IDENTIFICATION ET CARACTÉRISATION DE PROTÉINES ANTIGÉNIQUES DE BORRELIA BURGDORFERI SENSU LATO

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IMPRIMATUR POUR LA THESE

Identification et caractérisation de protéines antigéniques de *Borrelia Burgdorferi* sensu lato

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Le doyen :
P. Kropf

A mon père

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Mots clefs
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1 Résumé

Borrelia afzelii, *B. garinii*, et *B. burgdorferi* sont trois des cinq espèces de *Borrelia* définitivement reconnues comme responsables de la borréliose de Lyme en Europe. Cette maladie infectieuse est transmise par les tiques et se caractérise par des symptômes multiples en plusieurs étapes touchant le derme, les articulations, le système neurologique et cardiaque. Le but de cette étude est de révéler de nouvelles protéines antigéniques spécifiques de *B. burgdorferi* sensu lato et de les caractériser. L''intérêt serait d'améliorer la détermination sérologique, la PCR de routine, et le diagnostic médical (symptômes / antigènes particuliers).

Dans cette étude, deux approches globales ont été utilisées pour étudier les protéines antigéniques d'intérêts: une démarche protéomique et une démarche génomique.

La partie protéomique consiste à étudier l'immunoprotéome de chaque espèce pathogène. Les fractions antigéniques des lysats protéiques totaux de *B. burgdorferi* sensu stricto VS215, *B.garinii* VS102 et *B. afzelii* VS461 ont été préparées en utilisant différentes colonnes d'immuno-affinités ayant une réactivité sérologique spécifique. Les protéines ont ensuite été séparées par électrophorèse bidimensionnelle pour obtenir des cartes de références spécifiques à chacune des espèces. 4 spots spécifiques ont été observés pour *B. afzelii* et 2 pour *B. burgdorferi.*

La partie génomique consiste à étudier le protéome prédit de chaque espèce. Des algorithmes bioinformatiques ont été utilisés et une méthode a été décrite pour sélectionner les protéines potentiellement sécrétées par *B. afzelii*, *B. burgdorferi* et *B.garinii* (méthode rapide, facile et librement disponible à partir d'internet). Cette sélection a mis en évidence 3 candidats pour *B. afzelii*, 7 pour *B. burgdorferi* et 2 pour *B.garinii*.

Ces deux approches donnet une vue de l'ensemble des antigènes de *B. burgdorferi* s.l.

Finalement un exemple d'identification et de caractérisation de candidat a été faite. L'objectif de cette étude était d'identifier une protéine de 12 kDa de *B. garinii* réagissant avec l'anticorps monoclonal D6. La protéine a été extraite et soumise à une analyse de séquence LC-MS/MS. Cette analyse a révélé trois séquences polypeptidiques analogues à BB0477 (30S ribosomique S10), à BB0061 (thiorédoxine A), et à BB0390 (50 S ribosomique L7/L12).

Les analyses génétiques ont été réalisées et deux polypeptides de la thiorédoxine A et un de la protéine ribosomique 50S ont ainsi été identifiés comme des épitoques potentiels.

L'expression dans le vecteur PQR9 de *E. coli* suivie d'immunoblots a permit de montrer que les résidus 7-12 de la thiorédoxine A. sont reconnu par le D6. Cela a été confirmé par des expériences de compétition avec un peptide synthétique.

Abstract

Borrelia afzelii, *B. garinii*, and B. *burgdorferi* are three of the five Borrelia species definitely recognized as responsible for Lyme borreliosis in Europe. This infectious disease is transmitted by ticks and is characterized by multistage skin, joint, neurological and cardiac manifestations. The aim of the present study is to reveal new species specific proteins of *B. burgdorferi* sensu lato and to characterize them. The points of interest consist in serologic determination, routine PCR, chips technology and in medical diagnostic (symptoms / particular antigen).

In this study, two global approaches were used to study antigenic proteins of interest: the proteomic way and the genomic way.

The proteomic part consists to study the immune-proteome of each pathogenic genospecies. Antigenic fractions of total bacterial protein lysate of *B. burgdorferi* sensu stricto VS215, *B.garinii* VS102 and *B. afzelii* VS461 were prepared using different immune-affinity columns with specific serological reactivity. Proteins were then separated by two-dimensional electrophoresis to obtain specific reference map for each species. 4 specific spots were observed for *B. afzelii* and 2 for *B. burgdorferi*.

The genomic part consists to study the predicted proteome of each genospecies. Bioinformatic algorithms were used and a method was described to select potential secreted proteins from *B. afzelii*, *B. burgdorferi* and *B.garinii* proteome in a simple way (fast, easy and freely available from internet). At the end of selection, 3 candidates were found for *B. afzelii*, 7 for *B. burgdorferi* and 2 for *B.garinii.*

These two approaches resume the antigenic state of *B. burgdorferi* s.l.

Finally an example of identification and characterisation of such candidates was made. The objective of this study was to identify a12 kDa protein from *B. garinii* reacting with the D6 monclonal antibody. Protein was extracted and submitted to LC-MS/MS sequence analysis. This analysis revelead three polypeptide sequences analogous to BB0477 (30S ribosomal protein S10), BB0061 (thioredoxine A), and BB0390 (50 S ribosomal protein L7/L12).

Genetic analyses were performed and two polypeptides from thioredoxin A and one from 50S ribosomal protein were thus identified as potential epitopes. Expression in *E.coli* PQR9 followed by by immunoblotting identified residues 7-12 of thioredoxin A as the D6 mab epitope. This was confirmed by competition experiments with a synthetic peptide.

2 Introduction

2.1 *Borrelia burgdorferi*

2.1.1 Classification

Borrelia burgdorferi sensu lato appartient à l'ordre des Spirochaetales, comme les genres Leptospira et Treponema. Le genre *Borrelia* comprend une trentaine d'espèces, dont *Borrelia burgdorferi* sensu lato qui regroupe 19 espèces et 5 sont des espèces pathogènes majeures, *B. burgdorferi* sensu stricto, *B. garinii, B. afzelii, B.bavaiensis* et *B. spielmani* responsable de la borréliose de Lyme (BL) (Baranton et al. 1992; Canica et al. 1993). Il contient aussi au moins huit espèces étroitement apparentées qui ne provoquent que très rarement des infections humaines.

Borrelia burgdorferi sensu lato est abrégé dans cette thèse comme Bb ou *B. burgdorferi, Borrelia burgdorferi* sensu stricto comme Bb s.s., *Borrelia garinii* comme Bg et *Borrelia afzelii* comme Ba.

2.1.2 Structure et exigences de croissance in vitro

Bb est une bactérie Gram-négative hélicoïdale qui mesure 15-25 µm de long sur 0,2-0,5 µm de large, qui est dotée de 7-11 flagelles périplasmiques (Barbour, Hayes 1986), qui permettent la motilité et lui confèrent sa forme. D'une manière analogue aux autres bactéries Gram-négatives, Bb a une membrane externe qui entoure l'espace periplasmique et une membrane cytoplasmique interne qui protège le cytoplasme (Barbour, Hayes 1986). Exceptionnellement, aucun lipopolysaccharide (LPS) n'est présent dans la membrane externe de Bb. Bb peut être visualisé sans coloration par champ noir ou par microscopie à contraste de phase (Preac-Mursic et al. 1993). L'organisme peut être détecté par microscopie après coloration de Wright, Giemsa ou argent, ainsi que par microscopie à fluorescence suite à un marquage immunocytochimique.

Bb peut être cultivé *in vitro* dans des conditions microaérophiliques à 33 °C dans un milieu liquide appelé Barbour-Stoenner-Kelly (BSK II) (Barbour 1984). Lors de conditions défavorables, tel que pH bas ou sous l'influence d'antibiotiques, le spirochete développe des blebs de membrane (Preac-Mursic et al. 1986).

2.1.3 Génome

Bb a un chromosome linéaire d'une taille approximative de 1Mb (Fraser et al. 1997), et il contient généralement au moins quatre plasmides linéaires et plusieurs plasmides circulaires. La plupart du génome de la souche B31 de Bb s.s. a été publié en 1997 (Fraser et al. 1997) et

le génome complet a été publié en 2000 (Casjens et al. 2000). Le génome contient un grand nombre de gènes qui codent pour des lipoprotéines qui incluent des protéines de surface externes (Osps) de A à F (Fraser et al. 1997). Par contre, le génome de Bb ne code que pour peu de protéines impliquées dans la biosynthèse, signifiant que le spirochète est dépendant de l'hôte pour les exigences alimentaires.

2.1.4 Les protéines de surfaces externes

Les protéines de la surface externe OspA et OspB sont deux lipoprotéines majeures de surface de la membrane externe de Bb. Dans le génome de la souche B31 de Bb s.s, les gènes codant OspA et OspB sont localisés sur le plasmide linéaire 54 (lp54). Les deux gènes partagent un promoteur et sont transcrits de manière coordonnée (Howe et al. 1986; Bergström et al. 1989). Bb est transmis aux êtres humains par les tiques et les études ont montré que Bb régule l'expression de beaucoup de protéines de surface lors de la phase de transmission. OspA et OspB sont exprimés par Bb dans l'intestin moyen de la tique. Peu après l'entrée dans l'hôte vertébré, l'expression est diminuée, et l'expression d'OspC est augmentée (Schwan et al. 1995; Schwan, Piesman 2000). Des anticorps dirigés contre OspA et OspB sont détectables chez quelques malades lors de la phase précoce de la maladie, et lors de l'étape tardive pendant l'arthrite (Kalish et al. 1995; Chen et al. 1999), ainsi que lors de traitement-réfractaire à l'arthrite de Lyme (Lengl-Janssen et al. 1994; Chen et al. 1999) suggérant qu'OspA et OspB sont aussi exprimés lors de certaines étapes de l'infection persistante. De plus, des antigènes OspA (Coyle et al. 1993) ainsi que ces anticorps (Schutzer et al. 1997) ont été identifiés dans le fluide cérébrospinal de malades atteints de neuroborréliose. Dans une étude publiée par Batsfor, approximativement 80% de sera de malades ayant une arthrite ou une acrodermatite chronique atrophiante (ACA) et 23% des sera de malades ayant un érythème migrant (EM) reconnaissent les antigènes OspA ou OspB (Batsford et al. 1998).

2.2 Borréliose de Lyme

2.2.1 Epidémiologie

Les spirochètes Bb vivent dans un cycle enzootique impliquant des tiques et une grande gamme d'animaux comprenant des mammifères, en particulier des souris, des oiseaux et même des reptiles (Anderson, Magnarelli 1984; Gern et al. 1998; Xu et al. 2007). La borréliose de Lyme apparaît comme l'affection vectorielle la plus fréquente aux USA et probablement en Europe. Les vecteurs sont de la famille des *Ixodidae*. Selon le lieu géographique l'espèce diffère : *Ixodes ricinus* en Europe occidentale, *I. persulcatus* en Europe

de l’Est et Asie, *I. ovatus* au Japon, et *I. scapularis* et *I. pacificus* pour l’Amérique du Nord, respectivement à l’est et à l’ouest. Le risque de contamination dépend directement de la densité en tiques et de leurs pourcentages d’infestations, ainsi que des facteurs géographiques et climatiques (Magnarelli, Anderson 1988; Wittenbrink et al. 1994).

Le cycle d’*Ixodes* comprend trois stades : larve, nymphe et adulte. Le passage d’un stade au suivant nécessite un repas sanguin. La larve se fixe généralement sur des insectivores ou petits rongeurs, la nymphe sur des vertébrés de taille moyenne ou des oiseaux et finalement l’adulte sur des mammifères de plus grande taille. Aux trois stades, l‘homme est un hôte accidentel, mais Bb est habituellement transmis par les nymphes et les tiques adultes (femelles).

Les études sur les animaux de laboratoire ont montré que la transmission de la maladie exige généralement un attachement de la tique d'une durée de 48-72 heures (selon l’espèce), signifiant que si la tique a été attachée durant moins de 24 heures, le risque d'infection peut être considéré comme bas (Piesman et al. 1987; Piesman 1993; Des Vignes et al. 2001).

2.2.2 Pathologie

Pour maintenir son cycle enzootique, Bb doit s'adapter aux environnements des différents hôtes. À l'intérieur de la tique, Bb exprime la protéine de surface externe A (OspA) qui reste attaché au récepteur OspA de la tique (TROSPA) au niveau de l'intestin moyen (Pal et al. 2004). Lors du repas sanguin de la tique, Bb change l'expression de plusieurs gènes, y compris la régulation du gène qui code pour l’OspA. Simultanément, l'expression de l’OspC est augmentée, ce qui est nécessaire pour infecter les mammifères (Steere et al. 2004; Hu et al. 1996).

Il a été démontré que l’OspC lie la protéine salivaire Salp15 de la tique, Salp15 ayant des propriétés immunosuppressives (Hovius et al. 2008).

Plusieurs jours à semaines après la transmission au niveau de la peau du mammifère, Bb peut migrer par voie hématogène vers plusieurs organes. Pour faciliter cette dissémination, Bb adhère aux intégrines, protéoglycanes, ou glycoprotéines des cellules hôtes ainsi qu'aux matrices extracellulaires (Steere et al. 2004).

Bb peut se lier à des plasminogènes et des urokinases activatrices de plasminogènes, pour mieux pénétrer à travers les couches cellulaires endothéliales. D’autres protéines importantes de *Borrelia* interviennent, tel BBK32 (47 kDa), qui a été démontrée comme se liant aux fibronectines; p66 (66 kDa), une protéine de surface externe qui se lie aux récepteurs du fibrinogène et au récepteur du vitronectine; p26 (26 kDa), qui se lie aux glycosaminoglycanes

des cellules endothéliales et neuronales. De plus, les protéines dbp A et B lient un collagène associé aux décorines du protéoglycane (Steere et al. 2004).

2.2.3 Réponse immunitaire à *B. burgdorferi*

Une fois que Bb est dans la peau, les premiers facteurs de l'hôte que rencontrent les bactéries sont des composants du système du complément et les cellules du système immunitaire inné. Selon l'espèce de Bb, la lyse médiée par le complément contre les spirochètes peut être le premier mécanisme de défense de l'hôte (Breitner-Ruddock et al. 1997). Le premier symptôme clinique, la lésion EM, est la réponse des lymphocytes, cellules dendritiques (DCs), macrophages et un petit nombre de cellules du plasma (Müllegger et al. 2000). Cependant, seulement très peu de neutrophiles sont présents, ce qui est atypique lors d'une infection bactérienne (Steere et al. 1983a). Les cellules inflammatoires dans l'EM produisent des cytokines pro-inflammatoires, les plus abondantes étant TNFα et INFγ. Une réponse Th1 optimale dans la phase précoce de la maladie a été associée avec de bons résultats (Sjöwall et al. 2005). L'infection peut être limitée à la peau et peut se résorber même sans traitement, mais pour des raisons encore inconnues, dans quelques cas la bactérie envahit la circulation sanguine et se dissémine vers plusieurs organes. Les symptômes peuvent paraître même des années après l'infection primaire. Il semblerait que dans la phase précoce de la maladie, la réponse Th1 serait importante dans la défense contre les bactéries, alors que la réponse Th2 serait importante dans la phase plus tardive de la borréliose de Lyme (Oksi et al. 1996; Sjöwall et al. 2005).

2.2.4 Manifestations cliniques

La borréliose de Lyme est caractérisée par un polymorphisme clinique, auquel il faut de plus distinguer la forme européenne de l'affection américaine (Stanek, Strle 2003; Nadelman, Wormser 1998).

Le développement de la maladie peut être divisé en infection locale précoce, disséminée et chronique tardive (Steere et al. 2004). Cependant, les trois étapes se chevauchent et leurs présence n'est pas claire dans tous les cas de BL. Le premier symptôme dermatologique de l'infection, l'érythème migrant (EM), a lieu habituellement 3-32 jours après l'infection. Cette phase primaire peut s'accompagner de symptômes apparentés à la grippe, tel que malaise, fatigue, maux de tête, arthralgies, myalgies et fièvre (Steere et al. 1983b). Cet EM est parfois suivi d'une phase dite secondaire, le plus souvent neurologique, tel que des névropathies crâniennes et périphériques (un exemple typique étant une paralysie du nerf facial),

accompagné de méningite lymphocytaire, plus précisément de meningoradiculite lymphocytaire.

Les manifestations typiques au niveau de la peau de la BL disséminée sont des lésions EM multiples, des lymphocytomes cutanés et des Acrodermatites chroniques atrophiantes (ACA) (Hansen, Asbrink 1989) qui sont des manifestations tardives de la BL. L'arthrite de Lyme est généralement une manifestation tardive de la BL mais elle peut se produire quelquefois plus tôt lors de la maladie. L'arthrite est caractérisée typiquement par des attaques périodiques au niveau des (grosses) articulations. Les manifestations neurologiques tardives de la BL incluent méningites, et radiculites.

Il y a un grand nombre d'autres manifestations disséminées et tardives engendrées par la BL : au niveau du cœur il peut y avoir des cardites, plus précisément des endomyocardites ou péricardites (Steere 1989) ; au niveau des yeux on trouve des conjonctivites, des kératites et uvéites (Lesser 1995); au niveau de foie des hépatites (Kazakoff et al. 1993), des splénomégalies au niveau de la rate (Cimmino et al. 1989) ; ou encore des orchites et des hématuries microscopiques (Steere 1989). L'infection Bb a une forte tendance à devenir chronique (Berger et al. 1983; Steere et al. 1983b; Steere et al. 1983a).

Il existe des différences cliniques et épidémiologiques entre l'expression de la BL entre l'Europe et les USA. Aux USA le pathogène n'est représenté que par *Borrelia burgdorferi* sensu stricto alors que les trois espèces pathogènes sont représentées en Europe. Les différences cliniques s'expliquent par l'organotropisme existant entre les différentes espèces de *Borrelia*. Ainsi les complications rhumatologiques sont dans la plupart des cas causées par Bb s.s., alors que Bg est responsable des symptômes de type neurologiques, et que les manifestations dermatologiques tardives sont souvent associées avec les infections de Ba (Assous et al. 1993; Péter et al. 1997; Wang et al. 1999).

2.2.5 Diagnostic et traitement

Dans la phase précoce de la BL, le diagnostic est basé habituellement sur des conclusions cliniques typiques, la plus évidente et spécifique étant la rougeur de l'EM, et dans ce cas le diagnostic de laboratoire n'est pas recommandé. Si une rougeur EM est suspecte, le malade devrait être traité aux antibiotiques. L'agent causal étant de la famille des Spirochaetacea, les Beta-lactamines et les tétracyclines sont les antibiotiques de choix et les nouveaux macrolides peuvent être utilisés en deuxième intention.

Au stade tardif de la maladie, le diagnostic sérologique est recommandé en aide au diagnostic clinique. La production d'anticorps IgM a lieu durant les deux premières semaines suite à

l'infection et atteint un maximum après deux mois. Progressivement, la production d'anticorps IgG commence en même temps que le déclin des IgM. Une approche en deux étapes est généralement utilisée dans la sérologie de la BL (Wilske 2003). La première étape est un test ELISA sensible qui est suivi par un test immunoblot lors d'un positif ou cas positif limite. Bb peut être mis en culture dans un milieu liquide BSK II (Barbour 1984). Le temps de génération de Bb étant long, les cultures nécessitent d'être incubées quelques semaines. Une méthode moléculaire consiste à détecter l'ADN de Bb dans des échantillons de biopsie ou fluides corporels (par exemple fluide synovial, fluide cérébrospinal, sang) en utilisant la technique de la « polymerase chain reaction » (PCR) (Stanek, Strle 2003). En général, les sensibilités des cultures de Bb et des PCR ne sont pas des méthodes de choix dans le diagnostic de laboratoire de la BL, et sont donc négligeables en dehors d'études cliniques.

2.3 Problématique et objectifs

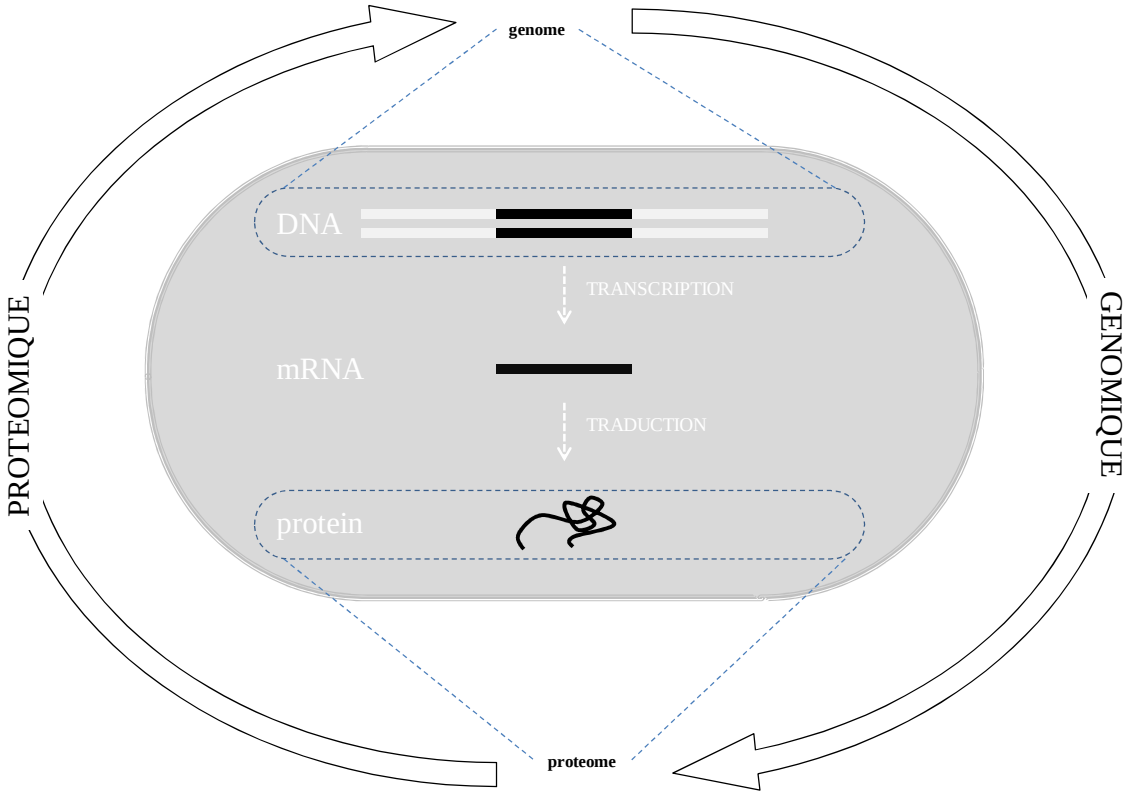
BL est l'une des infections bactériennes persistantes les plus communes en Europe qui n'offrent pas encore de sérologie adéquate. Ceci paraît être lié aux réponses immunes uniques résultant des stratégies de fuite de la bactérie par rapport au système immunitaire de l'hôte. Cette thèse a pour but d'identifier de nouveaux antigènes pertinents pour le diagnostic.

