epitopes it contained, individually. Unexpectedly, the epitope-specific titer showed that position within the construct influenced each epitope's immunogenic activity, with a progressive decrease in titer response from N to C termina. When tested against intact *B.burgdorferi* cells the antibodies bound well and caused bacterial death, even among the targets associated with less prolifically produced C terminal epitope. (Earnhart, et al. 2007)

V. Next Steps

Animal response tests on dogs exposed to *B. burgdorferi* after immunization with canine-specific OspC tetravalent immunogen demonstrated successful antibody production (Napreny, et al. 2015). Outside the lab, Doctor Richard Marconi of Virginia Commonweath University Medical School, has developed a canine Lyme disease vaccine that was licensed by the U.S. Department of Agriculture in January and is currently being marketed as VANGUARD® CRLYME. He is continuing his work toward a human OspC-based chimeric vaccine, using human specific OspC types (Rhodes, et al, 2013). The epitope-selection technology has also been back-engineered to provide new and effective immunoassays for use in diagnostics by setting epitope fragments in a microarray. Such arrays allow detection and characterization of Borrelia infection based on genotype which may help in diagnosis, and lead to strain-specific treatment of Lyme disease in the future. (Gomes, et al. 2000).

They have built a better spirochete trap.

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