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Faculty of Science

Cloning of the gene coding for Outer surface protein C from the Lyme borreliosis spirochetes

Bachelor thesis

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Annotation:

Outer surface protein C (OspC) is an immunogenic lipoprotein produced by *Borrelia burgdorferi* during their migration from tick midgut into the salivary glands. Aims of this work were to clone the gene for OspC, prepare the recombinant OspC protein and purify it using different affinity chromatography techniques. Last step involved LC-MS analysis of the rOspC.

Anotace:

Vnější membránový protein C (OspC) je imunogenní lipoprotein, který je produkován spirochétami *Borrelia burgdorferi* během jejich migrace ze střeva klíštěte do jeho slinných žláz. Cílem této bakalářské práce bylo zaklonovat gen kódující OspC, připravit tento rekombinantní protein a následně ho purifikovat pomocí různých affinitně-chromatografických metod. Posledním krokem byla LC-MS analýza takto připraveného rOspC.

Affirmation:

I hereby declare under oath that the submitted bachelor thesis has been written solely by me without any third-party assistance. Additional sources or aids are fully documented in this paper, and sources for literal or paraphrased quotes are accurately credited.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my dissertation thesis, in full / in shortened form resulting from deletion of indicated parts to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.

České Budějovice, 2010

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Adriana Walnerová

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Table of Contents:

1. List of Abbreviations:

APS = ammonium persulphate

CoMAC = cobalt metal affinity chromatography

DAB = diaminobenzidine

DNA = deoxyribonucleic acid

dNTP = deoxyribonucleotide Triphosphate

DTT = dithiothreitol

EDTA = ethylenediaminetetraacetic acid

ESI = electron spray ionization

FT-MS = Fourier transform mass spectrometry

His = Histidine

HPLC = high performance liquid chromatography

IPTG = isopropyl β-D-1-thiogalactopyranoside

LB medium = lysogeny broth medium

LC = liquid chromatography

MAC = metal affinity chromatography

NiMAC = nickel metal affinity chromatography

OspC = Outer surface protein C

PBS = phosphate buffered saline

PCR = polymerase chain reaction

PVDF = polyvinylidene fluoride

rOspC = recombinant Outer surface protein C

SDS-PAGE = sodium dodecyl-sulphate polyacrylamide gel electrophoresis

SwAM/Px = swine anti-mouse antibody labeled with peroxidase

TB medium = terrific broth medium

TEMED = tetraethylmethylenediamine

NTA = nitrotriacetic acid

2. Main objectives

- cloning of the *ospC* gene

- preparation of expression system for production of rOspC

- pilot expression of the protein

- purification of the protein using different affinity chromatography methods

3. Literature research

3. 1. Lyme disease

Lyme disease is the most common vector-borne disease in Europe as well as in USA. Initial manifestations of Lyme disease were first described by Afzelius in 1910. It was described as skin lesion, erythrema migrans, which was for the first time associated with tick bite [1]. In 1976, epidemic of juvenile rheumatoid arthritis in Lyme (Connecticut, USA) was recognized as a cluster of symptoms known as Lyme disease or Lyme borreliosis [2].

The causative agent of Lyme disease, *Borrelia burgdorferi* was isolated from *Ixodes tick* in 1982 [3]. Later, three major genospecies causing infection in humans were determined and classified as *Borrelia burgdorferi* sensu lato: *B. burfdorferi* sensu stricto (s.s.), *B. garinii* and *B. afzelii*. All three genospecies are found to be pathogenic in Europe while *B. garinii* and *B. afzelii* are common in Asia and *B. burgdorferi* s.s. is known to cause infection in USA. [4]. Spirochetes of *Borrelia* live in enzootic cycles and are associated with ticks from *Ixodes* complex containing fourteen tick species [5].

In Europe, the major vector is *Ixodes ricinus* which feeds on more than three hundred different species. The hosts of these parasites are mammals as well as reptiles and birds. *B. afzelii* is associated mainly with rodents while *B. garinii* is found mainly in birds [6]. Greatest incidence of this disease is in middle Europe – Austria, Slovenia, and Germany [7].

In USA, most cases are reported from Northeast and Upper Midwest. In these regions, spirochetes are transmitted from larval or nymphal *Ixodes scapularis* to rodent host especially white-footed mice and chipmunks [8].