Le diagnostic de l'infection de *Borrelia burgdorferi* sensu lato, agent étiologique de la BL, est basé sur les manifestations cliniques et est confirmé par tests sérologiques. Ces tests ont néanmoins pour défauts leurs manques de sensibilité et spécificité. L'usage de nouveau marqueurs spécifiques et fortement antigéniques pourraient améliorer ces limitations.

• La première partie de cette thèse a pour but d'identifier de nouveaux antigènes de *B. burgdorferi* en employant une approche protéomique.

• La deuxième partie de cette thèse a pour but d'identifier de nouveaux antigènes de *B. burgdorferi* en employant une approche génomique.

• La troisième partie est un exemple d'identification et de caractérisation d'une protéine antigénique.



Résumé des deux démarches : L'étude protéomique se faisant à partir des protéines (approche pratique : détection physique grâce aux anticorps) pour aboutir aux gènes, alors que l'étude génomique (approche informatique : prédiction grâce à des algorithmes) est à l'inverse : on part du gène pour aboutir aux protéines. Ceci permet donc d'avoir une vision globale et complète des antigènes potentiels de *Borrelia*.

3 Articles

3.1 Comparison of antigens of *Borrelia afzelii, B. burgdorferi* and *B. garinii* isolated with immuno-affinity columns on two-dimensional electrophoresis maps

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3.1.1 Introduction

The bacterium *B. burgdorferi* sensu lato (s.l.), the causative agent of Lyme borreliosis (LB), belongs to Gram-negative, spirochetal bacteria (Paster et al. 1991) characterised by a spiral morphology and flagella that function as motility organs.
B. burgdorferi s.l. is divided into different genospecies of which three have been identified as major human pathogens: *B. burgdorferi* sensu stricto (s.s.), *B. garinii* and *B. afzelii* (Baranton et al. 1992; Marconi, Garon 1992; Canica et al. 1993). All three species can be found in Europe and Asia whereas in the USA only *B. burgdorferi* s.s. occurs.
The genome of *B. burgdorferi* s.l. consists of a linear chromosome and numbers of linear and circular plasmids (Barbour 1988; Casjens et al. 2000). The large number of plasmids may enable an extensive antigen variation to adapt to the different environments the bacterium may encounter. Outer surface proteins exposed on the cell surface interact with the host and contribute to the pathogenesis of LB.
Lyme borreliosis, the most prevalent and widespread vector-borne infectious disease in the northern hemisphere, is a multisystem disease involving many organs, mainly the skin, nervous system, joints and heart (Steere 1989; Pfister et al. 1994; Stanek, Strle 2003).
Different clinical forms are found and are directly correlated to phenotype and genotype heterogeneity of the pathogen (Pachner et al. 2004). Arthritis and carditis are preferentially associated with *B. burgdorferi* sensu stricto, the degenerative skin disorder acrodermatitis chronica atrophicans (ACA) with *B. afzelii* and neuroborreliosis with *B. garinii* (Demaerschalck et al. 1995; Balmelli, Piffaretti 1995).
If there are no pathognomonic symptoms such as a typical erythema migrans, clinical diagnosis of Lyme borreliosis usually requires confirmation by means of a laboratory-diagnostic assay. Antibody detection methods mainly are used for this purpose, whereas detection of the causative agent by culture isolation and nucleic acid techniques is confined to special situations.
Antibody detection is made by serological tests, currently using a two step approach: a screening assay, mostly ELISA, confirmed by immunoblot analysis (Wilske et al. 2007). Recently, immunoblots have been improved by addition of recombinant antigens (i.e. VlsE and DbpA) (Goettner et al. 2005; Schulte-Spechtel et al. 2003). Despite recent improvements, the limitations regarding sensitivity and specificity of the tests still restrict their use as routine diagnostic tools (Aguero-Rosenfeld et al. 2005). To date, the cross-reactivity of borrelia antigens, the delayed appearance or even lack of measurable immune responses in the early stage of LB and the absence of a marker for persistent or active infections are the main challenges for serodiagnosis (Brouqui et al. 2004). Identifying and characterizing antigenic components that are involved in the pathogenesis of *B. burgdorferi* s.l. will permit a better understanding of the disease and lead to more effective diagnosis and treatment.
A way to detect and identify specific antigens is to work with complete immunoproteomes of each pathogenic genospecies. The method consists of two-dimensional electrophoresis (adequate resolution for microorganisms proteome) followed by blotting on membranes and overlaying the protein pattern with patient's sera (Krah, Jungblut 2004).
Generally, prior to electrophoresis, proteins are enriched in proteins of interest to increase the resolution of the separation.
For Borrelia various techniques have been attempted (Bledsoe et al. 1994): surface labelling using I-125 or biotin (Luft et al. 1989), antibody labelling (Barbour et al. 1984), accessibility to proteases (Norris et al. 1992) , and differential solubilisation with detergents (Sambri, Cevenini 1991; CUNNINGHAM et al. 1988).
However, the techniques are subject to artefacts, and consider only a fraction of protein of interest for diagnosis purpose.

Here we isolated first the antigenic proteins to obtain directly immunoproteome by 2d-electrophoresis. Antigenic fractions of the total bacterial protein lysate of *B. burgdorferi* s.s. VS215, *B. garinii* VS102 and *B. afzelii* VS461 were prepared using different immune-affinity columns with specific serological reactivity. The following immunoblot step is used to validate the results.

The study allowed identifying new species-specific antigens of *B. burgdorferi* s.l., and obtaining antigenic maps for *B. burgdorferi* s.s., *B. garinii* and *B. afzelii*. Such studies were already made by American groups, but focalised only on B.b.ss(Nowalk et al. 2006a). Maps of all 3 genospecies were elaborated by Jungblut (Jungblut et al. 1999), but the antigenic characteristics were focused on *B. garinii* exclusively.

3.1.2 Material and methods

Bacteria cultures

Low passage Borrelia strains, *B. burgdorferi* s.s. VS215, *B. garinii* VS102 and *B. afzelii* VS461 were used for antigen preparation (Péter et al. 1997). All strains were isolated from ticks (*I. ricinus*) (Péter, Bretz 1992). Spirochetes were cultured in BSK II medium. During the late logarithmic phase of growth, the culture was centrifuged at 14,000g for 15 min and washed twice in phosphate-buffered saline (pH 7.2) to which MgCl2 (0.05 M) was added. After sonication, the protein concentrations of the suspensions were determined by the Biuret method and adjusted to 1 mg/ml in distilled water.

Sera

A minimum of three sera were mixed to constitute a pool. Selection consisted of sera from patients with characterized late LB symptoms, i.e. with neuroborreliosis, acrodermatitis or arthritis (stage III). Each serum was highly reactive in ELISA and presented strong species specific IgG reactivities in immunoblots (Ryffel et al. 1999). They were pooled according to their reactivities.

Affinity chromatography

Hitrap protein G columns (Amersham bioscience) were equilibrated with 10 volume of binding buffer (B-buffer; 20mM Sodium Phosphate, pH7.0). Each pool of sera was diluted 1:1 with B-buffer, filtered (0.45µm) and titration of IgG was carried out by nephelometry. At least 25mg IgG of each elaborated pool of sera were incubated for 30 min in the corresponding column. The Ab-bead conjugates were washed with eight volumes of B-buffer and with eight volumes of washing buffer (W2-buffer; 20mM Sodium Phosphate buffer pH8.2). The antibodies were then covalently linked for one hour to the protein G beads using dimethylpimeliminidate (DMP) solution (0.2M Triethanolamine pH8.2 + DMP (40mg/10ml)). After an eight volume wash with 0.2M Triethanolamine pH8.2, remaining protein G sites were blocked with fifteen volumes of saturation buffer (0.1M Ethanolamine buffer pH8.2). Columns are ready for use after a pre-elution with four volumes of elution buffer (E-buffer; 0.1M Glycine-HCl pH2.8) and equilibration with ten volumes of W2-buffer. For long term storage, H_2O with 10% ethanol was used.

Immunoprecipitation

Columns were washed with lysis buffer (L-buffer; 150mM NaCl, 1%NP-40 (v/v), 0.1% SDS (w/v), 50mM Tris-HCl pH8.0) containing protease inhibitors. Two mg of each *Borrelia* antigen were resuspended in a final volume of 10ml L-buffer, sonicated, incubated 15 min on a rotating wheel, and centrifuged at 18000g. Supernatants were incubated in the corresponding columns during 30 min with continuous running. Columns were then washed with eight volumes of L-buffer and followed by elution with eight volumes of E-buffer. Elutions were stabilized with 175µl 2.0M Tris-HCl pH 8.0 for a final volume of 1.5 ml. Columns were regenerated with ten volumes W2-buffer and finally stored in 10% ethanol.

Two-dimensional Gel Electrophoresis

Volumes of 100µl (for gel staining) or 50µl (for immunoblots) of eluted material were concentrated and precipitated with trichloroacetic acid (20%). Individual 7cm ReadyStrips IPG strips (Bio-Rad), pH 5–8 were rehydrated with 125 µl of urea buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (v/v) Np40, 0.2% (v/v) Bio-Lytes, 1% (w/v) dithiothreitol, 0.002% (v/v) bromophenol blue) containing the sample. Isoelectric focusing was carried out using a Protean IEF Cell according to the instructions from the manufacturer (Bio-Rad): 14

hour 50V active rehydration; 20 min 250V linear slope; 120 min 4000V linear slope; 4000V 12000VH rapid slope. Prior to the second dimension, the IPG gel strips were equilibrated twice in 2.5 ml of equilibration solution (50 mM Tris-HCl buffer, pH 8.8, containing 6 M urea, 30% (v/v) glycerol, 1% (w/v) SDS) for 20 min, first in the presence of dithiothreitol (65 mM) and then in the presence of iodoacetamide (87 mM). Second dimension gels consisted in a 12% tris-glycine SDS-PAGE. Gels were run using the Protean II xi Cell (Bio-Rad), at a constant current of 15 mA/gels. The analytical two-dimensional gels were stained with zinc (Hardy, Castellanos-Serra 2004), with silver (Shevchenko et al. 1996; Yan et al. 2000) or were transferred for immunoblots and scanned with G-700 from Bio-rad.

Immunoblots

After electrophoresis the proteins were transferred to a polyvinylidenedifluoride membrane with constant voltage (120V) during one hour. Before further use, the membranes were blocked for 1 h at 37°C with Tris-buffered saline (TBS; pH 7.2) with 5% gelatine and were washed three times for 5 min each time with washing buffer (W-buffer; TBS with 0.1% gelatine and 0.05% Tween 20) at room temperature. The following steps were also performed at room temperature. Each membranes were incubated for 2 h with pools of human sera diluted 1/200 in D-buffer (TBS with 1% gelatine and 0.05% Tween 20). For monoclonal antibodies dilutions were 1:1500 for LA114 ZS7 (93 kDa protein), 1: 100 for LA 18 (66 kDa protein), 1:1000 for H9724 (41 kDa protein; flagellin), LA112 ZS7 (39 kDa protein), LA222B8 (OspA) and LA31 (OspC) (kindly provided by A. G. Barbour, University of California, Irvine; R. Wallich, Ruprecht-Karls-Universität, Heidelberg, Germany; and B. Wilske, Max von Pettenkofer Institut, Munich, Germany) (Ryffel et al. 1999). The membranes were washed three times for 5 min with W-buffer. After the washing, rabbit anti-human IgG (Sigma, St-Louis, USA) conjugated to alkaline phosphatase diluted 1/1000 in D-buffer was added for human sera. The secondary antibodies for monoclonal antibodies were goat anti-mouse polyglobulin (Sigma, St-Louis, USA) conjugated to alkaline phosphatase diluted 1/1000. At an incubation of 2 hours, two washes were done with W-buffer and one was done with TBS. The bound conjugate was visualized by addition of the chromogenic substrate 5-bromo-4-chloro-3-indolylphosphate-Nitro Blue Tetrazolium (Kirkegaard & Perry Laboratories). The reaction was stopped 30 min later by two rinses in distilled water.

Image analysis

Image analyses were performed with Flicker version 0.83.6 (Lemkin, Thornwall 1999), a program belonging to the Open2Dprot Software, with ImageMaster 2D Platinum 6.0 (GE Healthcare) and TopSpot (Pleissner et al. 2002).

3.1.3 Results

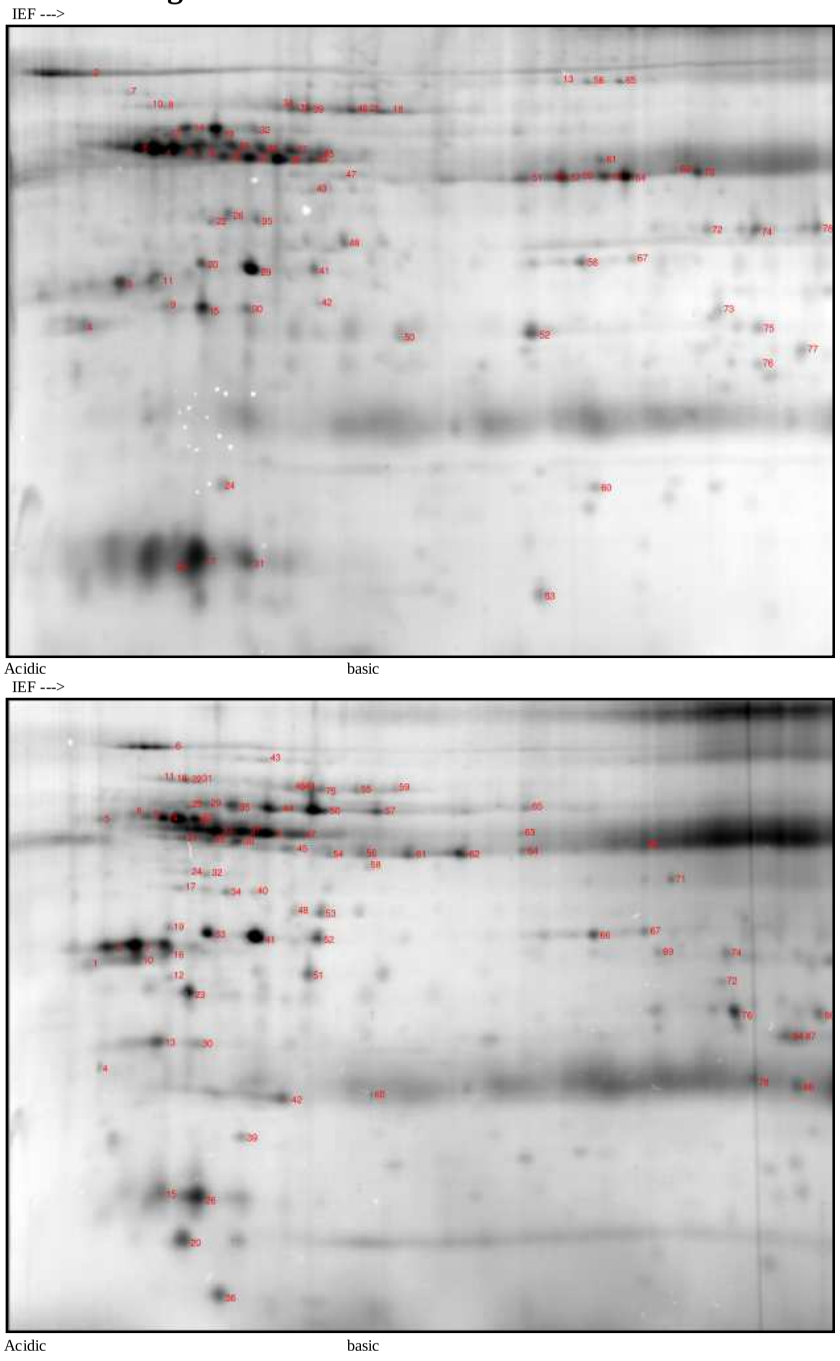
On every gel (Figure 1), two horizontal diffuse streaks (~50kD and ~25kD) are observed that represent a small amount of eluted IgG from the column (heavy and light chains). These streaks were used to align gels together.
After two-dimensional electrophoresis, different staining techniques (zinc and silver) were used to visualize proteins on gels. The general pattern (Figure 1) between species is very similar. Staining differs in sensitivity, in the increasing order from silver to zinc. Less spots are present with silver staining. This gives a semi-quantitative aspect of the visualized proteins.
With all the landmarks and in comparison with the literature it was possible to identify the main spots (listed on Table 1). For *B. burgdorferi s.s.*, 90 spots were detected with zinc staining, 22 were well identified and 2 were described as species specific: spot n°23 and n°51. For *B. afzelii*, on a total of 78 spots, 16 were identified and 8 were species specific: spot n°13, 56, 65, 22, 26, 35, 52 & 75. No species specific spots were observed for *B. garinii* among the 71 spots (19 with identity).
More spots seem to be seen for zinc maps, but they appear more distinct with silver staining. With identical parameters (area; separation), more spots are detected on silver maps: 98 for *B. burgdorferi* (36 identified); 102 for *B. afzelii* (23 identified) and 76 for *B. garinii* (17 identified). Species specific spots are similar to spots observed on zinc maps, just one supplementary isoform for *B. afzelii*: spot n°84 is dectectable. Every data are visualized on Figure 1 and data are referenced on Table 1.
The proteins p83/100, Oms66, ErpB, OppA, FlaB, BmpA, OspC and LA7 are detected in all species. Some spots are only present in one species like Hsp90, ErpA and OspB for *B. burgdorferi* or GADPH for *B. garinii*. Some proteins are represented by one spot like p83/100, whereas others with spot string like Oms66 or FlaB (Table 1). Differences could also be observed between staining methods. Hsp 90; OspB or RevA are only detected on zinc maps of *B. burgdorferi s.s*. On the contrary, OspA and OspB are well represented on silver maps.
Most of identified spots are concentrated in acidic range of the gel. Some proteins like p83/100, Oms66, ErpB and FlaB are similar in MW and PI between species. Other proteins differ in PI like OspC or in MW and PI like OspA (not present for *B. garinii*).
Finally some proteins are only represented in one species: spots 23, 51 for *B. burgdorfer*i, (13, 56, 65), (22, 26, 35), 52, 75 for *B. afzelii*. Corresponding respectively to 14, 46 for *B burgdorferi* s.s silver map and (67; 75; 83; 84); (15; 21; 28); 66; 97 for *B. afzelii*. No species specific spots were detected for *B. garinii.* To confirm the antigenic properties of the proteins, immunoblots were performed with monoclonal antibodies (p100, p66, p41, p39, OspA, OspC) in order to localize the major proteins, to scale gels and to align them together (data not shown). This standardisation was made with *B. burgdorferi* s.s only. Recognition of the corresponding proteins for the other species was deduced since these known common proteins have identical patterns and molecular mass, excepted OspA. All proteins detected on the 2D gels were also present on immunoblots (data not shown)