A novel alternative source for the derivation of potent pharmacological agents is tick saliva. The saliva retrieved from various tick species contain great number of physiologically active compounds. For example in *Ixodes ricinus*, a B cell-inhibitory protein and T-cell inhibitors, Iris and Salp15, can be found. The latter immunosuppressor, Salp15, acts via binding to OspC in the tick salivary glands and thus protects the spirochetes from antibody-mediated killing [9].

3. 2. Borrelia burgdorferi

Borrelia is a genus of parasitic Gram negative bacteria. These motile, helical shaped cells contain up to twenty periplasmic flagella. Usually, the cells are of length of 3 – 20 μm and width of 0.2 – 0.5 μm [10]. The location of flagella in periplasmic space helps spirochetes to avoid the immune reaction since flagella are highly antigenic [11].

The flagella rotate in the periplasmic space thus enabling spirochetes to move in a wave-like manner. This feature corresponds to their ability to move through very viscous media and even various tissues. The ability of spirochetes to penetrate tissues is essential for their dissemination in host organism. As much as 6% of the borrelial genes code for proteins which are involved in the motility and chemotaxis [12, 13].

3. 3. General aspects of the B. burgdorferi infection

The ticks of the genus *Ixodes* feed once during each stage. These include larva, nymph and adult. The larval ticks acquire the spirochetes by feeding on infected vertebrate such as a small rodent. Spirochetes multiply in the midgut of the infected tick. *Borrelia burgdorferi* persists in the tick during the moult to the next active stage. This can take up to one year. In nymphal stage, this tick can eventually infect the naïve mammal – for instance deer or human. When the tick starts to feed on a host, the spirochetes migrate from the tick midgut to the salivary glands from which they are delivered to the host via the saliva [13].

A model of spirochetal infection was developed in a mouse. It showed, that spirochetes form a localized infection in the skin (the site of tick bite) known as erythrema migrans at first. Afterwards, *B. burgdorferi* disseminates via the bloodstream to various types of tissues. In the last stage of the infection, the spirochetes establish persistent infection in diverse organs such as skin, joints, bladder and heart [14].

Moreover, experiments on primates showed that the infected host can develop the infection of central nervous system as well, which is not seen in rodents [15].

3. 4. Outer surface proteins

During the tick feeding, *B. burgdorferi* spirochetes change the composition of their surface proteins. In unfed ticks, only OspA is expressed on the surface of spirochetes. During the feeding, changes in the protein expression occur and OspC is found on the surface of spirochetes instead of OspA. This alteration of outer surface protein expression is activated by two factors – the tick feeding and the temperature shift. It was proved, that OspC is not produced at 24°C but starts to be expressed at temperatures in the range of 32-37°C. This increase in temperature corresponds to the environmental change in the tick midgut which is caused by ingestion of the warm blood when the tick starts to feed on a host [16].

Thus, OspC expression is enhanced by higher cultivation temperature together with the co-cultivation with tick cells. On the other hand, it is supressed by lower temperature [17].

Two other outer surface proteins, OspA and OspB, have an essential role in the invasion and survival of *B. burgdorferi* spirochetes within the tick midgut. Adherence of spirochetes to midgut tissue is mediated particularly by OspA [18].

OspA is expressed once the spirochetes get into the tick. OspA binds specifically to proteins in the gut of *Ixodes scapularis* – a tick receptor for OspA ("TROSPA") was identified and proved to be essential in colonizing the tick gut by spirochetes [19]. Its expression is suppressed during the feeding to enable spirochetal migration from the gut to the salivary glands [20].

A new model which is in disagreement with the previous findings was proposed by Ohnishi et al. in 2000. In the early period of the tick blood meal, the spirochetes in the tick gut produce only OspA. As the feeding proceeds, diverse borrelial populations can be found in the midgut. First group of bacteria produces OspA, the second one OspA and OspC, the third one produces only the OspC and the last one none of these proteins. Concerning the migration of bacteria into the tick salivary glands the populations producing no OspA are involved. After 53 hours of tick feeding, group of borrelia expressing only a little OspC was able to cause a stable infection in the host organism. Bacteria with no production of outer surface proteins prevailed. The blood meal also triggers a recombination of other borrelial protein vlsE [21].

VlsE is an outer surface lipoprotein which is very important in the bacterial invasion of a host. It has six variable regions on its outer surface which serve as a kind of protection for the conserved parts - these are shielded from the antibodies [22]. The number of combinations in variable regions in this locus is huge, thus giving the borrelia a very potent tool for defending

themselves from the host immune response [23]. VlsE is required for persistence of spirochetes in mammal host [24].