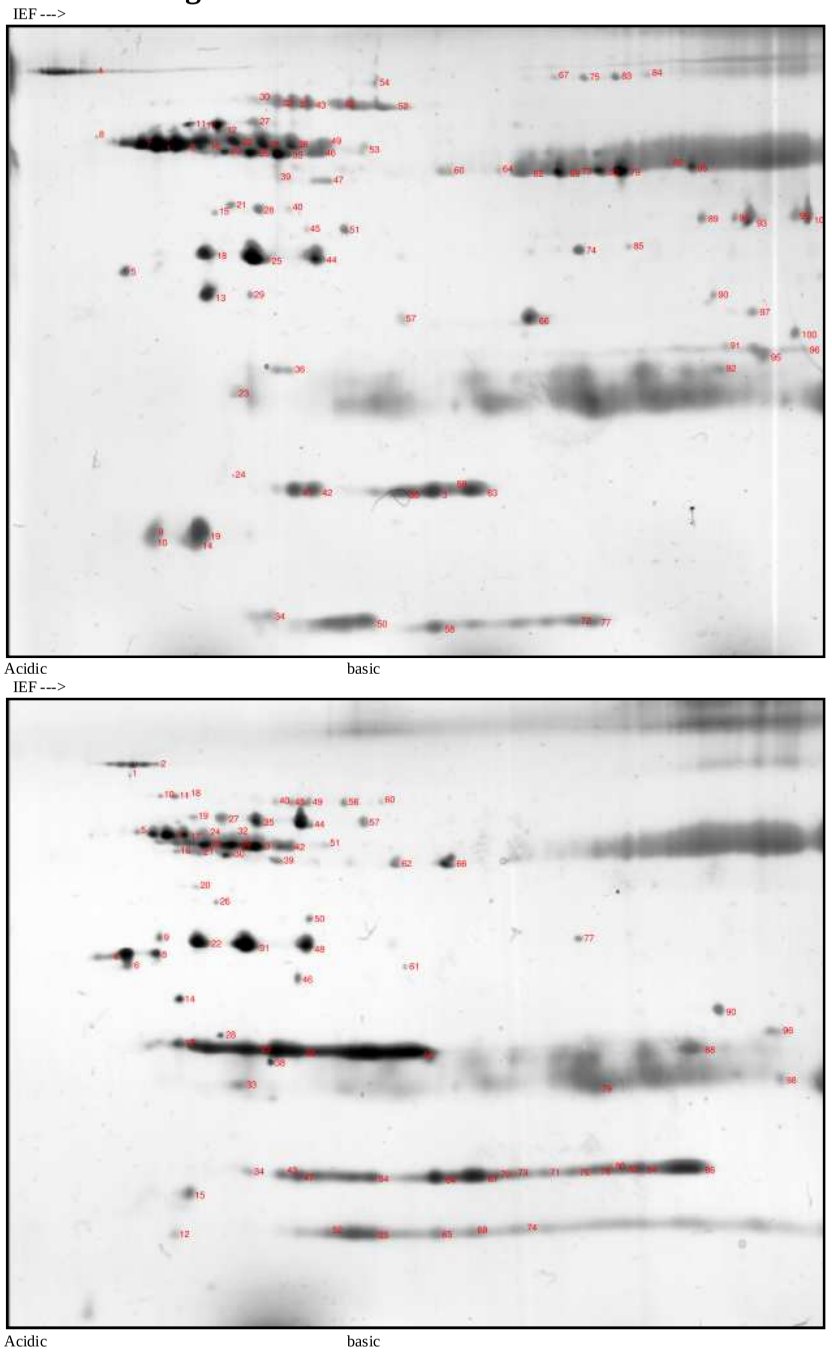
Zinc staining

Silver staining

B. afzelii

B. burgdorferi





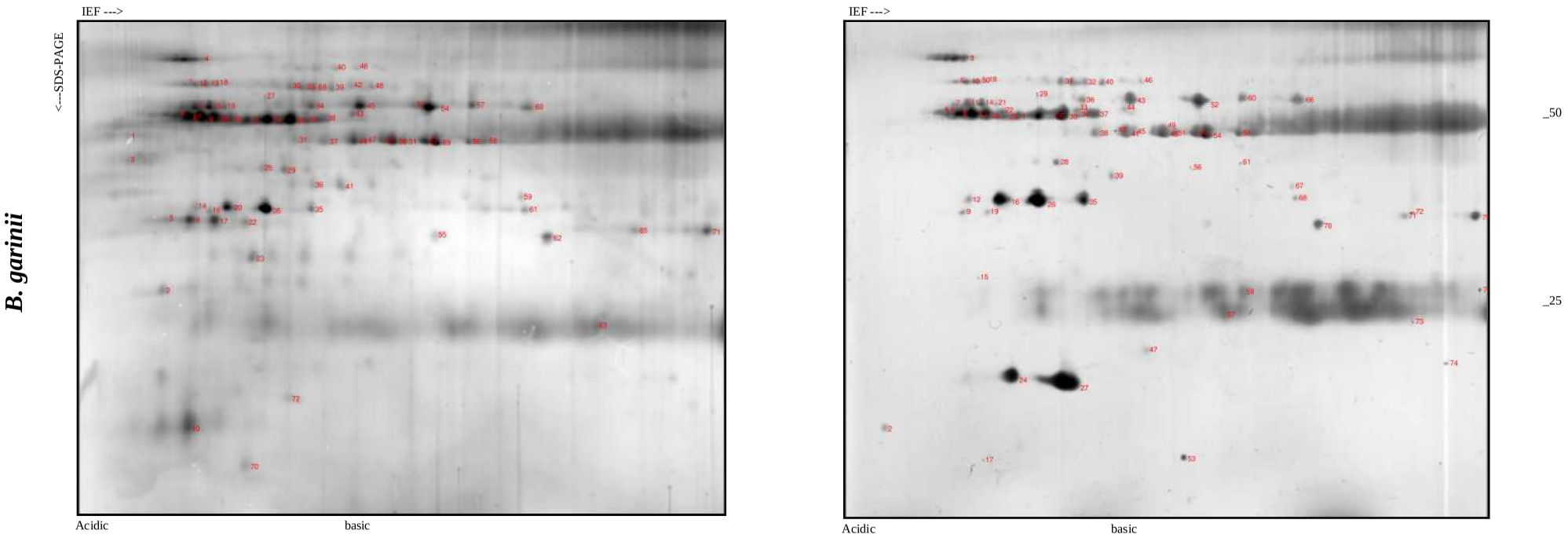


Fig. 1: 2D reference maps of *B. afzelii* , *B. garinii* and *B. burgdorferi*. Extracted proteins were separated by IEF in the pH range 5 to 8, followed by SDS-PAGE gel (12%). Spot numbers indicate identified proteins by TopSopt program.

Table 1: List of spots of interest for zinc and silver reference maps

		----------ZINC STAINING----------			----------SILVER STAINING----------		
		B. afzelii	*B. burgdorferi*	*B.garinii*	*B. afzelii*	*B. burgdorferi*	*B.garinii*
Short-Name	**Ref**	**spot n°**	**spot n°**	**spot n°**	**spot n°**	**spot n°**	**spot n°**
p83/100	monoAB; (Nowalk et al. 2006b)	2	6	4	4	2	3
Hsp90	(Nowalk et al. 2006a)		43				
Oms66	(Jungblut et al. 1999)	14; 19; 32	35; 44; 50	11; 15; 19	11; 17; 27	27; 35; 44	7; 11; 14; 21
ErpB	monoAB; (Nowalk et al. 2006a)	6; 1; 3	9; 14; 76	67; 9; 66	7; 1; 2	5; 7; 17	8; 13; 20
OppA	(Nowalk et al. 2006a)	23; 27; 36	73; 37; 3	21; 24; 29	20; 26; 35	29; 3; 42	25; 30; 34
FlaB	monoAB; (Nowalk et al. 2006a)	20; 29; 41	33; 41; 52	20; 26; 35	18; 25; 44	22; 31; 48	16; 26; 35
BmpA	monoAB; (Nowalk et al. 2006a)	5; 11	2; 7; 16	5; 8	5	4; 8	9
GAPDH	(Jungblut et al. 1999)			62			70
OspB	(Nowalk et al. 2006b)		76				
OspA	monoAB;				36	13; 23; 36; 53	
OspD	(Nowalk et al. 2006b)		24		23	33	
OspC	monoAB;			72	41; 42; 55; 3; 59; 63	43; 47; 54; 64, 67; 70; 73; 71; 76; 78; 80; 82; 84; 86	27
LA7	(Nowalk et al. 2006a)	17	26; 15	10	19	15	
ErpA	(Nowalk et al. 2006a)		20			12	
RevA	(Nowalk et al. 2006a)		36	70			
unknown sp spot		13; 56; 65	23		67; 75; 83; 84	14	
		22; 26; 35	51		15; 21; 28	46	
		52			66		
		75			97		
Statistic		**nb spot**	**nb spot**	**nb spot**	**nb spot**	**nb spot**	**nb spot**
nb total of spot		78	90	71	102	98	76
nb unknown spot		62	68	52	79	62	59
known spot		16	22	19	23	36	17
known prot		7	12	10	10	11	9

Table 2: Characteristics of proteins identified and cited in the literature

Protein	MW (kDa)	Known function or localisation	Importance in diagnosis
p83/100	80/100	Protoplasmic cylinder or flagellum-associated ?	+++
Hsp90		heat shock proteins role in iron metabolism	
Oms66	66	Membrane-associated porin	+
ErpB	60		no
OppA	59	Oligopeptide permease homolog A	
FlaB	41	flagellin	+
BmpA	39	Membrane-associated	+++
GAPDH	37	Glyceraldehyde 3-phosphate dehydrogenase	no
OspB	34-36	Outer membrane protein	no
OspA	31-33	Outer membrane protein	+++
OspD	28	Outer membrane protein	no
OspC	21-24	Outer membrane protein	+++
LA7	22	Lipoprotein	no
ErpA	19		no
RevA	17	Outer membrane protein	

3.1.5 Discussion

By definition, the proteome is the totality of all proteins in a genome, or more precisely in two gel analysis the total of all proteins at a particular place under certain environmental conditions (Wasinger et al. 1995). The method of choice for the study of the proteome is two-dimensional polyacrylamide gel electrophoresis (2-DE) (Rabilloud 2002), (Klose 1975; O'Farrell 1975). Complex mixtures of proteins are separated by isoelectric point (pI) and molecular weight (MW). The result is a pattern of dots (spots), representing single protein (Jungblut et al. 1996).
The 2-DE is in their high-resolution version capable of separating (identifying) approximately 5000 different proteins (spots) (Jungblut et al. 1996). This is largely sufficient for prokaryotes (Jungblut 2001), *B. burgdorferi* s.s. having for example 1556 predicted proteins (Casjens et al. 2000).
To visualise only antigenic proteins, two-dimensional blots were revealed with antibodies from patient sera. The resulting spots permit to identify specific antigens (vaccine candidates), their pathogenicity and association with disease (diagnostic markers).
Immunoblots have with regard to the antigen-recognition a limitation: through the separation of proteins using 2-DE using high concentrations of urea, the proteins partially unfold. This destroys the native three-dimensional structure irrevocably for many proteins.
Antibodies are directed not only against sequential but also against conformational epitopes, so not all epitopes can be detected with immunoblots. Here we isolated antigens first under semi-native conditions with an immunoprecipitation (IP) step. The IP is based on the relationship of antibodies with sepharose beads coupled with protein G, which have a high affinity for the Fc fragment of immunoglobulins. An additional advantage of IP is that no restriction occurs in terms of pI and MW precipitable proteins. A series of studies already used IPs successfully in connection with 2-DE (Chang et al. 2001; Houry et al. 1999; Imam-Sghiouar et al. 2002; Stancato, Petricoin 2001).
Another advantage of enrichment with IP is that *Borrelia burgdorferi* s.l.produces excessive amounts of membrane lipoproteins such as OspA. So, when grown *in vitro*, many low or moderately abundant proteins are underrepresented when cell lysates are examined by 2-DE (Schulte-Spechtel et al. 2003). Enrichment with IP advantages soluble proteins and minimizes strikes on gel due to high level of lipoproteins.
To validate the method, immunoblots were performed using the same sera as used to conjugate to the sepharose beads. All spots present on gels (silver staining) were identified on immunoblots, meaning that all proteins have antigenic properties. Separation of spots was better on gel maps, where fewer strikes were present. Coloration of proteins is more homogeneous than immunologic staining of immunoblots, resulting in more exact and proper reference maps.
IP allowed us to have nice reference results, but we have to keep in mind that collected proteins depend on solubilisation and elution buffer. The method has to be strong enough to avoid to have proteins from secondary interaction (solubilisation) and not to strong, but enough to elute highly antigenic proteins (elution).
Many studies were done to investigate proteomes of *Borrelia* using 2-DE (Nowalk et al. 2006a; Norris et al. 1992; Norris 2006), chromatography (Jacobs et al. 2005; Angel et al. 2010), or proteome array (Barbour et al. 2008), but only with *B. burgdorferi* s. s. Maps of all 3 genospecies were elaborated by Jungblut (Jungblut et al. 1999), but the antigenic characteristics were focused on *B. garinii* exclusively.
The general profile between the three species seems similar. Most of the identified spots are present on each map. A few spots are present in one species only, explaining the low numbers of unknown specific spots.

Proteins between 60 and 41 kDa are normaly conserved between species (Wilske et al. 1988), but here 3 specific spots are found for *B. afzelii*. For lower weight it is known that profiles are variables between species, that explain the two species specific spots found for *B. burgdorferi* and the two smaller proteins of *B. afzelii*.

If we consider the genome of the three species, it is known that *B. afzelii* appears to be the more divergent compare to *B. garinii* and *B. burgdorferi* s.s. (Wang et al. 1999). It could explain why we found more species specific spots for *B. afzelii*.

This study serves as antigenic reference maps for each pathogenic species of *Borrelia*. Next step could be to present a universal map of antigenic protein of Borrelia or maps of specific antigens for each genospecies. In the first case, 2DE has to be made with the elution of antigens from columns built with a pool made with each species specific human hyperimmune serum. In the second case, the idea is to pass the elutions of each species through the two other columns to eliminate cross-antigenicities. Proteins of the last method could be directly studied using chromatographic separations coupled with tandem mass spectrometry (Angel et al. 2010).

These references maps were made for the three human pathogenic genospecies of *B. burgdorferi* s.l. and not only with *B. burgdorferi* s.s.. Another point of interest is to work directly with antigens and to obtain antigenic maps in a one step procedure.

3.1.6 Bibliography

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3.2 Analysis of putative extracellular proteins of *Borrelia afzelii* (strain PKo), *B. burgdorferi* (strain B31) and *B. garinii* (strain PBi)

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3.2.1 Introduction

Borrelia burgdorferi sensu lato (s.l.) belongs to the order *Spirochaetales* along with Leptospira and Treponema. The complex *B. burgdorferi* s.l. currently includes three major pathogenic species, *B. burgdorferi* sensu strict (s.s.), *B. garinii* and *B. afzelii*, which cause Lyme borreliosis (LB), transmitted by ticks of the *Ixodes* species (Anderson 1989; Steere et al. 2004; Piesman, Gern 2004). A particular sub-group of *B. garinii* OspA type 4 has been described as *B. bavariensis* and is associated with neurological cases of Lyme borreliosis. Other species not belonging to the Bb complex cause tick- and louse-borne relapsing fever (e.g. *B. recurrentis*, *B. duttonii*).
Bb is an helicoid bacterium with some periplasmic flagella, which mediate the motility and shape of *Borrelia* (Goldstein et al. 1996). Similar to Gram-negative bacteria Bb has an outer membrane surrounding the periplasmic space and an inner cytoplasmic membrane surrounding the cytoplasm. However, no lipopolysaccharide (LPS) is present in the outer membrane of Bb (Barbour, Hayes 1986; Takayama et al. 1987). This labile outer membrane also spontaneously generates extracellular vesicles or blebs during *in vitro* growth (Barbour, Hayes 1986; Radolf et al. 1994; Preac-Mursic et al. 1986).
Bb is an obligate parasite, not able to survive outside its arthropod or vertebrate host. This natural life cycle of *Borrelia* is associated with a relatively small genome with only limited biosynthetic pathways. Based on the genome of the sequences of strain B31 (Casjens et al. 2000; Fraser et al. 1997), *B. burgdorferi* s.s. has an unusual genome structure, consisting of a 0.91 Mb linear chromosome and 21 plasmids (12 linear and 9 circular) that comprise an additional 0.61 Mb.
Potential proteins released by the Gram-negative spirochete bacteria *Borrelia* to the extracellular environment could be responsible to induce protective immunity (in some bacteria) and could be involved in pathogenesis (Kuehn, Kesty 2005; Shoberg, Thomas 1993). Recognition of *Borrelia* secreted proteins by the immune system may lead to early detection of the infection and to a better control of the disease.
While exported proteins are those proteins that cross membranes and keep subsequent covalent attachment to the bacterial cell wall (and possibly released into the culture medium over time), secreted proteins are proteins that are directly released into the culture supernatant and remain not associated with the bacteria.
Prokaryotes have a number of pathways dedicated to the process of protein secretion. In general, these organisms translocate the majority of their secreted proteins via the Sec protein-translocation pathway (Pugsley 1993). A protein is tagged for export by a signal peptide at its N-terminus. Immediately following this signal peptide, there is a characteristic cleavage site recognized by a signal peptidase that cleaves this peptide following the “threading” of the secreted protein through the Sec complex in the cytoplasmic membrane (Song et al. 2009). There are at least 6 different pathways for protein secretion known in Gram-negative bacteria (Thanassi, Hultgren 2000). Four of these pathways release proteins with cleavable amino-terminal signal sequences to the extracellular space in a two-step process that requires the Sec pathway for translocation across the inner membrane. The four pathways are autotransporters, chaperone/usher, type II and type IV secretion. The other pathways are Sec-independent and capable of exporting substrates in one step to the extracellular space. These pathways are type I and type III secretion. The type I pathway secretes proteins directly from the cytoplasm across the outer membrane (Binet et al. 1997; Delepelaire 2004). Type III is activated by bacterial contact with host cells and is capable of translocating anti-host factors into the cytosol of target eukaryotic cells (Hueck 1998). An alternate secretion mechanism, the twin-arginine translocation (Tat) pathway (Santini et al. 1998) is based on the Tat signal peptides

that are similar to Sec signal peptides, but they contain a highly conserved twin-arginine motif.

Various approaches have been used to study secretion and secreted proteins from bacteria.

Analysis of *in vitro* culture filtrates allow to detect putatively secreted proteins, but it represents not exactly the *in vivo* situation (Angel et al. 2010; Tokarz et al. 2004; Meinke et al. 2004).

A second approach for studying secreted proteins utilizes comparative genomics and is readily applicable to study *Borrelia* secreted proteins. For example, genes known to encode secreted proteins in *Treponema pallidum* can be used to search the genome of *B. burgdorferi* s.l. for sequences with strong DNA sequence similarities. If these regions of similarity appear to encode genes with potential for secretion, then the *B. burgdorferi* s.l. genes can be analysed.

Bioinformatics can also be used to support comparative genomic studies by providing tools for predicting properties of proteins, including cellular location. Computer algorithms have been developed that predict whether a protein is located within the cytoplasm, the cell membrane or exported from the cell. Methods to predict subcellular localization are based on N-terminal sequence motifs directing proteins to the secretory pathway and other sequence properties.

In this study, two different programs were used to identify proteins having secretory signal peptides but lacking additional membrane attachment domains: PrediSi was used to predict the presence and location of signal peptide cleavage sites in amino acid sequences and Transmembrane Hidden Markov Model (TMHMM) was used to predict the location and orientation of transmembrane helices in protein sequences (Hiller et al. 2004; Krogh et al. 2001). Additional algorithms were used to eliminate protein containing lipoprotein signal peptides and to detect false cellular localization. Finally comparative genomic was used between *Borrelia* genospecies to identify species specific candidates.

The proteome of *B. afzelii* and *B. garinii* contains 1262 protein sequences and 1556 for B. *burgdorferi*. At the end of the analysis 3 candidates were found for *B. afzelii*, 7 for *B. burgdorferi* and 2 for *B. garinii*. The method described here is fast, easy and freely available from internet.

3.2.2 Material and methods

Proteomes of *B. afzelii* (strain PKo) (Glöckner et al. 2006), *B. burgdorferi* s.s. (strain ATCC 35210 / B31 / CIP 102532 / DSM 4680) (Casjens et al. 2000); (Fraser et al. 1997) and *B. garinii* (strain PBi) (Glöckner et al. 2004) were obtained from Expasy Proteomics Server from the Swiss Institute of Bioinformatics (SIB) (Gasteiger et al. 2003) (http://us.expasy.org/) that contains entries from UniProtKB/Swiss-Prot and UniProtKB/TrEMBL.
Whole proteome datasets were first analyzed with JvirGel standalone version (Hiller et al. 2003) that allows prediction and visualization of secretomes and membrane proteomes. Jvirgel use the PrediSi (PREDIction of SIgnal peptides) algorithm (Hiller et al. 2004) to predict signal peptide sequences and their cleavage positions and TMHMM 2.0 algorithm (Krogh et al. 2001) to predict transmembrane helices. Only predicted proteins with a signal peptide sequence, a cleavage site (prob > 0.5) and with ≤1 transmembrane domains were kept. ProtCompB version 3, a program for identification of sub-cellular localization, was used to select secreted proteins. LipoP (Juncker et al. 2003) was used to discriminate between lipoprotein signal peptides and other signal peptides. Only “unknown” candidates were kept, and cross homologues between species were dismissed.

To characterize the putative secreted proteins different programs were used: BLAST (Basic Local Alignment Search Tool) from UniProt; the Pfam database (http://pfam.sanger.ac.uk/) (Finn et al. 2008); Program using the method of Kolaskar and Tongaonkar (Kolaskar, Tongaonkar 1990) to predict antigen determinants ; MineBlast (a literature presentation service supporting protein annotation by data mining of BLAST results) (Dieterich et al. 2005).

3.2.3 Results

The analysis was started with a total of 1262 *B. burgdorferi* sensu stricto, 1556 *B. afzelii* respectively 1262 *B. garinii* predicted proteins, for, and from each complete proteome.
Two different programs were used to identify proteins having secretory signal peptides, but lacking additional membrane attachment domains: PrediSi used to predict the presence and location of signal peptide cleavage sites in amino acid sequences permitted to isolate 87, 104 and 84 proteins with a mean S score ≥ 0.5 (Figure 1). JCaMelix, which excluded proteins containing more than one f transmembrane helice domain in the protein sequences, resulted in 63, 79, and 59 sequences (Figure 1).
To discriminate between secreted and exported proteins, ProtCompB allowed identifying 17, 32 and 20 sequences potentially encoding extracellular proteins. To focus on extracellular proteins, a selection was made on proteins that pass through the outer membrane. To exclude proteins anchored on the surface of the outer membrane, the algorithm LipoP reduced the candidates to 12, 21 and 10 proteins. The selection was so far limited to 6, 11 and 4 unknown proteins and finally only 3, 7 and respectively 2 proteins were retained that did not show cross-homologies (Table 1).
These secreted proteins represent 1,4% for *B. afzelii*, 2,1% for *B. burgdorferi* s.s and 1,6% for *B. garinii* of the corresponding proteome and three are coded by plasmids.
Functional analysis made with Pfam database (Table 2) gives 2 predicted proteins with no annotation; 3 contain only Pfam-B domains (structural units); 1 with a repeat (short unit which is unstable in isolation but forms a stable structure when multiple copies are present) additional to Pfam-B domain; 2 have defined domains and 4 have families (collection of related protein regions). Domains are DUF329, Laminin_G_3 and fn3, and family names are DUF2147 and Exonuc_VII_S.
Signal peptides were predicted for all candidates using Pfam database, and no transmembrane domain was found.

3.2.4 Figures and tables

	BORAP	BORBU	BORGA
Expasy	1262 (100%)	1556 (100%)	1262 (100%)
PrediSi	87 (6.9%)	104 (6.7%)	84 (6.7%)
JCaMelix	63 (5.0%)	79 (5.1%)	59 (4.7%)
ProtCompB	17 (1.4%)	32 (2.1%)	20 (1.6%)
LipoP	12 (1.0%)	21 (1.4%)	10 (0.8%)
Hypothetical	6 (0.5%)	11 (0.7%)	4 (0.3%)
w/o Cross-homologues	3 (0.2%)	7 (0.5%)	2 (0.2%)

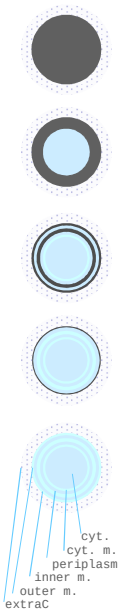
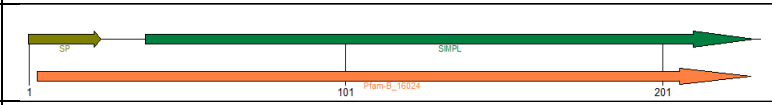
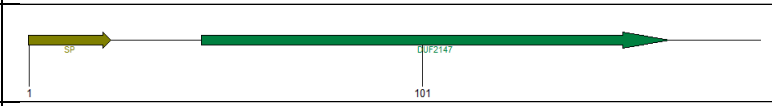
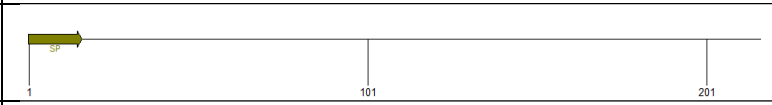
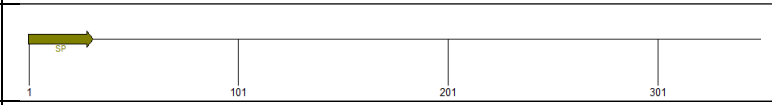
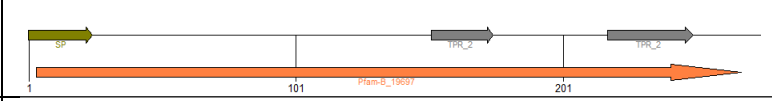
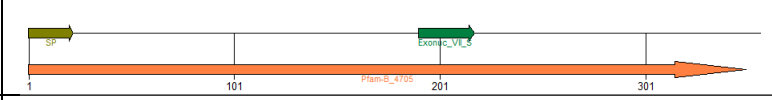
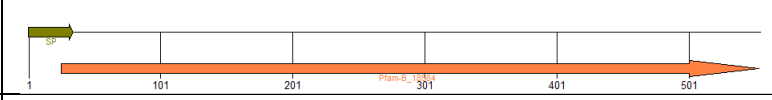
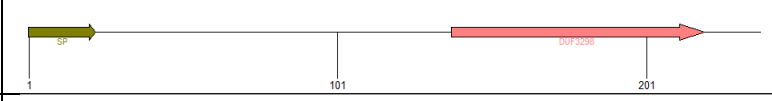
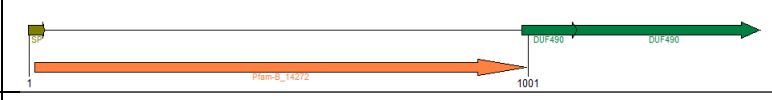
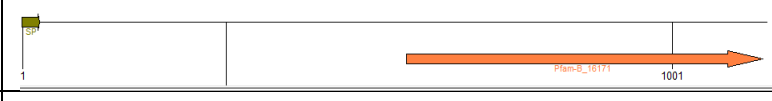
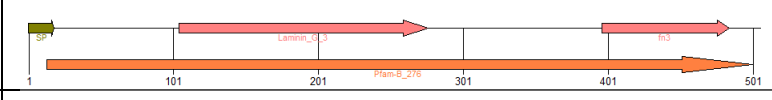
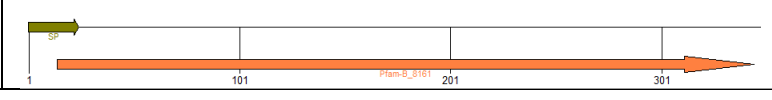


Figure 1. Bioinformatic strategy to select potential secreted proteins from *B. afzelii* (BORAP), *B. burgdorferi* (BORBU) and *B. garinii* (BORGA) proteome. On the left are schematic representations of protein localization in Gram- bacteria (cyt: cytoplasm; m: membrane; extraC: extracellular). Proteins localisations are represented in black.

Table 1. List of candidates. Description of the 3 candidates for *B .afzelii* (BORAP); 7 candidates for *B. burgdorferi* (BORBU) and 2 for *B. garinii* (BORGA). Proteins are described with name and molecular weight (kDa), and genes with name, percentage of identity with the two other genospecies and gene localisation in the genome.

	Protein		Gene		
	Name	**kDa**	**Name**	**%Identity**	**Localisation**
BORAP	Q0SLI8	26.6	BAPKO_3034	93.94 BGP175 56.03 BB_0223	Plasmid lp34
	Q0SM78	21.3	BAPKO_0825	84.95 BG0800 87.10 BB_0776	Chromosome
	Q0SNG6	24.0	BAPKO_0355	95.83 BG0347 94.44 BB_0346	Chromosome
BORBU	O51059	39.7	BB_0028	89.97 BG0028 90.26 BAPKO_0027	Chromosome
	O50778	31.2	BB_J23	42.80 BAPKO_6004	Plasmid lp38
	O50789	39.7	BB_J34	63.51 BAPKO_3540	Plasmid lp38
	O51183	66.3	BB_0161	95.50 BG0159 97.20 BAPKO_0162	Chromosome
	O51416	28.2	BB_0460	80.69 BG0471 82.55 BAPKO_0486	Chromosome
	O51734	168.8	BB_0794	90.18 BG0820 91.10 BAPKO_0847	Chromosome
	O51778	134.4	BB_0838	90.64 BG0863 91.25 BAPKO_0891	Chromosome
BORGA	Q661G1	58.15	BG0467	96.63 BAPKO_0482 95.45 BB_0458	Chromosome
	Q661K0	39.47	BG0421	90.86 BAPKO_0434 88.86 BB_0418	Chromosome

Table 2. Functional properties of candidates

Seq id	Start - end	hmm name	Type	Structure	Liteature
Q0SLI8	4-229 38-230	Pfam-B_16024 SIMPL	Pfam-B Family		(Vig et al. 2001) (Pfeuffer et al. 2000)
Q0SM78	45-164	DUF2147	Family		
Q0SNG6					
O51059					
O50778	4-268 152-175 218-250	Pfam-B_19697 TPR_2 TPR_2	Pfam-B Repeat Repeat		(Lamb et al. 1995) (Das et al. 1998)
O50789	1-350 191-218	Pfam-B_4705 Exonuc_VII_S	Pfam-B Family		(Vales et al. 1982)
O51183	26-554	Pfam-B_18584	Pfam-B		
O51416	138-220	DUF3298	Domain		(Wang et al. 2007)
O51734	14-998 989-1100 1086-1465	Pfam-B_14272 DUF490 DUF490	Pfam-B Family Family		
O51778	593-1141	Pfam-B_16171	Pfam-B		
Q661G1	14-501 105-277 397-485	Pfam-B_276 Laminin_G_3 fn3	Pfam-B Domain Domain		(Bazan 1990) (Little et al. 1994) (Kornblihtt et al. 1985)
Q661K0	15-345	Pfam-B_8161	Pfam-B		
: signal peptide ; :Pfam-B ; : Domain ; : Family ; : Repeat					

3.2.5 Discussion

All tools described here are directly available online on the web. The package JVirGel allows working with whole proteomes rapidly (Hiller et al. 2004). It facilitates selection of all proteins containing the faculty to be exported, with prediction of Sec-dependent signal peptides (PrediSi) and a-helical membrane helices (JCaMelix). This first step allows us to pursue the analysis with only 5% of each predicted proteome. This lets us use the algorithms for cellular location, that are enabled for restricted for light-volume use and are limited in numbers per day (ProtCompB, LipoP).
The choice to exclude proteins with a name, in opposition of "putative uncharacterized proteins", is to work with a reduced number of candidates and to optimize the chance to find new good antigenic secreted proteins. Hypothetical proteins (HPs) are predicted proteins from nucleic acid sequences that have not been shown to exist by experimental protein characterization. New HPs may be serving as markers and pharmacological targets. This step legitimates our procedures, because all proteins with a name are known as exposed on outer surface and having antigenic properties. The names of these proteins are: P26, P35, p66 (Ojaimi et al. 2005); (Goettner et al. 2005); (Wilske 2005); (Valentine-Thon et al. 2007); (Glatz et al. 2008), p83/100 (Honegr et al. 2001; Guerra et al. 2000), BapA (Miller, Stevenson 2003), RlpA, revA (Brissette et al. 2010), Sensor protein (Rogers et al. 2009), Basic membrane protein (Nowalk et al. 2006). Proteins like p26, p35, p66 and p83/100, are well described and used in diagnosic.
The Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990; Altschul et al. 1997) is the most frequently used tool for calculating sequence similarity. It was used to eliminate candidates with homologues in the other genospecies. Functional analysis of HPs was made by domain analysis using Pfam databases. Domains are evolutionary units and most proteins consist of one or more domains.
A structural domain may be defined as one or several segments of a polypeptide that forms a compact and stable structure with an associated hydrophobic core and that can fold and function independently of other parts of the sequence. Structural domains are often associated with identifiable cellular/biochemical functions (Orengo et al. 1999).
Motive analysis is an obligatory step in the identification and characterisation of HPs. A series of signature databases are publically available and are often combined with sequence cluster and domain databases such as Pfam (Finn et al. 2008).
Pfam is a large curated collection of protein multiple sequence alignments and profile hidden Markov models. Predictions of non-domain regions are also included and contain active site residue mark up (Bateman et al. 2002). Pfam was useful for indication of domains in six out of twelve HPs (Table 2). In addition, each family has associated annotation, literature references, and links to other databases.
SIMPL family was found for Q0SLI8, it function is unknown in bacteria, but it is thought to be located in the periplasm or outer membrane (Pfeuffer et al. 2000). Fn3 (Fibronectin type III), found in Q661G1, is an evolutionary conserved protein domain that is found in a wide variety of extracellular proteins (Little et al. 1994). These informations give us tracks to confirm or to follow investigation with these candidates
In order to confirm bioinformatic data and validate protein secretion, *PhoA* fusion of the different *Borrelia* corresponding genes could be used to confirm if encoding genes exported and secreted proteins in *E. coli* (Giladi et al. 1993). Recombinant proteins could then be purified to check antigenic properties.

3.2.6 Bibliography

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3.3 Characterization of the protein recognized by monoclonal antibody D6 specific for *Borrelia garinii* isolates

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3.3.1 Abstract

We previously reported on the D6 monoclonal antibody reactive with a 12 kDa protein from Borrelia garinii isolates but not from other Borrelia species. The objective of this study was to identify the D6-reacting protein. Protein purification was performed using preparative tube gel electrophoresis followed by SDS-page of a *B. garinii* isolate protein extract. The D6-reactive protein band was submitted to LC-MS/MS sequence analysis. This analysis revelead three polypeptide sequences analogous to BB0477 (30S ribosomal protein S10), BB0061 (thioredoxine A), and BB0390 (50 S ribosomal protein L7/L12). Three PCR assays were developed to amplify and sequence the corresponding ortholog genes from 28 isolates of *B. burgdorferi* sensu lato in an attempt to identify potential epitopes based on their presence only in *B. garinii* isolates. Two polypeptides from thioredoxin A and one from 50S ribosomal protein were thus identified as potential epitopes. Expression in *E.coli* PQR9 followed by by immunoblotting identified residues 7-12 of thioredoxin A as the D6 mab epitope. This was confirmed by competition experiments with a synthetic peptide.

3.3.2 Introduction

Borrelia burgdorferi is responsible for Lyme borreliosis (LB) throughout the temperate regions of Northern hemisphere. *Ixodes ricinus* is the principal vector of *B. burgdorferi* to humans in Europe. Since the discovery of *Borrelia burgdorferi*, several species have been identified. Currently, *I. ricinus* is known to contain *B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, *B. valaisiana, B. lusitaniae,.B. spielmanii* and *B. garinii* OspA type 4 referred as *B. bavariensis* [1, 2].
In 1992, we produced monoclonal antibodies and one of them named D6 allowed to classify different Borrelia isolates [3]. All isolates that reacted with this antibody yielded specific OspA profiles. The resulting classification was later confirmed at the genetic level [4-7]. It also led to the discovery of a new group of bacteria called VS116, later named *B. valaisiana* [8]. Interestingly, mab D6 has been shown to react with an estimated 12 kDa protein in *B. garinii* isolates but not in other species. One exception was observed with a *B. garinii* isolate (named 935T) from Korea unreactive with mab D6 [9].
LB is a multistage condition which starts with erythema migrans, a typical skin lesion at the site of tick bite. In absence of antibiotic treatment, Borrelia can disseminate from the bite site and establish persistent or chronic infections [10]. Detailed examinations have shown that different Borrelia species tend to be more frequently associated with specific chronic manifestations of the disease. Thus, *B. burgdorferi* ss is preferentially found with Lyme arthritis, *B. garinii* and the newly described *B. bavariensis* with neuroborreliosis, and *B. afzelii* with Acrodermatitis chronica atrophicans. Although pathogenicity of *B. valaisiana*, *B. spielmanii* and *B. lusitaniae* is established [11-14], human infections are far less frequent than those linked with *B. burgdorferi* ss, *B. garinii, B. bavariensis* and *B. afzelii*.
B. burgdorferi has an unusual genome, unlike that of any other characterized bacterium. It is constituted of a linear chromosome and of a large number of plasmids essential for its viability and virulence [15-17]. In 1997 the genome of *B. burgdorferi* strain B31 was published [16] and completed in 2000 [15]. Since then whole genome sequences of many Borrelia species and isolates [18-23] are available. Knowledge of these complete genomic sequences has led to a number of studies, such as gene expression profiles in response to modifications in the environment [24-26], or differential expression of particular genes in cultures at different temperatures and pHs [27, 28]. Gene expression in Borrelia was found to be influenced by CO_2 concentrations [29]. Sequence information has also made it possible to identify, characterize and compare proteins in different Borrelia species [30] and even related bacteria, such as *Treponema* species for the *tpr* gene family [31]. The search for antigenic proteins has been greatly stimulated either for new serologic markers or vaccine candidates [32-34]. In this context, our objective was to identify the protein reactive with mab D6 and to search for species specific domain with potential applications in specific PCR or antigens for serology.

3.3.3 Material and methods

Borrelia isolates and antigen production

The following Borrelia isolates (N=28) were used in this study:
B. burgdorferi ss: B31 (ref. strain), Geho, IP1, VS215, VS123
B. afzelii : VS461 (ref. strain), ACA1, A26s, A38s, Bo23,
B. garinii: 20047 (ref. strain), 387, 935T, A19s, A77c, FAR01, G25, HP3, M63, NT29, VS102, VS BP, VS BM,
B. valaisiana : VS116 (ref. strain), UK, AG1, FRANK, F_10-08-94
The data concerning the biological and geographical origin of these isolates have been described earlier [9].
For antigen production, they were grown in BSK II medium at 34°C and harvested during the late log phase by centrifugation at 10'000g for 10 min at room temperature. The bacterial pellet was washed twice in phosphate-buffered-saline containing 5mM $MgCl_2$**,** and finally resuspended in distilled water and adjusted to 2 mg protein / ml as determined by turbidimetry in an Integra 500 apparatus. These preparations were stored at -80°C until use.

Protein purification and analysis

A suspension of washed *B. garinii* isolate (VS 102), was dissolved (1:1) in 200 µl protein sample buffer containing sodium dodecyl sulfate to final concentration of 0.6%, and 50 mM dithiothreitol. The sample was boiled for 5 minutes and the proteins separated by 12% T (37.5:1) SDS-polyacrylamide preparative tube gel electrophoresis (mini Prep cell, BioRad, Hercules, CA USA). Running conditions were as follows: voltage, 200V constant, current, 20 mA, and approximate total run time, 6 hours. The separated proteins migrating off the bottom of the gel were collected in 250 µl fractions. Proteins from each fraction were analyzed by Western blotting after separation in a 12% (37.5:1) SDS-PAGE and transfer to a PVDF membrane (Immobilon, Millipore, Betford, Mass. USA) [9]. Immunoblotting was performed with mab D6 diluted 1:200 for the first incubation, followed by an anti-mouse IgM conjugated to alkaline-phosphatase diluted 1:1000 (Sigma, St-Louis, MO, USA) and subsequently by the substrate BCIP/NBT (KPL, Gaithersburg, MD, USA). In order to get sufficient amount of protein this preparative procedure had to be repeated 5 times. All the D6-reactive fractions were again pooled, concentrated using a concentrator evaporator (Jouan, Saint-Herblain, France) and submitted to 16% T (37.5:1) polyacrylamide preparative tube gel electrophoresis. Because the concentrated sample was heavily charged with SDS and in order to improve migration, SDS was omitted in the sample and running buffers. The eluted proteins were collected as described above. Proteins from each fraction were then submitted again to a 12% T (37.5:1) SDS-PAGE and analyzed by Western blotting as described above. D6 reactive fractions were pooled, concentrated as described above and separated one final time in a Tris-tricine 16% T (37.5:1) polyacrylamide gel without SDS (voltage:150V constant, current: 27 mA, run time: 45 min). The gel was stained with unfixing Coomassie blue. The unique visible protein band, with an apparent molecular mass of 12kDa, was excised from the gel using a scalpel and prepared for LC-MS/MS sequence analysis as described previously [35].

PCR and DNA sequencing

The 3 polypeptide sequences determined by LC-MS/MS were reverse translated to yield degenerate DNA sequences. These sequences were aligned against the known complete genome sequence of *B. burgdorferi* to identify its corresponding ortholog genes. PCR primers were then designed from the respective upstream and downstream genes of *B. burgdorferi*, and used to produce specific amplicons for nucleotide sequence determination. For example,

primers for the ortholog gene of BB0390, were designed at the 3'end of BB0389 (the upstream gene) and at the 5' end of BB0391 (the downstream gene) (Table 1). PCR was performed with a Biometra Tgradient apparatus (Biometra, Göttingen, Germany) as follows: 95°C for 15', followed by 45 cycles at 95°C for 1 min, 44-55°C (Table 1) for 1min and extension for 1 min at 72°C. Hotstart mastermix (Qiagen, Hilden, Germany) was used for DNA amplification. Amplicons were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining. DNA sequencing was performed using BigDye 1.1 chemistry (Applied Biosystems), ABI310 or 3130 instruments (Applied Biosystems) and appropriate PCR primers.

Bacterial strains and plasmids

Borrelia garinii VS102 was grown in MKP medium [36] at 34°C. The M15 (pREP4) Escherichia coli was grown in Luria-Bertani medium at 37°C. For selection of transformants, Luria-Bertani medium was supplemented with 100 µg/ml ampicillin and 25 µg/ml kanamycin as required.

DNA extraction

Extraction of DNA from Borrelia strain was performed as described previously [9]. Plasmid DNA was extracted from M15 (pREP4) with the QIAGEN mini-prep kit according to the manufacturer's instructions (QIAGEN, Hilden,Germany).

Plasmid constructs.

For cloning/sequencing, genes were amplified by PCR with Thermo-Start PCR Master Mix (ABgene). The primers used for amplification of the gene BG0060 and BG0391 were BG0060_5'_SalI (5'-ACGCGTCGACGCTGTTTCTTTAACCAAAGAAGA) and BG0060_3'_PstI (5'-AACTGCAGCTAAAAACCAAAAAAATCCTTAATTA) (underlined sequences represent SalI and PstI restriction sites, respectively), and BG0391_5'_SalI (5'-ACGCGTCGACGCACTAAGTAAAGAAGATATTTTAAC) and BG0391_3'_PstI (5'-AACTGCAGTTATTTAACTTCAACTTTTGCGCCAA).

Amplified products were digested with the appropriate restriction enzymes, ligated to linearized pQE-9 and transformed into E. coli M15(pREP4) as indicated in the QIAexpressionist manual Qiagen. The plasmid sequence inserts were confirmed by DNA sequencing.

Protein expression and purification

BG0060 (*TrxA*) and BG0391 (*rpIL*) were expressed as hexahistidyl fusion proteins and purified with nickel affinity resin. Briefly, E. coli M15(pREP4) cells were grown to mid-log phase and expression of the recombinant protein induced with a final concentration of isopropyl-ß-D-thiogalactopyranoside of 1 mM. After 4 hours cells were harvested by centrifugation (4,000 x g for 20 min) and then lysed in 8 M urea buffer (8 M urea, 0.01 M Tris, 0.1 M NaH2PO4, pH 8.0) with gentle rocking for 1 h. Cell debris was pelleted by centrifugation at 15,000 x g for 30 min. 0.25 volume of a 50% Ni-nitrilotriacetic acid bead slurry (QIAGEN) was added to the supernatant, and mixed gently for 1 h. Bead-bound proteins were collected (15,000 x g for 10 sec) and washed twice with 8 M urea buffer (pH 6.3). Loading buffer was directly added to beads and the suspension was heated 5 min at 95°C to release the proteins. Proteins were then analyzed by SDS-page and western blot, as described above.

Peptide synthesis and absorption of reactivity of the mab D6

Peptide (NH2-Asn-Asn-Lys-Tyr-Asp-Phe-Val-Lys-Ala-Val-Phe-Asp-Glu-Lys-Thr-COOH)) including the 7-12 residues (underlined) of trxA protein was ordered at Polypeptides (Strasbourg, France). It was dissolved in a small volume of distilled water and adjusted to 1 mg/ml with TBS (Tris buffer saline, 0.1M, pH8.3) containing 1% gelatin and 0.05% Tween-20. The mab D6 was diluted 1/100 (condition A) or 1/200 (condition B) in this mixture and incubated overnight at 4 °C. D6 incubated in normal TBS 1%gelatin and 0.05% Tween-20 at the same dilutions was used as positive control. Western blot strips of isolates (*B. garinii*) VS 102 were used for immunoblot analysis as described above (condition A).
Better conditions were obtained in diluting peptides in TBS alone for overnight incubation at 4°C with the mab D6 diluted 1/200. Gelatine and Tween-20 (1% and 0.05% final concentrations respectively) were added just before immunoblot analysis (condition B).

Phylogenetic analysis

After alignment with CLUSTAL W [37], nucleotide sequences were edited to match the shortest sequence. MEGA 2.1 software [38] was then used to infer neighbor-joining (Kimura-corrected p-distance), minimum evolution with close neighbor-interchange with a search level of 1 (Kimura-corrected p-distance), as well as parsimony trees (standard parsimony).

Accession Numbers

Sequences of each isolate specific gene corresponding to BB0061 were deposited in Genbank under accession numbers DQ986202 to DQ986223, sequences of each isolate specific gene corresponding to BB0390 were deposited under accession numbers AY737686 to AY737712, partial sequences of each isolate specific gene corresponding to BB0477 were deposited under accession numbers EU363467 to363479.

3.3.4 Results

Protein and DNA sequencing

Three different proteins were identified by LC-MS/MS sequencing of the *B. garinii* purified protein band (see Materials and Methods): thioredoxin (trxA), 50 S ribosomal protein L7/L12 (rpIL) and 30 S ribosomal protein S10 (rpsJ). These proteins probably failed to separate owing to their closely related molecular weight during the sequential purification steps. PCR amplification products of the corresponding 3 ortholog genes were obtained for each of the 13 isolates that were initially studied (3 ***B. burgdorferi ss:*** B31, VS123, VS215, 3 *B. afzelii*: A26s, ACA1, VS461, 4 *B. garinii*: 935T, 387, NT29, VS102, 3 *B. valaisiana*: UK, VS116, AG1). Sequence were performed and compared (see below).

Putative D6 epitope

To identify the D6 epitope, two assumptions were made. Firstly, we assumed that it would be located within a conserved polypeptide sequence of one of the three identified proteins. Secondly, it ought to be absent from the orthologs not recognized by D6, including those from *B. garinii* isolate 935T. Indeed, this particular isolate from Korea contains a D6-unreactive protein with a similar electrophoretic mobility and divergent peptide sequences as is the case for the other *B. burgdorferi* species not recognized by D6. The D6-reactive candidate proteins were thus tentatively identified after an alignment of in-frame translations of the obtained amplicon sequences and the identification of a conserved motif among all *B. garinii* isolates with the exception of *B. garinii* isolate 935T.

30S ribosomal protein S10 (rpsJ)

Alignment of the 13 partial DNA sequences of the gene corresponding to *rpsJ* revealed a highly stable gene with only 1-2% divergence (Fig 1). In-frame deduced amino acid sequences were almost identical within all isolates. Only one amino acid substitution out of 103 amino acids was observed in one isolate of *B. valaisiana* (VS 116). The estimated molecular mass of this protein is 11.7 kDa.

Thioredoxin A (trxA)

The sequences of the gene identified as *trxA* obtained from the 13 initial isolates as described above were much more variable than *rpsJ*. The complete deduced protein is composed of 117 amino acids with an estimated molecular mass of 13.4 kDa . Protein alignment revealed several regions with species-specific amino acid substitutions, mainly between residues 7 to 28 and 74 to 81. In order to increase the discriminating power of the phylogenetic analysis of these variable regions, 9 additional isolates of *B. garinii* were analyzed (20047 (ref. strain), A19s, A77c, FAR01, G25, HP3, M63, VS BP, VS BM, see Fig 2). Because the sequences in the two regions spanning amino acids 7-12 and 74-81 were different in 935T and in isolates belonging to Borrelia species other than *B. garinii*, they were considered as likely candidates as the mab D6 epitope. It should also be noted that the phylogenetic analysis of the complete nucleotide sequences (data not shown) as well as that of the amino acid sequences generated similar robust trees with significant bootstrap values (Fig 3). However, in the second region (residues 74-81) a high variability was also observed among the D6-reactive *B. garinii* isolates. It was thus unlikely that this region was in fact the epitope recognized by D6. The other candidate region (residues 7-12) displayed only one to two amino acid substitutions in the 13 *B. garinii* isolates. The KEDFVA sequence was present in 11 *B. garinii* isolates, KEDFIA in 1 isolate (FAR01) and KENFVA in isolate 935T (the non reactive control). The

valine (V) to isoleucine (I) substitution in FAR01 isolate is conservative: these 2 amino acids have similar, hydrophobic-aliphatic properties and structures. In contrast, the substitution of aspartate (D) to asparagin (N) is clearly significant. N is an uncharged amino acid with a potential of conformation changes (N is present in isolate 935T, the non reactive isolate) whereas D is an acidic amino acid negatively charged at neutral pH with strong electrostatic interactions (D is present in all other *B. garinii* isolates). These residues 7-12 were highly suspected to correspond to the epitope recognized by the D6 mab.

50 S ribosomal protein L7/L12 (rpIL)

In frame deduced amino acid sequences were aligned and compared between 28 isolates belonging to the four Borrelia species (Fig 4). The complete protein contains 124 amino acids (123 aa for one *B. valaisiana* isolate, FRANK) with an estimated molecular mass of 12.9 kDa. Six amino acids (A, E, K, V, G and S) represent 60 to 64.5% of the total amino acid composition of the protein with specific regions that are species-dependent. Phylogenetic analysis of nucleotide sequences generated robust trees with significant bootstrap values (Fig 5). The topology was quite similar to that of the neighbor-joining tree inferred from the amino-acid sequences and from both minimum evolution and parsimony trees (data not shown). Intriguingly, the four *B. garinii* isolated from human cerebrospinal fluid (VS BP, VS BM, 387, A77s) were restricted to a small sub-cluster. One tick isolate from Germany, G25, was also found in this cluster.

A high variability was observed within the region spanning residues 34 to 50. The amino acid sequence of this particular region displayed Borrelia species-specific characteristics (Fig 5). This segment of the protein comprises only 5 amino acids: alanine (A), glycine (G), valine (V), serine (S), threonine (T), with a sixth one, isoleucine (I), encountered in all B. garinii isolates and in one *B. afzelii* isolate. The central part of this segment (residues 40-43) is strikingly composed of stretches of two to four glycine residues. The presence of alanine, valine and isoleucine confers to this region a highly hydrophobic nature. This region also differed in the 935T isolate in comparison with all the other *B. garinii* isolates, making residues 34-39 a potential candidate as the mab D6 epitope.

Recombinant proteins, reactivity with mab D6 and epitope mapping

TrxA and rpIL proteins expressed in *E. coli* PQE9 were assessed by SDS-page and Coomassie blue staining. Due to difficulties in purifying these recombinant proteins, we used crude lysates of cells expressing them for immunoblotting. D6 was was weakly reactive with expressed trxA (dilutions ½ and 1/20) while no reaction was observed with recombinant rpIL or with an extract of cells transformed with the pQE9 vector alone (Fig 6). Competition of D6 was performed with a synthetic peptide whose composition corresponded to sequences of the region spanning from amino acid 6 to 20 of trxA protein of VS102 isolate (*B. garinii)*. A clear reduction (50-63%) of the mab D6 reactivity was observed as compared with positive control strip (condition A). Finally total extinction was obtained with trxA peptide with adjusted incubation (conditions B) (Fig 7).

3.3.5 Figures and tables

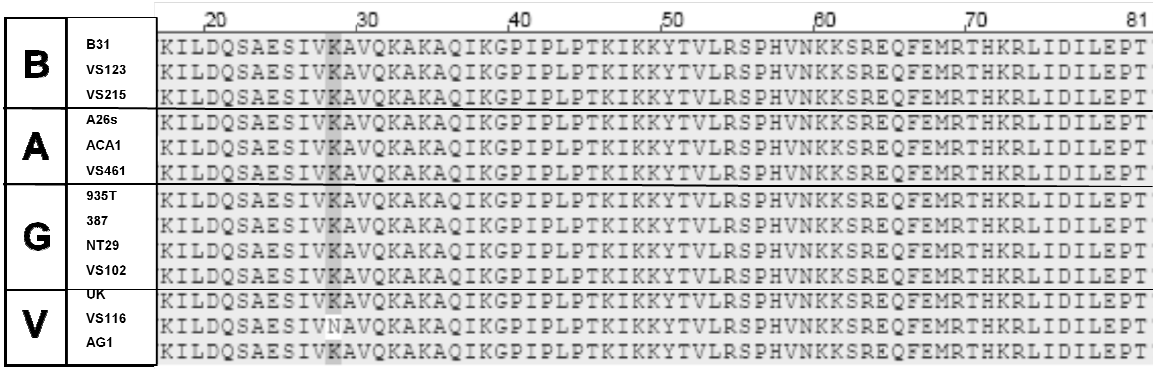

Fig 1: Amino acid sequence alignments of the rpsJ partial sequences
B: *B. burgdorferi* sensu stricto, A: *B. afzelii*, G: *B. garinii*, V: *B valaisiana* followed by the name of each isolate (N=13).

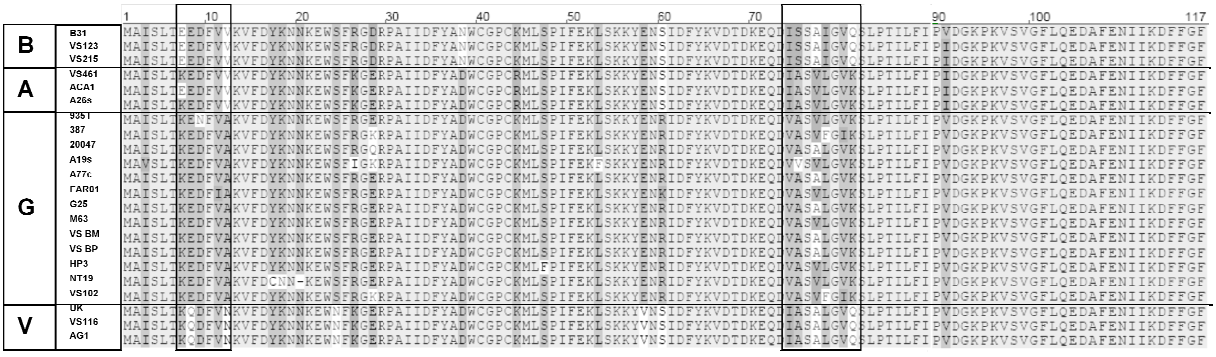

Fig 2: Amino acid sequence alignments of the trxA whole sequence
B: *B. burgdorferi* sensu stricto, A: *B. afzelii*, G: *B. garinii*, V: *B valaisiana* followed by the name of each isolate (N=22). Frame indicates area of interest.

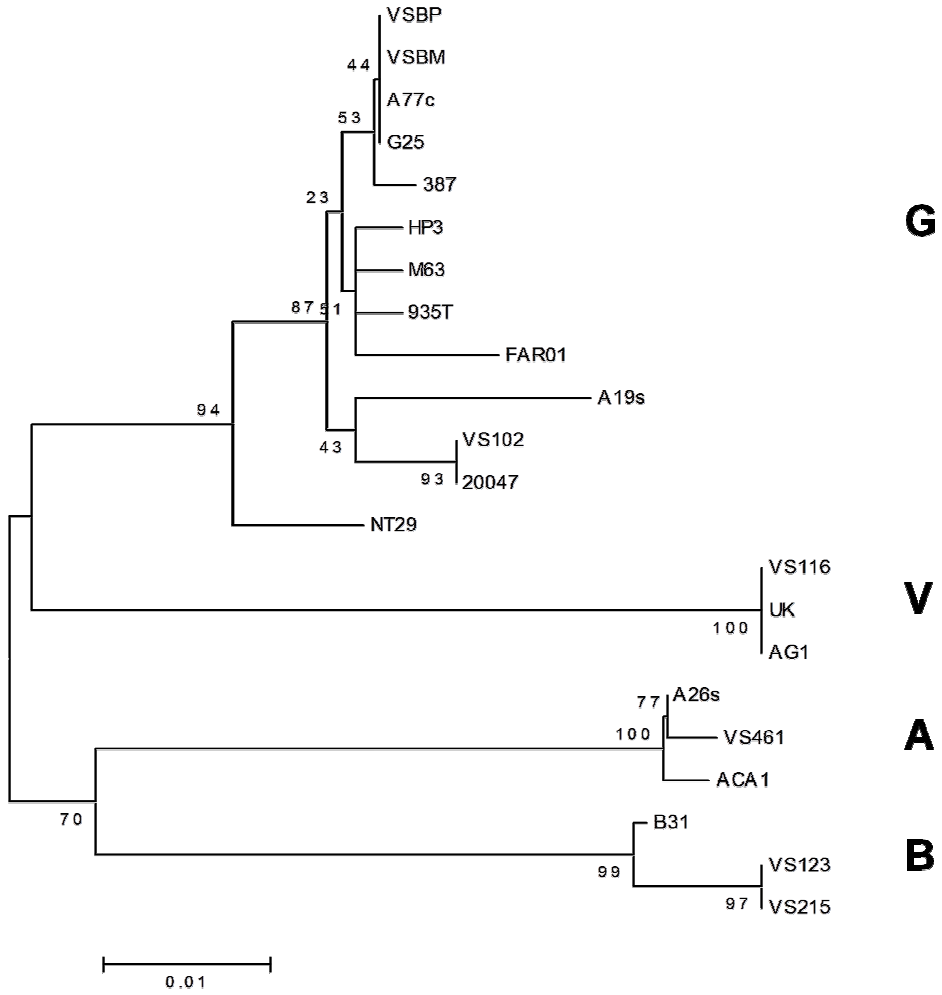

Fig 3: Phylogenetic analysis of the whole amino acid sequences of the trxA infer from 22 Borrelia isolates using neighbor-joining (Kimura-corrected p-distance). Bootstraps values are shown at each nodes.
B: *B. burgdorferi* sensu stricto, A: *B. afzelii*, G: *B. garinii*, V: *B valaisiana*

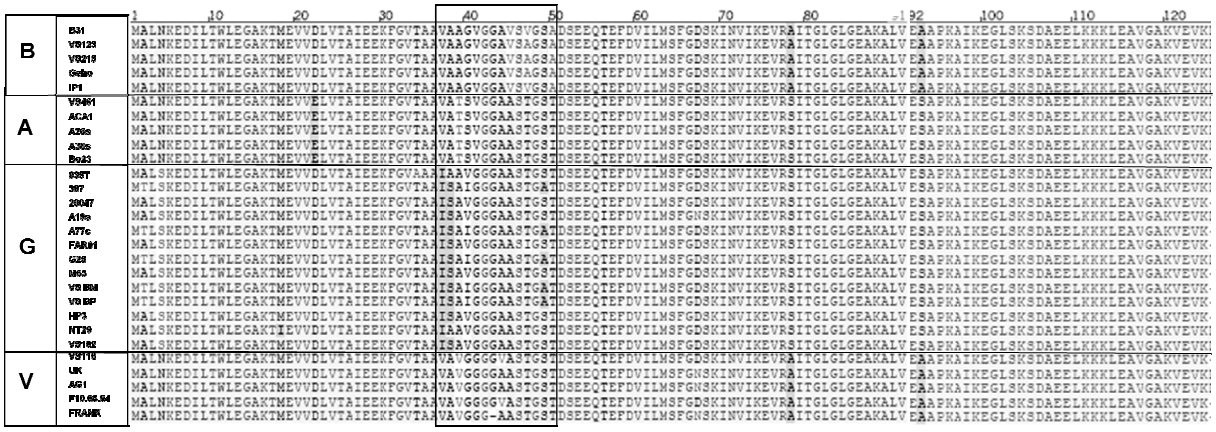

Fig 4: Amino acid sequence alignments () of the rpIL whole sequence
B: *B. burgdorferi* sensu stricto, A: *B. afzelii*, G: *B. garinii*, V: *B valaisiana* followed by the name of each isolate (N=28). Frame indicates area of interest.

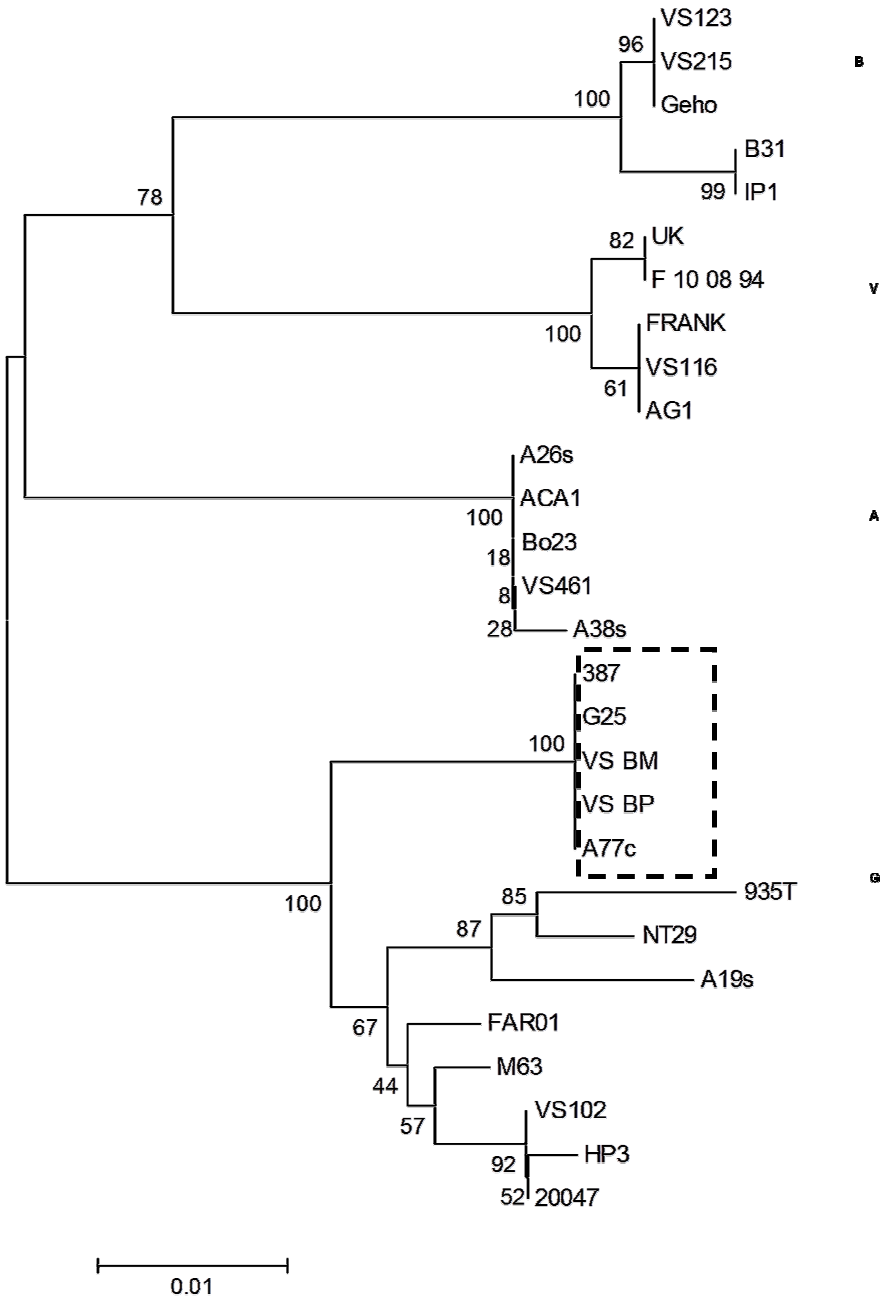

Fig 5: Phylogenetic analysis of the whole amino acid sequences of the 50S ribosomal protein L7/L12 ortholog of *B. burgdorferi* (Bb0390) infer from 28 Borrelia isolates using neighbor-joining (Kimura-corrected p-distance). Bootstrap values are shown at each node.
B: *B. burgdorferi* sensu stricto, A: *B. afzelii*, G: *B. garinii*, V: *B valaisiana.* Frame indicates area of interest.

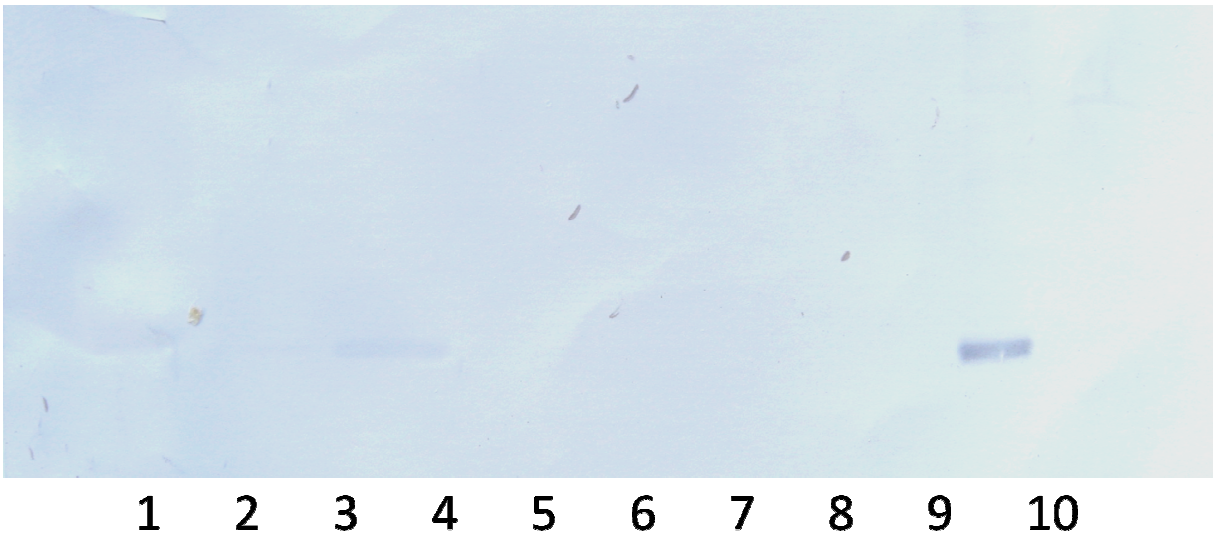

Fig 6: Immunoblots of recombinant *trxA* and *rpIL* strips incubated with mab D6 1/100
1-4 corresponds to *trxA* diluted (1/2000, 1/200, 1/20, ½) and 5-8 to *rpIL*, diluted (1/2000, 1/200, 1/20, ½). 9 VS 102 as positive control, 10 Vector *E.coli* PQE9.

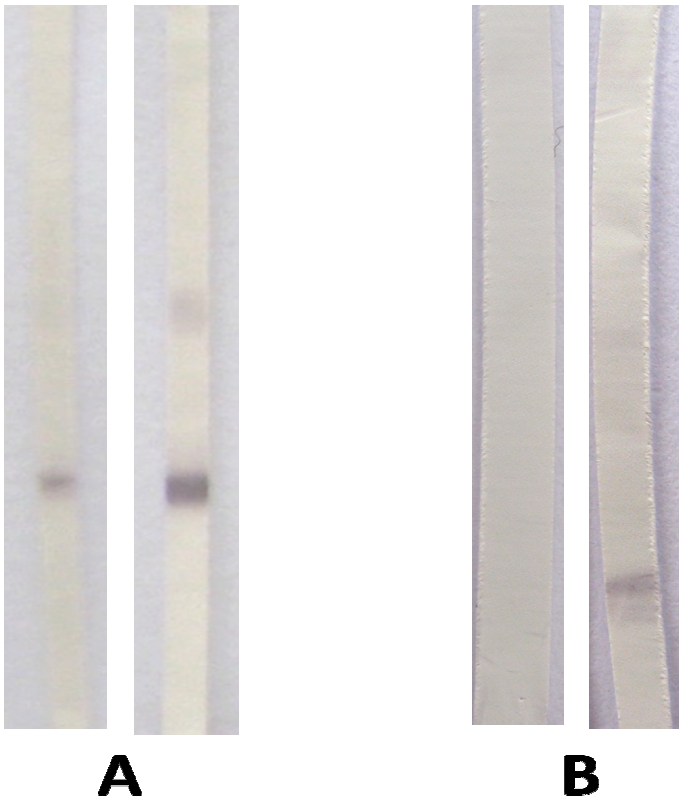

Fig 7: Immunoblot with strips of VS 102 incubated with mabD6 1/100 (positive control) or with mab D6 1/100 incubed with peptide. A : first experiment. B : similar experiment with optimized conditions and mab D6 1/200.

Table 1: Primers and annealing temperatures used for amplification of the corresponding genes from isolates of *Borrelia burgdorferi* sensu lato

Genes	Primers	Annealing temperature
trxA	F 5’ GTATGATTAATTGCTATAGC 3’	52°C
	R 5’ GCTATTTCTTTAACCGAAGAAG 3’	
	R 5’ GTATCATTGAGTGAAATTCCTG 3’	44°C
rpIL	F 5’ AAT CTC ATC AGC TCT TCC TTG 3’	55°C
	R 5’ GTT CAA GCT TAC AGC AAG CT 3’	
rpsJ	F 5’ GATTGCTAAAGATAAGATACGC 3’	53°C
	R 5’ CTGTTTAATATCTACCTCAACAC 3’	

3.3.6 Discussion

We have identified three polypeptides that are potential targets of the species-specific mab D6. The 30 S ribosomal protein (rpsJ) was quickly eliminated from candidate list of proteins potentially reactive with the mab D6 based on our starting hypothesis for the putative epitope. A Genbank search showed that whole amino acid sequence of this protein is fully conserved in *B. burgdorferi* ss (B31), *B. afzelii* (PBi) and *B. garinii* (PKo) isolates confirming our observation reported here among Borrelia isolates and species.
The other two polypeptides shared characteristics compatible with observed species-specificity of the antibody. They comprised residues 7-12 and 74-81 from thioredoxin protein ortholog of *B. burgdorferi* (BB0061) or of *B. garinii* (BG0060) and residues 34-50 from 50s ribosomal protein L7/L12 ortholog of *B. burgdorferi* (BB0390) or of *B. garinii* (BG0391).
The 50s ribosomal protein has been and still is extensively investigated in many bacteria. Studies conducted on *E.coli* [39] and *Haloarcula marismortui* [40] indicate that the 50S ribosomal protein plays an essential role in stabilizing RNA during assembly and translation [41]. The C-terminal part of the protein identified in our work is composed of numerous lysine (K) residues which confer to this region a strong electrostatic potential. This type of domain is believed to be involved in protein-RNA interactions that prevent RNA from falling into kinetic folding traps [39, 40, 42]. Just adjacent to the potential epitope (residues 34-39), the multiple glycine alignment (residues 40-43) found in the central part of the variable region suggests the presence of a loop. Indeed, this specific residue is known to influence the conformation of polypeptides by conferring a high degree of local flexibility. The presence of this highly hydrophobic site may indicate a globular domain that extends over the surface of the 50S subunit. The region constituted of residues 34-50 confer to this protein a degree of variability sufficient for species determination. Intriguingly the sub-cluster observed among *B. garinii* isolates and constituted mainly of CSF isolates (4/5), could be associated, this is an hypothesis, to the newly described species *B. bavariensis* [2]. Indeed isolates belonging to this new species were all derived from species *B. garinii* and are associated to neuroborreliosis in human. We know that mab D6 do not differentiate *B. garinii* from *B. bavariensis*, since PBi assigned to *B. bavariensis* is reactive to mab D6 [11]. Of course additional studies are needed to confirm that isolates (VS BM, VS BP, 387, A77s and G25) belong to *B. bavariensis*.
Thioredoxin A (trxA) is a member of a protein family found in all living organisms. It plays an essential role in redox reactions by facilitating thiol-disulfide exchanges with disulfide bridges of target proteins. Classification of thioredoxins is based on the presence of one (trxB) or two (trxA) cysteines in active site [43]. Two regions, spanning residues 7-12 and 74-81, displayed a high degree of variability among the studied isolates and thus may explain the differential reactivity with mab D6. In these short sequences, at least 1-3 amino acid substitutions were found.
Identification of trxA as the D6 reactive protein was achieved by recognition of the recombinant protein. The weak reactivity of this recombinant protein with D6 is not fully understood. We know that codon usage in *E. coli* and *Borrelia* is different [44] . Is it the explanation? Or is it a posttranscriptional modification? Synthetic peptides (region 6-20 of trxA) provided final clue. As predicted trxA protein synthetic peptide was able to reduce (63-100%) reactivity of D6 in our competition assay. This last experience defines the epitope recognized by mab D6 as the region spanning from amino acid 7 to 12 of trxA protein of *B. garinii* isolates.
Proteins that contain species-specific domains, such as rpIL or trxA, should be particularly useful for precise identification of Borrelia species using PCR and DNA chip technology.

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4 Discussion

Borrelia burgdorferi sensu lato (Bb) est une bactérie particulière, complexe et très intéressante à investiguer. Cette bactérie vit dans la tique vectrice appartenant au complexe *Ixodes ricinus* et dans les hôtes réservoirs. Son épidémiologie est complexe avec plusieurs espèces de Borrélies transmises par le même vecteur. Lors de son passage dans un hôte vertébré (essentiellement mammifères et oiseaux), les borrélies doivent s'adapter rapidement à leur nouvel environnement. Certains hôtes sont connus pour être des réservoirs, ainsi certains oiseaux sont décrits comme des réservoirs de *B. garinii* et *B. valaisiana*, (Humair 2002) les petits rongeurs sauvages sont associés à *B. afzelii* (Hu et al. 1997), le lérot à *B. spielmanii* (Richter et al. 2004) Bb est capable de survivre des années dans ses hôtes, malgré une forte réaction immune. Bb est transmis accidentellement à l'homme chez qui elle peut provoquer différents symptômes. Le premier de ces symptômes est l'érythème migrant, une lésion cutanée caractéristique d'une infection à Borrelia (Steere et al. 1983a). Toutes les lésions cutanées suite à une piqûre de tique ne sont pas aussi typiques et toutes les autres manifestations cliniques (dermatologiques, neurologiques, articulaires, oculaires ou cardiaques…) sont aspécifiques et ne peuvent être diagnostiquées d'emblée comme une borréliose de Lyme. Elles nécessitent des examens de laboratoire. La mise en évidence directe et la culture sont laborieuses et d'un rendement inutilisable pour le diagnostic de routine. Le diagnostic par biologie moléculaire (PCR en particulier) est d'un usage modéré, en raison de la très faible présence de bactéries dans les tissus et liquides biologiques (LCR, liquides articulaires…). C'est d'ailleurs ce qui rend si difficile le diagnostic de cette infection. Seule la sérologie, par la recherche d'anticorps est véritablement utilisable, mais la montée des anticorps est lente. Au fil du temps les tests sérologiques se sont améliorés aussi bien en termes de spécificité que de sensibilité, mais le diagnostic reste difficile. La circulation de plusieurs espèces de borrélies rend difficile la standardisation des tests sérologiques en Europe. D'autre part la population des zones d'endémie (zones tempérées de l'hémisphère nord) est soumise à de fréquentes piqûres de tiques et par conséquent est en contact avec *B. burgdorferi*. La grande majorité de ces personnes ne tombent pas malades, et certaines développent des anticorps. La séroprévalence varie beaucoup d'une région à l'autre. Une séroprévalence élevée dans la population relativise l'utilité d'un résultat positif confirmé pour la prise en charge du patient. Actuellement nous manquons cruellement d'un ou de plusieurs marqueurs d'activité de la maladie. Ce travail s'inscrit dans cette perspective par la recherche de nouveaux marqueurs antigéniques.

Deux approches ont été entreprises, une approche protéique et une approche génomique. L'article final intègre ces 2 approches protéomique – génomique avec l'identification et la caractérisation d'une protéine.

Par définition, le protéome est la totalité de toutes les protéines d'un génome, ou plus précisément, lors d'électrophorèse, de toutes les protéines exprimées à un temps donné et selon des conditions données (Stanek, Strle 2003). La méthode de choix pour l'étude du protéome est l'électrophorèse à deux dimensions (2 DE) (Anderson, Magnarelli 1984), (Wittenbrink et al. 1994; Magnarelli, Anderson 1988). Les mélanges complexes de protéines sont séparés par leurs points isoélectriques (pI) puis selon leurs poids moléculaires (MW). Des cartographies sont alors obtenues, chaque points représentant une protéine (Nadelman, Wormser 1998).

Une façon de détecter et d'identifier des antigènes spécifiques est de travailler avec l'immunoprotéome complet de chacune des trois espèces pathogènes, et de les comparer. La méthode consiste en électrophorèses à deux dimensions (résolution adéquate pour le protéome des micro-organismes), suivi de Western blot réalisés avec des sera de patients (Coyle et al. 1993).

Avant l'étape de migration, les protéines sont généralement enrichies en protéines d'intérêt pour augmenter la résolution de la séparation.

Chez Borrelia, plusieurs techniques ont été tentées (Lengl-Janssen et al. 1994): marquage de surface avec de l'I-125 ou de la biotine (Schutzer et al. 1997), marquage à l'aide d'anticorps (Batsford et al. 1998), traitement aux protéases (Xu et al. 2007), ou alors solubilisation avec divers détergents (Piesman et al. 1987; Piesman 1993). Cependant, toutes ces techniques sont victimes d'artefact, et ne considèrent qu'une fraction des protéines d'intérêts pour le diagnostic.

Dans l'approche protéomique de ce travail, la fraction antigénique a été isolée dans un premier temps afin de séparer dans un deuxième temps par électrophorèse l'immunoprotéome uniquement. Les fractions antigéniques des lysats protéiques totaux de *B. burgdorferi* s.s. VS215, *B. garinii* VS102 et *B. afzelii* VS461 ont été préparés grâce à des colonnes d'affinité élaborées avec des sera sélectionnés pour leurs fortes réactivités spécifiques dirigées contre les espèces respectives. Les antigènes ont été isolés sous conditions semi-natives avec une étape d'immunoprécipitation (IP). L'IP est basée sur la caractéristique des protéines G, associées à des billes de sépharose, de pouvoir lier le fragment Fc des immunoglobulines G

(IgG). De tel travaux ont déjà été réalisés avec succès comme préliminaire à la 2 DE (Assous et al. 1993; Wang et al. 1999; Wilske 2003; Gern et al. 1998).

Beaucoup d'études ont été faites au niveau du protéome de Borrelia en utilisant la 2 DE (Steere et al. 1983b; Xu et al. 2007; Canica et al. 1993), la chromatographie (Péter et al. 1997; O'Connell et al. 1998), ou les protéomes arrays(Baranton et al. 1992), mais seulement avec *B. burgdorferi* s. s. Des cartographies des 3 espèces ont été élaborées par Jungblut (Müllegger et al. 2000), mais l'aspect antigénique n'a été abordé que pour *B. garinii*.

Le profil général des cartographies présenté dans ce travail entre les trois espèces paraît semblable. La plupart des points identifiés sont présents sur chaque carte. A l'aide d'anticorps monoclonaux les protéines p83/100, ErpB, FlaB, BmpA OspA et OspC ont pu être identifiées chez *B. burgdorferi*. On retrouve ces protéines chez les autres espèces, à l'exception d'OspB pour *B. garinii*. En se basant sur la littérature [Nowalk ; Jungblut ; Nowalk], certaines protéines identifiées sont trouvées chez chaque espèce, telles Oms66, OppA et LA7. D'autres chez l'une ou l'autre des espèces tel GAPDH pour *B. garinii*, OspD pour *B. burgdorferi* et *B. afzelii*, OspB et ErpA pour *B. burgdorferi* uniquement et RevA pour *B. burgdorferi* et *B. garinii*.

Ces cartographies d'antigènes sont précises et riche en informations. 102 points sont définis pour *B. afzelii*, 98 pour *B. burgdorferi* et 76 pour *B. garinii*. Parmi ces antigènes, 9 points sont spécifiques à *B. afzelii* et 2 pour *B. burgdorferi*. Ce qui correspond à 4 protéines spécifiques pour *B. afzelii* et 2 pour *B. burgdorferi.*

Si l'on considère le génome des trois espèces, il est connu que *B. afzelii* est plus divergent comparé à *B. garinii* et *B. burgdorferi*. (Humair 2002). Ceci pourrait expliquer pourquoi nous avons trouvé plus de points spécifiques pour *B. afzelii*.

Cette étude sert de cartographie de référence des antigènes pour chaque espèce pathogène de Borrelia. Par la suite, une carte universelle de protéines antigéniques de Borrelia ou des cartes d'antigènes spécifiques pour chaque espèce pourraient être élaborée. Dans le premier cas, la 2 DE devrait être réalisée avec l'élution d'antigènes de colonnes construites avec un groupement de sera humain présentant une réactivité spécifique contre chaque type de borrélies**.** Dans le deuxième cas, l'idée est de passer l'élution de chaque espèce dans les deux autres colonnes afin d'éliminer les protéines antigéniques communes. Les protéines contenues dans cette dernière élution pourraient être directement étudiées par séparation chromatographique lié au spectromètre de masse (O'Connell et al. 1998).

L’approche génomique initiée dans ce travail est parti de l’hypothèse, que *B. burgdorferi* excrète des protéines antigéniques en vue d’échapper au système immunitaire de l’hôte. Cette hypothèse vient de l’observation clinique où l’on décrit des symptômes très importants chez l’homme, alors que le nombre de bactéries présentes dans les tissus et les liquides biologiques (sang, LCR, liquides articulaire…) est très faible. Même les techniques de biologie moléculaire comme la PCR très sensible, n’arrive pas dans toutes les situations, et le loin, à découvrir une infection à Borrelia. Nous imaginons donc que les symptômes observés chez les patients ne sont pas provoqués uniquement par ces quelques bactéries, mais par des protéines qui seraient excrétées en grande quantité par ces bactéries. Les protéines potentielles sécrétées par les spirochètes vers l'environnement extracellulaire pourraient être responsables d'induire une réaction immunitaire et pourrait être impliquées dans les mécanismes de pathogénie (Stanek, Strle 2003; Anderson, Magnarelli 1984). La reconnaissance par le système immunitaire de protéines sécrétées pourrait mener à la découverte marqueurs d’activité de la maladie.

L'analyse *in vitro* des filtrats de culture permettrait de détecter les protéines sécrétées, mais il ne représente pas exactement la situation *in vivo* (Angel et al. 2010; Tokarz et al. 2004; Meinke et al. 2004). De plus, le milieu de culture de *B. burgdorferi* est très riche et contient déjà un grand nombre de protéines qui rendraient l’analyse difficile. C’est probablement la raison pour laquelle ces recherches n’ont pas progressées.

B. burgdorferi est un parasite obligatoire, incapable de survivre à l'extérieur de son hôte arthropode ou hôte vertébré, mais avec une capacité à s’adapter très rapidement à des changements d’environnement. Ce cycle de vie est associé à un relativement petit génome avec des voies de biosynthèses très limitées. Le séquençage du génome de la souche B31 (Schwan et al. 1995; Schwan, Piesman 2000) a démontré une structure exceptionnelle du génome, constituée d’un chromosome linéaire de 0.91 Mo et de 21 plasmides (12 linéaires et 9 circulaires) représentant 0.61 Mo d’ADN supplémentaire.

Dans cette approche génomique, l’outil principal est la bioinformatique. Des algorithmes informatiques ont été utilisés afin de prédire si une protéine est localisée dans le cytoplasme, la membrane cellulaire ou exporté vers le milieu extracellulaire. Ces méthodes de prédiction de localisation subcellulaire sont basées sur les motifs N-Terminal de la séquence protéique et sur d’autres propriétés de la séquence.

Dans cette étude, deux programmes différents ont été utilisés pour identifier les protéines qui contiennent des signaux peptidiques de sécrétions mais qui ne sont pas doté de domaines d’attachement à la membrane. PrediSi a été utilisé pour prédire la présence et l’emplacement

du signal peptidique ainsi que la présence ou non d'un site de scission. La comparaison des séquences en acide aminé avec le modèle Markov Model Caché (TMHMM) a été utilisée pour prédire l'emplacement et l'orientation d'hélices transmembranaires (Coyle et al. 1993; Schutzer et al. 1997). Des algorithmes supplémentaires ont été utilisés pour éliminer les protéines qui contiennent des signaux lipidiques et détecter les fausses localisations cellulaires. Finalement la génomique comparative a été utilisée entre espèces pour identifier les candidats spécifiques.

Les protéomes de *B. afzelii* et *B. garinii* contient 1262 protéines et *B. burgdorferi* 1556. À la fin de l'analyse 3 candidats ont été trouvés pour *B. afzelii*, 7 pour *B. burgdorferi* et 2 pour *B. garinii*.

L'étude des domaines pfam a mis en évidence une famille SIMPL pour Q0SLI8. La fonction de cette famille est inconnue chez les bactéries, mais il est trouvé dans les protéines localisée dans le périplasme ou la membrane externe (Hu et al. 1997). Le domaine Fn3 (Fibronectin type III) est présent chez Q661G1, ce domaine est conservé au cours de l'évolution et est retrouvé dans une large variété de protéines extracellulaires (Little et al. 1994). Ces informations nous donnent des indices confirmant la légitimité de la procédure.

Pour confirmer les résultats bioinformatiques, des fusions PhoA pourraient être utilisés sur les différents gènes de Borrelia pour confirmer si les candidats sont secrétés chez *E. coli*. (Piesman 1993). Les protéines recombinantes pourraient alors être purifiées afin de vérifier le caractère antigénique des candidats.

La méthode décrite ici est rapide, facile et librement disponible d'internet.

La troisième partie de ce travail représente la concrétisation des 2 approches protéomique et génomique pour la caractérisation d'une protéine inconnue mais dont une région est spécifique à l'espèce *B. garinii*. En effet cette protéine de petie taille (12kD environ) est reconnue spécifiquement par un anticorps monoclonal (D6) réactif uniquement avec l'espèce *B. garinii.* Le but était d'identifier cette protéine et si possible l'épitope reconnu par l'anticorps monoclonal D6. Après avoir purifié la protéine d'une souche de *B. garinii* par plusieurs étapes d'électrophorèses, il a été possible de réaliser une analyse par maldi-tof. L'identification de 3 protéines potentielles est sortie de cette analyse. Grâce à la connaissance du génome complet de *B. burgdorferi* (Fraser et al. 1997; Casjens et al. 2000), puis de *B. afzelii* et *B. garinii* (Glöckner et al. 2004) le design d'amorces a été possible, suivi de l'amplification de ces 3 protéines par PCR. L'alignement des séquences obtenues de 28 souches de borrélies appartenant à 4 espèces (*B. burgdorferi*, *B. afzelii, B. garinii* et *B.*

valaisiana) a permis d'éliminer une des protéines candidates. Les 2 autres protéines potentielles présentaient des régions spécifiques de chaque espèce. Elles ont été clonées dans *E.coli* pQE-9. La réactivité de l'une de ces protéines, la TrxA a identifié cette protéine avec 2 régions hypervariables, dont l'une était compatible avec la spécificité de l'anticorps monoclonal. Afin de vérifier cette analyse prédictive, nous avons réalisé une expérience de compétition en incubant l'anticorps monoclonal D6 avec un peptide synthétique qui couvrait la région hypervariable spécifique. L'extinction totale de réaction de l'anticorps monoclonal D6 confirmait que l'épitope sur la protéine Trxa était identifié.

Ce dernier travail démontre la complémentarité des approches protéomiques et génomiques pour l'identification de protéines particulières. Les 2 premières parties de ce travail ont permis d'identifier un certain nombre de protéines spécifiques de *B. burgdorferi, B. afzelii* et *B. garinii.* Le champ est ouvert pour poursuivre ce travail à l'identification et la caractérisation de ces protéines.

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7 Annexes

Annexe_I :

Supplement data to the article: “Comparison of antigens of *Borrelia afzelii*, *B. burgdorferi* and *B. garinii* isolated with immuno-affinity columns on two-dimensional electrophoresis maps”.

Protein sequencing of a spot (older experiment) corresponded to spot n°4 on zinc map of *B. afzelii*. Mass spectrometry results were not significant, so it cannot be consider as identification. It serves here as an example of how to start to characterise a spot of interest: protein sequence, gene sequence, gene comparative, phylogeny.

Annexe_II:

Supplement data to the article: “Comparison of antigens of *Borrelia afzelii*, *B. burgdorferi* and *B. garinii* isolated with immuno-affinity columns on two-dimensional electrophoresis maps”.

All immunoblots are represented in a summary table.

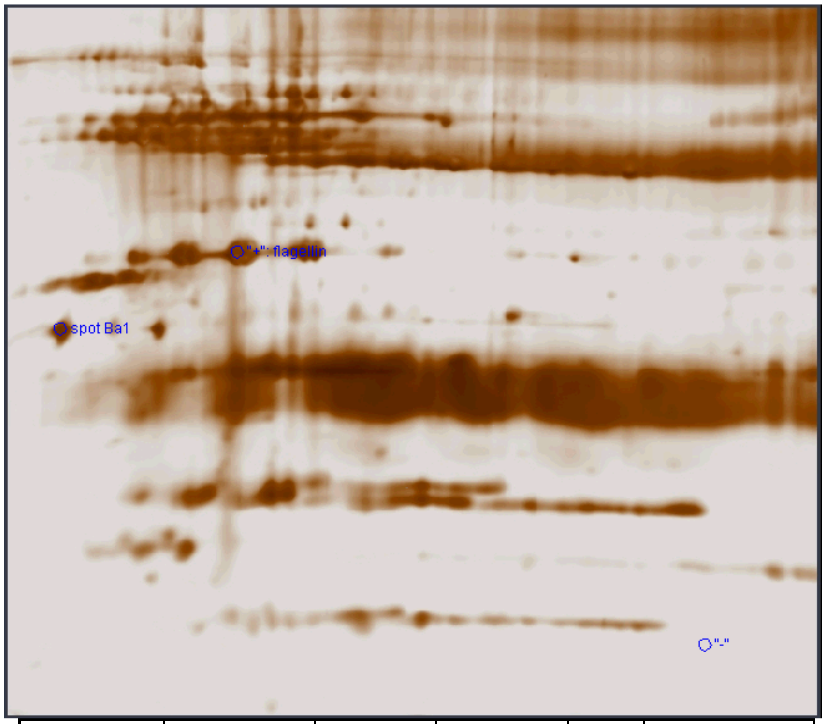
Annexe_III:

Supplement data to the article: “Analysis of putative extracellular proteins of *Borrelia afzelii* (strain PKo), *B. burgdorferi* (strain B31) and *B. garinii* (strain PBi)”.

Here the analysis to detect putative extracellular proteins was made before all three genomes were sequenced. At the date of these experiments (2006) *B. burgdorferi* s. s. genome was complet, *B. garinii* genome was partial and *B. afzelii* genome was inexistent. The parameter of transmembrane domain (≤ 1) was different relative to the article (<1) during the bioinformatic analysis.

Candidates were cloned to obtain recombinant proteins in order to test their antigenic properties.

7.1 Annexe_I: BB0112



Sample	Sample ID	Species	Staining	pI	MW (kDa)
2-DE	Ba1	B. afz	silver	5.2	36
Ctrl+ (fla)	"+ "	B. burg	silver	5.9	39
Ctrl -	"-"	B. burg	silver	7.5	16

Samples provide from affinity chromatography: proteins are in 0.1M glycine-HCl pH2.8 / 230mM Tris-HCl pH 8.0 (final pH ~7-8).

TCA precipitation

- add 1 volume of TCA 100% (w/v) stock to 4 volumes of protein sample
- incubate sample at -20ºC for 15 min
- centrifuge at 20,000 g for 10 minutes at 4ºC and discard supernatant
- wash pellet with 500 µl of 90% acetone and centrifuged at 20,000 g for 10 minutes at 4ºC and discard supernatant
- repeat last step
- air-dry pellet until moist to remove excess acetone (do not overdry)

Solubilization in sample rehydration buffer

- add 140µl urea/thiourea buffer (7M Urea, 2M Thiourea, 4% CHAPS, 1% (w/v) DTT, 1% NP-40, 0,2% Ampholytes pH 3-10, 0.002% bromophenol blue) to pellet
- solubilization on rotating wheel
- centrifuge at 20,000 g for 10 minutes at room temperature and collect supernatant as protein extract

IEF procedure

- load 125µl of protein extract
- ReadyStrip IPG Strip (BioRad): 7cm ph 5-8
- conditions: 20°C, active rehydration 14h 50v, linear slope 20' 250v,linear slope 2h 4000v, rapide slope 12000vh 4000v.

Equilibration of the ipg strips

- incubation 15' in 2.5 ml equilibration buffer (50 mM Tris-Cl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.01%bromophenol blue) plus 25mg DTT
- incubation 15' in 2.5 ml equilibration buffer (50 mM Tris-Cl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.01%bromophenol blue) plus 62.5mg iodoacetamide

- rise with SDS cathode buffer

Transfert onto the sds gels

-seal with agarose solution (0.5% agarose C, 0.01% bromophenol blue, SDS cathode buffer)
-Tris-Glycine SDS-PAGE 1mm x 8mm x 7.5mm, 10mA/gel 15', 20mA/gel ~60'

MS-compatible silver staining procedure

http://www.unil.ch/Jahia/site/paf/cache/off/pid/12983
Adapted from Blum et al., Electrophoresis 8, 93-99, 1987

Solutions:

-Solution A: prepare 100 ml of 50% MeOH, 10% acetic acid in bidistilled H2O (v/v).
-Solution B: prepare 100 ml of 5% MeOH in bidistilled H2O (v/v).
-Solution C: dissolve 0.2 g of sodium thiosulfate (Na2S2O3) in 1 l bidistilled H2O.
-Solution D: dissolve 200 mg of silver nitrate (AgNO3) in 100 ml bidistilled H2O.
-Solution E: mix 3 g sodium carbonate (Na2CO3), 50 µl of formaldehyde (HCOH 37%), 2 ml of solution C and complete to 100 ml with bidistilled H2O.
-Solution F: dissolve 1.4 g of Na2-EDTA in 100 ml bidistilled H2O.

Procedure:

-Fix 30 min in solution A.
-Wash 15 min (or O/N) in solution B.
-Wash 3 times 5 min with bidistilled H2O.
-Sensitize 2 min in solution C.
-Wash 3 times 30 sec with bidistilled H2O.
-Stain 25 min in solution D.
-Wash 3 times 1 min with bidistilled H2O.
-Develop 5min in solution E (development solution).
-Stop 10 min in solution F.
-Wash with bidistilled H2O.

Gel cutting

-Wear gloves and work under a laminar flow hood if possible to limit keratin contamination.
-Cut gel band/spot with a clean scalpel on a clean glass plate or Petri dish.
-Maximise protein concentration by cutting bands or spots where staining is the strongest.
-Transfer to Eppendorf tubes containing 10% ethanol.

Borrelia isolates

The Borrelia isolates used in this study were grown in BSK II medium at 34°C and harvested during the late log phase by centrifugation at 10'000g for 10 min at room temperature. The bacterial pellet was washed twice in phosphate-buffered-saline containing 5mM MgCl2, and finally resuspended in distilled water. The following Borrelia isolates (N=11) were used in this study:

B. burgdorferi ss: B31 (ref. strain), VS215
B. afzelii : VS461 (ref. strain), ACA1, A26s
B. garinii: 20047 (ref. strain), 935T
B. valaisiana : VS116 (ref. strain), UK, AG1, F_10-08-94

The data concerning the biological and geographical origin of these isolates have been described earlier {Bretz 2001 #1}.

PCR and DNA sequencing

The polypeptide sequence determined by LC-MS/MS was reverse translated to yield degenerate DNA sequences. These sequences were aligned against the known complete genome sequence of *B. burgdorferi* to identify its corresponding ortholog genes. PCR primers were then designed from the respective upstream and downstream genes of *B. burgdorferi*, and used to produce specific amplicons for nucleotide sequence determination (F BB0112 5' – gtc aca tca taa gtt cca aaa g – 3'; R BB0112 5' – aac ttg gtt ttg ata tag aga ga – 3'). Primers for the ortholog gene of BB0112 was designed at the 3'end of BB0112 (the upstream gene) and at the 5' end of BB0112 (the downstream gene) (F B0112/BB0111 5' – gct ttt tct gca cca tca – 3'; R BB0112/BB0113 5' – gca aga tat atg gct ttg ttg – 3'). PCR was performed with a Biometra Tgradient apparatus (Biometra, Göttingen, Germany) as follows: 95°C for 15', followed by 45 cycles at 95°C for 1 min, 5? °C for 1min and extension for 1 min at 72°C. Hotstart mastermix (Qiagen, Hilden, Germany) was used for DNA amplification. Amplicons were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining. DNA sequencing was performed using BigDye 1.1 chemistry (Applied Biosystems), ABI310 or 3130 instruments (Applied Biosystems) and appropriate PCR primers.

Phylogenetic analysis

Nucleotide sequences were translated to protein sequences using BioEdit software. All sequences were aligned with CLUSTAL W {Thompson 1997 #2}. Distance matrix from protein sequences were computed with Prodist algorithm {Retief 2000 #5} and data were convert to graphic representation using TreeView {Page 2002 #4} software to obtain phylogenetic tree.

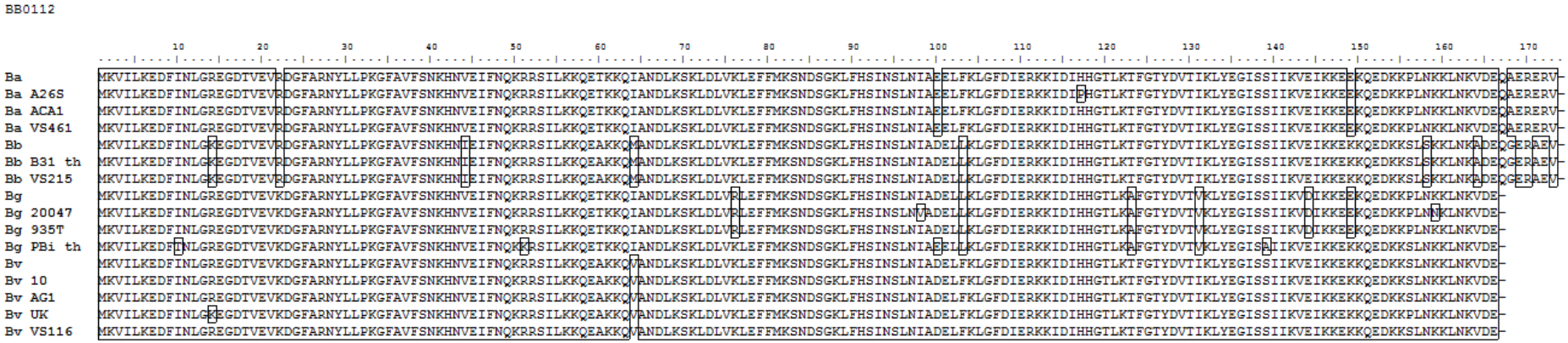


Figure1. Protein sequence alignement. Identical pattern are encountered with black lines

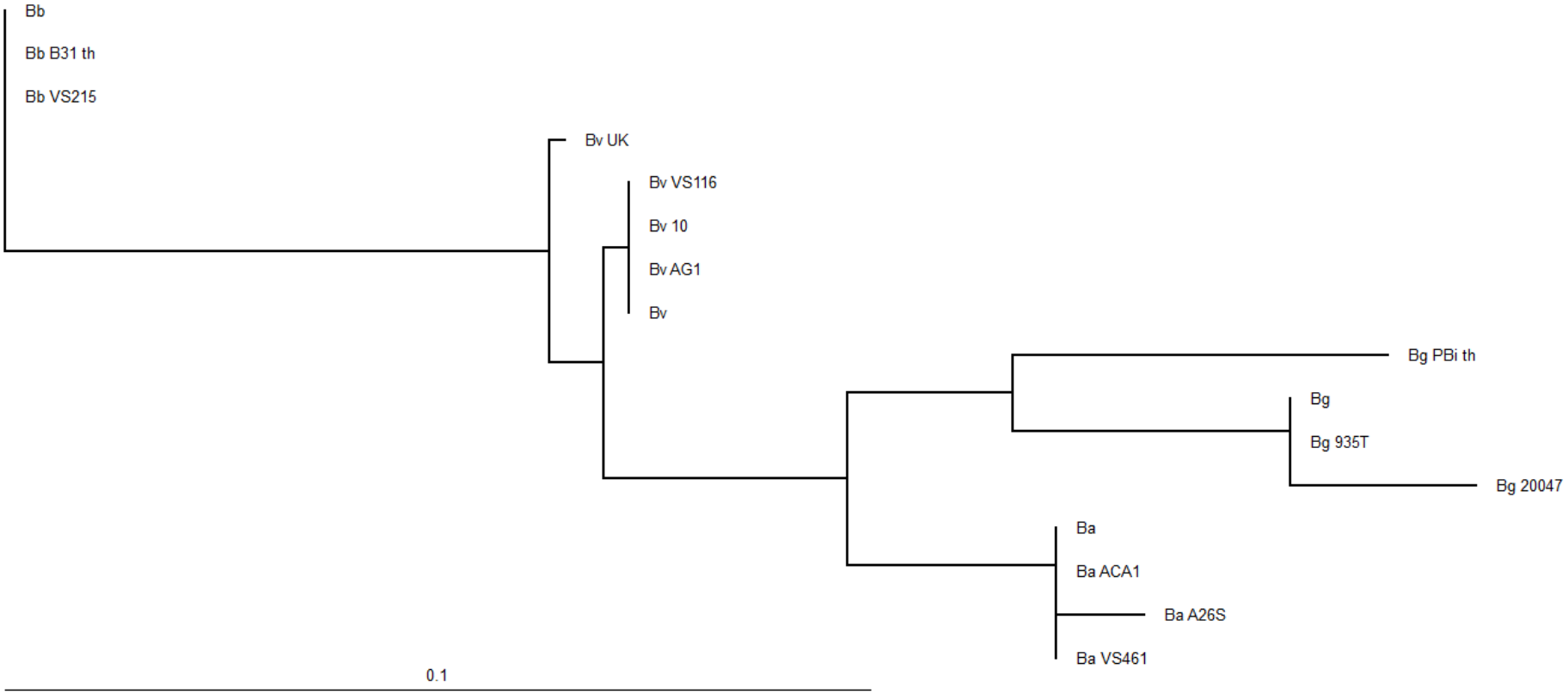
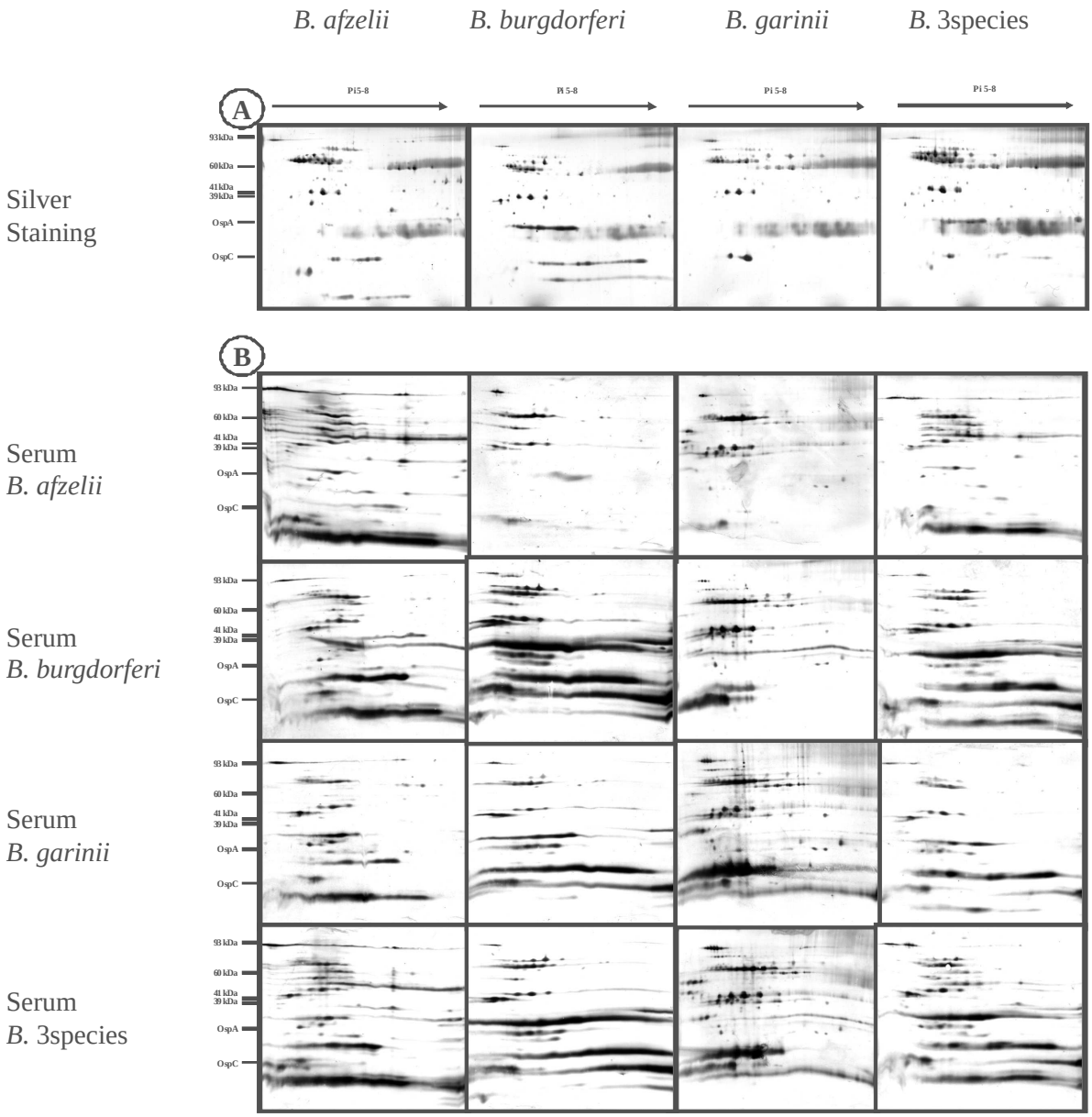


Figure 2. Phylogenetic distance between species for 50S ribosomal protein L9.

7.2 Annexe_II: 2D immunoblots



Maps of *Borrelia burgdorferi sensu lato*. Two dimensional maps of antigenic proteins were obtained after silver staining (A). Proteins were separated from pH 5-8 in the first dimension, and with a 12% SDS-polyacrylamide gel for the second dimension. Immunoblots (B) were reacted with pool of sera.

7.3 Annexe_III: Recombinant proteins

Bioinformatic

Proteomes of *B. burgdorferi* (strain B31) (Fraser et al. 1997) (1556 sequences) and *B. garinii* (strain PBi) (Glöckner et al. 2004) (928 sequences) were obtained from Expasy that contains entries from UniProtKB/Swiss-Prot and UniProtKB/TrEMBL.

Whole proteome datasets were first analysed with JvirGel standalone version (Hiller et al. 2003), that allow prediction and visualisation of secretomes and membrane proteomes. Jvirgel use the PrediSi (PREDIction of SIgnal peptides) algorithm (Hiller et al. 2004) to predict signal peptide sequences and their cleavage positions and TMHMM 2.0 algorithm (Krogh et al. 2001) to predict transmembrane helices. Only predicted proteins with a signal peptide sequence, a cleavage site (prob > 0.5) and without transmembrane domains were kept.

ProtCompB version 3, a program for identification of sub-cellular localization, was used to select-secreted proteins. LipoP (Rahman et al. 2008) was used to discriminate between lipoprotein signal peptides and other signal peptides. Finally only unknown candidates were kept.

To characterize the putative secreted proteins different programs were used: BLAST (Basic Local Alignment Search Tool) from UniProt; ClustalW (multiple sequence alignment programs); Program using the method of Kolaskar and Tongaonkar (Kolaskar, Tongaonkar 1990) to predict antigen determinants; MineBlast (a literature presentation service supporting protein annotation by data mining ofBLAST results).

Cloning primers for pQE-9 vector (Qiagen) were designed using GENtle program from Magnus Manske. Original gene sequences were obtain from NCBI (National Center for Biotechnology Information), and were manuely deleted from their signal peptide coding sequences. SalI restriction sites were added to foward primers and PstI to reverse primers (Table 3).

Molecular biology

Cell Culture

B. burgdorferi strain B31 and *B. garinii* strain PBi

were cultured in BSK II medium at 34°C, and spirochetes were harvested during the late log phase by centrifugation at 10,000 × *g* for 10 min. The pellet was washed twice in phosphate-buffered saline with 5 mM $MgCl_2$ and finally resuspended in distilled water and heat 10min at 95°C to be ready for DNA amplification.

Expression of 6xhis-tagged proteins

Protein expressions were performed with the QlAexpressionist system (Qiagen, Hilden, Germany). DNA encoding each protein was amplified by PCR using *B. burgdorferi* strain B31 or B. *garinii* strain PBi as a template. Primers were designed using PerlPrimer software (Marshall 2004). The DNA fragments were cloned into the expression vector pQE9. The accuracy of the constructs was verified by sequence analysis. Histidine-tagged proteins were expressed in *E. coli* Ml5 [pREP4] cells according to the manufacturer's instructions. Purified proteins were obtained by 6xHistagged protein minipreps under denaturing conditions using Ni-nitrilotriacetate (NTA) - agarose. Integrity of proteins was tested by SDS/P AGE (zinc staining, Figure 1; Coomassie staining not shown) and by Western (figure 1).

SDS-PAGE and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were performed as described previously (Péter et al. 1995). Briefly, samples were heated for 5 min at 95°C before undergoing electrophoresis on a polyacrylamide gel at 12.5% (constant voltage, 170 V, 58min). After electrophoresis the proteins were transferred to a polyvinylidene-difluoride membrane (constant voltage, 120V, 54min). Before use, the membrane was blocked for 1 h at 37°C with Tris-buffered saline (TBS; pH 7.2) with 5% gelatine and was washed three times for 5 min each time with washing buffer (W-buffer; TBS with 0.1% gelatin and 0.05% Tween 20) at room temperature. The following steps were also performed at room temperature. Each antigen strip was incubated for 2 h with patient serum diluted 1/500 in D-buffer (TBS with 1 % gelatin and

0.05% Tween 20). The strips were washed three times for 5 min with W -buffer. After the washing, rabbit anti-human IgG conjugated to alkaline phosphatase diluted 1/1000 in D-buffer was added. At the end of the second 2-h incubation, two washes were done with W -buffer and one was done with TBS. The bound conjugate was visualized by addition of the chromogenic substrate 5-bromo-4-chloro-3indolylphosphate Nitro Blue Tetrazolium (Kirkegaard & Perry Laboratories). The reaction was stopped 30 min later by two rinses in distilled water.

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Bacterial Culture Media

Medium		*Components per liter*	
LB		Tryptone	10 g
		Yeast extract	5 g
		NaCl	10 g
	Autoclave		
LB agar		Tryptone	10 g
		Yeast extract	5 g
		NaCl	10 g
		Agar (1.5%)	15 g
	Autoclave		

Prepare stock solutions of antibiotics separately from batches of liquid or solid media, sterilize by filtration (0.2µ), aliquot, and store in the dark at –20°C. Recommended stock and working concentrations for commonly used antibiotics are shown in Table 1.
Before adding antibiotics to freshly autoclaved medium, ensure that the medium has cooled to below 50°C.

Table 1. Concentrations of commonly used antibiotics

Antibiotic	*Stock solutions*	*Working concentration*	*Concentration*	*Storage (dilution)*
Ampicillin	50 mg/ml in water	–20°C	100 µg/ml	(1/500)
Chloramphenicol	34 mg/ml in ethanol	–20°C	170 µg/ml	(1/200)
Kanamycin	10 mg/ml in water	–20°C	50 µg/ml	(1/200) *
Streptomycin	10 mg/ml in water	–20°C	50 µg/ml	(1/200)
Tetracycline HCl	5 mg/ml in ethanol	–20°C	50 µg/ml	(1/100)
Carbenicillin	50 mg/ml in water	–20°C	50 µg/ml	(1/1000)

*for M15 cells 25µg/ml (1/400) is recommended (Qiaexpressionist)

Related protocol:

Qiagen
The Bench Guide
http://www1.qiagen.com/literature/BenchGuide/

Preparation of chemically competent cells ($CaCl_2$)

Have the following solutions at 0-4 °C:
a) 100 mM MgCl2
b) 100 mM CaCl2-15% glycerol
c) sterile 50ml Falcon and pre-cooled rotor

1. Grow a 5 mL overnight culture of bacteria M15 in LB containing 25µg/ml kanamycine.

2. Dilute 1:100 and shake at 37 °C (4x [250µl into 25 ml] in 50ml Falcon]).

3. After 1.5-2 hours (105min), A600= 0.5-0.6 (0.4 also works well).

4. When cells reach proper density, put one culture in an other to obtain two 50ml culture, spin down 3100 rpm (Sorvall RTH-750), 10 minutes at 4 °C.

5. Pour off supernatant and keep the cells on ice.

6. Resuspend in 2x [10 mL of 100 mM MgCl2 (ice-cold)] by pipetting up and down.

7. Incubate on ice 20-30 minutes.

8. Spin down cells at 3100 rpm for 10 minutes at 4 °C. Discard supernatant and keep cells on ice.

9. Prepare eppendorf tubes.

10. Resuspend in 2x [1 mL of 100 mM CaCl2-15% glycerol (ice-cold)].

11. Aliquot into tubes (100 µl/tube) and put at -80 °C (quick freezing not necessary).

Related protocol:

Fred Hutchinson Cancer Research Center
Preparation of chemically competent cells
http://www.fhcrc.org/science/labs/gottschling/bac/btrans.html

Other protocols:

Qiagen Benchguide, p2-3, competent cells and transformation, based on TFB1 and TFB2 solution (RbCl can be replace by KCl)

Current protocols in molecular biology, unit 1.8, introduction of plasmid DNA into cells, based on $CaCl_2$

Preparation of glycerol stocks

E. coli strains can be stored for many years at –70°C in medium containing 15% glycerol.
Prepare glycerol stocks of bacteria as follows:
1. Add 0.15 ml 100% glycerol to a 2 ml screw-cap vial and sterilize by autoclaving. (Or autoclave a bottle containing 100% glycerol and use 0.15ml before use. Sterilized eppendorf can also be used).
Vials of sterilized glycerol can be prepared in batches and stored at room temperature until required.
2. Add 0.85 ml of a logarithmic-phase (4-5 hours) E. coli culture (5ml preculture O/N, dilute 1/???) to the vial of pre-sterilized glycerol.
3. Vortex the vial vigorously to ensure even mixing of the bacterial culture and the glycerol.
4. Freeze in an dry ice–ethanol bath or liquid nitrogen and store at –70°C.
Avoid repeated thawing and re-freezing of glycerol stocks as this can reduce the viability of the bacteria.
For precious strains, storage of two stock vials is recommended.

Related protocol:

Qiagen
The Bench Guide
http://www1.qiagen.com/literature/BenchGuide/

Restriction with PstI and SalI

Important note: use first PstI (restriction sequence need two border nucleotides) and in a second time SalI (no border nucleotide required). Enzymes are from Promega.Two steps are needed to digest the vector (border nucleotides and buffer compatibility) and the inserts (buffer compatibility).

Restriction

8x inserts (all different) and 2x plasmid pQE-9

Reaction:	1x	12x
H2O	10.5µl	126µl
Buffer H 10x	2µl	24µl
PstI	1.5µl	18µl
DNA	6µl	

→ 14µl/tubes

Incubation 110min at 37°C
Purification with “MinElute PCR Purification Kit” from Qiagen, elution in 17.5µl EB buffer (1µl is lost during this step).

Reaction:	1x	12x
Buffer D 10x	2µl	24µl
SalI	1.5µl	18µl
Elution	16.5µl	

→ 3.5µl/tubes

Icubation 75min at 37°C
For plasmid: + 2.2µl CIP buffer 2x + 0.1µl CIP, 15min 37°C
For inserts: 15min 37°C

Purification with “MinElute PCR Purification Kit” from Qiagen, elution in 10µl EB buffer (1µl is lost during this step).

Ligation

"Subcloning Tips from ProtocolOnline - very important heating step

Source: Schimmelpenninck
Abstract: Put your insert and vector in an Eppendorff tube and then heat it for 5 minutes, then add ligation buffer and ligase

After isolating your digested fragments from the gel put digested vector and digested insert TOGETHER in an Eppendorff tube, then put this for about 5 minutes at 65 degrees celsius, then cool it and spin it down, then add your ligation buffer and ligase. The 65 degrees celsius step is VERY important.

Short explanation:
your insert is EcoRI/SalI
and vector is EcoRI/SalI
The heating step will break all the UNWANTED AND TOTALLY USELESS insert-insert and vector-vector bindings. After the heating step MANY good vector insert bindings will occur and your plates will be full of good transformants. "

Mix in eppendorf for each reaction: 1µl digested plasmid + 3µl digested insert
Heat 5' at 65°C

Reaction:	1x	9x
Ligase	1µl	9µl
Rapid ligase buffer 2x	5µl	45µl
(Insert	3µl)	
(Plasmid	1µl)	

→ 6µl/tubes

Incubation 1hour at RT

Related protocol

Transformation of competent M15 cells

1. Transfer an aliquot of the ligation mix (10 µl or less) into a cold sterile 1.5 ml microcentrifuge tube, and keep it on ice.
2. Thaw an aliquot of frozen competent M15[pREP4] cells on ice.
3. Gently resuspend the cells and transfer 100 µl of the cell suspension into the microcentrifuge tube with the ligation mix, mix carefully, and keep it on ice for 20 min.
4. Transfer the tube to a 42°C water bath or heating block for 90 sec.
5. Add 500 µl of LB to the cells and incubate for 60 min at 37°C.
Shaking increases transformation efficiency.
6. Plate out 200 µl aliquots on LB-agar plates containing 25 µg/ml kanamycin and 100 µg/ml. Incubate the plates at 37°C overnight.

Control to check transformation efficiency:

Positive control:	transformation with 1 µl of pQE-9 miniprep.
Ratio control:	transformation with pQE-9 digested and ligated
Negative control:	transformation with M15 cells alone

Related protocol:

Quiagen
The QIAexpressionist
http://www1.qiagen.com/literature/handbooks/PDF/Protein/Expression/QXP_QIAexpressionist/1024473_QXPHB_0603.pdf

PCR direct on colonies

Reaction:	1x	26x
H2O	10.5µl	273µl
Hotpol (Abgene) 2x	12.5µl	325µl
Primer pQE-9 5’	1µl	26µl
Primer pQE-9 3’	1µl	26µl

→ 25µl/tubes

PCR have only 25 tube positions

Touch a colony with an autoclaved toothpick, strike 5mm on an LB amp kan agar plate, and rotate it in a pcr tube with it mixture. Finally the toothpick can be put in liquid media for further experiments.

Program:

lid on
15’ 95°C
[30x_____
30’’ 94°C
30’’ 54°C
2’ 72°C
8’ 72°C
∞ 4°C

Related protocol

ABgene
Standard PCR
http://www.abgene.com/Static_Pages.asp?page=34

Rapid screening of small expression cultures

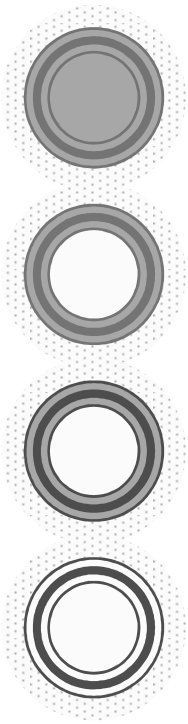
1. Pick single colonies of transformants into 5 ml of culture media containing both ampicillin (100 µg/ml) and kanamycin (25 µg/ml). Grow the cultures overnight.
2. Inoculate 5 ml of prewarmed medium (including antibiotics) with 250 µl of the overnight cultures, and grow at 37°C for 30-40 min, with vigorous shaking (until the OD600 is 0.5–0.7).
3. Induce expression by adding IPTG to a final concentration of 1 mM (stock solution 100mM).
4. Grow the cultures for an additional 4–5 h, and transfer to microcentrifuge tubes.
Harvest the aliquots of 1.5ml by centrifugation for 1 min at 15,000 x g, and transfert supernatants in new eppendorf.
5. Resuspend cells in 100 µl loading buffer and heat 5min at 95°C.
6. Precipitate supernatant with TCA (wash 1x with acetone), resuspend pellets in 100 µl loading buffer and heat 5min at 95°C.
7. Load 20µl of each samples on SDS-Page

Related protocol:

Quiagen
The QIAexpressionist, p46
http://www1.qiagen.com/literature/handbooks/PDF/Protein/Expression/QXP_QIAexpressionist/1024473_QXPHB_0603.pdf

Tables

Table 1. Bioinformatic strategy to select potential secreted proteins from *B. burgdorferi* and *B. garinii* proteome. On the left are schematic representations of protein localizations in gram-bacteria (Grey represent protein localization after selection after each steps).



Total proteins

Proterins with SP and cleavage site

Proteins w/o transmembrane domain

Pcompb, lipoP

Prediction de localisation

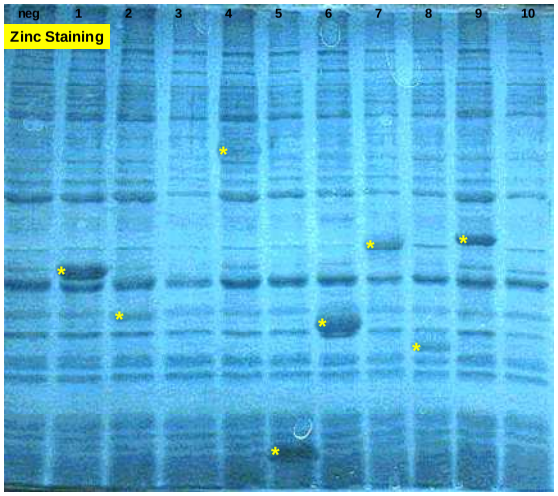
Borrelia burgdorferi (expasy) **1556** sequences protéiques prédites		Borrelia garinii (expasy) **928** sequences protéiques prédites
↓	JVirGel PrediSi	↓
104		**75**
↓	JVirGel TMHMM	↓
37		**34**
↓	pCompB	↓
25		**9**
↓	LipoP	↓
13	**Protéines sécrétées**	**7**

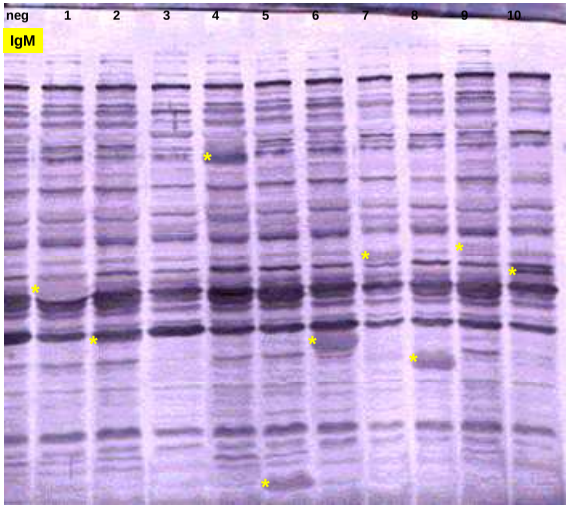
Table 2. Summary of candidates

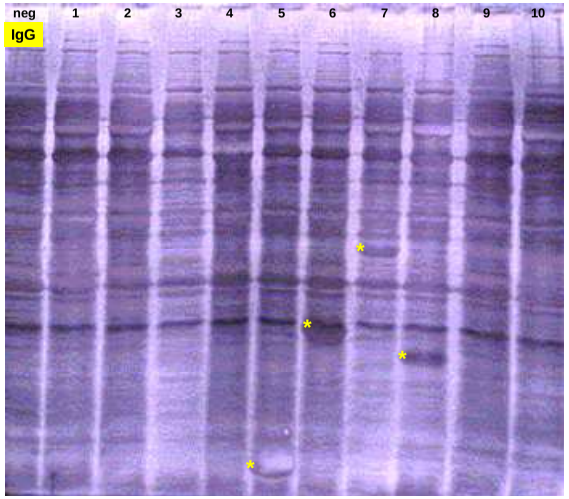
	Acc-No	name	pI	MW	Cleavage prob.	TM
B. burgdorferi	PR00038192	BB0028	9.03	39'700	0.64	0
	PR00038324	BB0161	5.96	66'248	0.65	0
	PR00038399	BB0236	8.96	75'341	0.59	0
	PR00038623	BB0460	9.17	28'188	0.53	0
	PR00039375	BBA32	11.07	7'790	0.64	0
	PR00039400	BBA57	5.03	47'418	0.66	0
	PR00039028	BBB14	9.78	19'520	0.54	0
	PR00039317	BBJ23	9.55	31'217	0.55	0
	PR00039328	BBJ34	5.14	39'645	0.51	0
B.garinii	PR00508527	BG0239	8.73	75'246	0.58	0
	PR00508634	BG0347	9.46	24'123	0.68	0
	PR00508640	BG0353	9.89	44'032	0.53	0
	PR00508705	BG0421	9.48	39'468	0.67	0

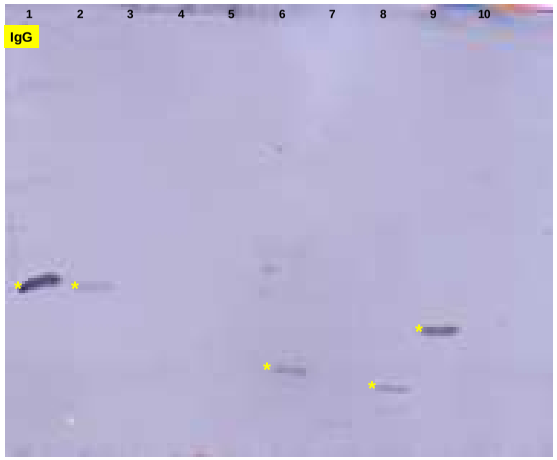
Table 3. List of primers used to clone respective genes in pQE-9

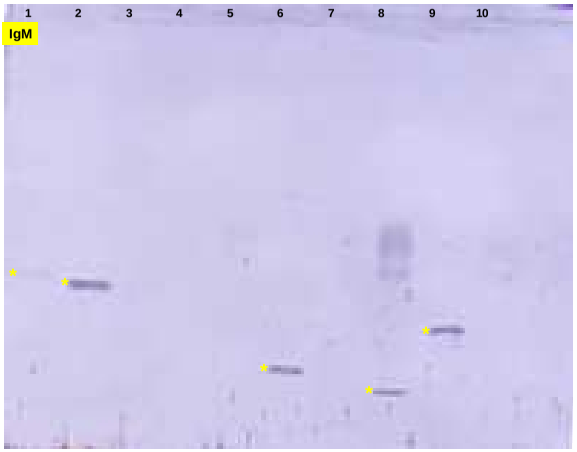
>BB0028-SP-3' pstI	AACTGCAGTAATTTTCTGTTTTCCAATTTCCAC
>BB0028-SP-5' SalI	ACGCGTCGACCAATTAGGAAATCTGCAAAAAATAA
>BB0161-SP-3' pstI	AACTGCAGTTAGATTAAATTACCCAAAGAATCAA
>BB0161-SP-5' SalI	ACGCGTCGACAAAGAAGTTTACTATAGGTTTGCAGA
>BB0236-SP-3' pstI	AACTGCAGCTAATTAATAAAATATGCAAACTCC
>BB0236-SP-5' SalI	ACGCGTCGACCAAGGAATAGTTACTAATAAAGATGC
>BB0460-SP-3' pstI	AACTGCAGTTAAGACTGAGAGTGAAGGTTTTTG
>BB0460-SP-5' SalI	ACGCGTCGACAAAGTTAATTCCGAATTTGAAATTA
>BBA32-SP-3' pstI	AACTGCAGTTAAAGCTTTCTGTTTCTACGATA
>BBA32-SP-5' SalI	ACGCGTCGACAAGTCGAGCAATAAAAGTTTATTG
>BBA57-SP-3' pstI	AACTGCAGTTATTGATAATTTTTTTCTACCAATA
>BBA57-SP-5' SalI	ACGCGTCGACGATACAAACGATAAAAACAAAGCC
>BBB14-SP-3' pstI	AACTGCAGTTAACTTTTATAATCTTTATTTTCAATC
>BBB14-SP-5' SalI	ACGCGTCGACGTTGAGCACGATCAATTTGGAAAAAC
>BBJ23-SP-3'pstI	AACTGCAGCTAATTTTTTATAGGAAAATCCATA
>BBJ23-SP-5' SalI	GCGTCGACTTTGATGTTTCAAGTAGAAAATTTTA
>BBJ34-SP-3' pstI	AACTGCAGTTATTTATCTTTATTTTTAGGCTTAA
>BBJ34-SP-5' SalI	ACGCGTCGACAGTGATGATACAAATAAAAAAAATAC
>BG0239-SP-3' pstI	AACTGCAGCTAATTAATAAAATATGCAAACTCTC
>BG0239-SP-5' SalI	ACGCGTCGACATAGTTACCAATAAAGATGCTCAAGA
>BG0347-SP-3' pstI	AACTGCAGCAGTTAATTTTTTTTAATATCATATAAA
>BG0347-SP-5' SalI	ACGCGTCGACCAAATATCTGCAAATCAATACTTTGA
>BG0353-SP-3' pstI	AACTGCAGTTAATTTTTATTTTTTTTTTGATTCT
>BG0353-SP-5' SalI	ACGCGTCGACAAAAATAAAAATATAATTGTACTAAC
>BG0421-SP-3' pstI	AACTGCAGTTAGTATTTATAAGTTACAGAGATTC
>BG0421-SP-5' SalI	ACGCGTCGACCAAAATCAACACGACTTAAAATTCAA
>pQE-9-3'	TTCTGAGGTCATTACTGGATC
>pQE-9-5'	GATAACAATTTCACACAGAAT











	(neg) pQE9	(1) BB00288	(2) BB04600	(3) BBA32	(4) BBA57	(5) BBB14	(6) BBJ23	(7) BBJ34	(8) BG03447	(9) BG03053	(10) BG04211
MW (kDa)	-	37.86	27.29	7.14	46.08	18.05	30.02	38.14	23.83	43.58	38.19
Coomassie	-	+	+		+	+	+	+	+	-	-
Zinc	-	+	+		+	+	+	+	+	+	-
IgM	-	?	+		+	+	+	+	+	+	++
IgG	-	-	-		-	?	+	+	+	-	-
Coo purif		+	+	-	-	-	+	-	+	+	-
IgM purif		(+)	++	-	-	-	++	-	++	++	-
IgG purif		++	(+)	-	-	-	+	-	+	++	-

Figure 1. Summary of recombinant proteins produced