When the spirochetes are under the host immune attack, they are able to alternate their surface by changing the expression levels of four lipoproteins: DbpA, OspC, BBF01 and VlsE. While the DpbA abundance is constant, OspC expression is radically reduced and VlsE with BBF01 are increased. This gives rise to new borrelia phenotypes making the spirochetes more resistant to the host immune reaction. DbpA (decorin binding protein A) is expressed in constant level in all tissues. When the immune response is absent, the spirochetes are present in many phenotypes dependent on the tissue type in which they occur. The OspC expression is increased in heart and decreased in various joints during the first four months of infection. OspC can be found on borrelia in skin and heart even in the case of no adaptive immune response. In the heart tissue it may be crucial for survival of spirochetes [25].

3. 5. OspC function

OspC is essential for the induction of infection in a vertebrate host. It is not required for the migration of *Borrelia burgdorferi* in the tick. An OspC deficient mutant of *Borrelia burgdorferi* was proved to fail in infecting the vertebrate host (mice). These modified borrelia were cleared from the host without any immune response. On the other hand, this mutant was able to colonize the tick and to move from the midgut to the salivary glands during the blood meal. This suggests, that the OspC is not the factor which is strictly required for borrelia to migrate from the midgut to salivary glands but is essential in establishing the infection in mammalian host [26].

This was also confirmed by further studies of the OspC requirement through the infection cycle. OspC is necessary in the initial stage of a mammalian infection in the range of days to weeks. At the same time, it is not needed in any other stage of the mouse-tick infection cycle [27].

However, previous studies showed that OspC expression is downregulated in mammal after the transmission from tick [28].

Borrelia with different OspC groups are able to infect different types of vertebrate hosts. 15 different groups of OspC were determined from which only four cause the infection in human

– A, B, I and K. Novel research demonstrates association of other OspC groups C, D and N with invasive strains of *B. burgdorferi* [29].

The highest levels of OspC expression arise when the spirochetes leave the tick gut and enter the salivary glands from where they invade the vertebrate host. OspC strongly binds the tick salivary glands. It plays an important role in the spirochete adhesion to this tissue. Such findings are in conflict with the conclusions from other studies [30].

3. 6. Crystal structure of OspC

The crystal structure of OspC was already resolved up to 2.5 Å [31]. Three-dimensional structure of OspC is mainly helical which is contrast with mainly β-sheets containing molecule of OspA. It consists of five helices – four long ones and a short one. Except helix 4 and 5, all are anti-parallel. Sequences of different OspC groups revealed variable regions located mostly in loops. It is assumed, that these variable regions form major antigenic sites. Big differences between OspC from invasive and non-invasive groups were found in their electrostatic potential. Electrostatic potential on the surface of OspC oriented to the extracellular space is highly negative in OspC from invasive strains. This characteristic may be involved in the binding of OspC to some positively charged ligand in the host. It is speculated, that this ligand can be fibronectin or some similar molecule. Indeed, the crystal structure of human fibronectin showed highly positively charged regions [32, 33].

3. 7. The genome of Borrelia burgdorferi

In 1997, the genome of *Borrelia burgdorferi* strain B31 was sequenced.

It consists of one linear chromosome containing 950 kilobases and more than 20 different linear and circular plasmids (twelve and nine, respectively) [34, 35]. Within the whole genome, more than one hundred sequences coding for different lipoproteins are present. Such large number is not present in any other organism [36].

The majority of genes encoding the spirochetal surface lipoproteins are located on linear plasmids - e. g. lp49 carries *ospA* and *ospB* in strain B31. This is not true for OspC which has its gene on a circular plasmid, cp27, in B31 strain [37 – 41].

The *ospC* genes in different strains (B31, PKo, PBi) are of different length and code for proteins composed of different number of amino acids. There are less conserved regions compared to other borrelial proteins such as flagellin [42].

The *ospC* gene is present also in the strains which do not express this lipoprotein [43].

The transcription of *ospC* gene was found to be dependent on environmental factors. Depending on the temperature, induction of OspC transcription is regulated by RpoS, an alternative sigma factor. RpoS is controlled by another alternative sigma factor, RpoN, which is activated by environmental signals [44, 45].

3. 8. Newly identified Outer surface proteins

Several new outer surface proteins have been identified in *Borrelia burgdoreri*. This group involves Bb0405, Bb0689, BbA36, BbA64, BbA66, BbA69 and BbI42. It was proved, that all these proteins are surface exposed. Except Bb0405, all other proteins are anchored to borrelial surface via N-terminal fatty acids. Experimental data suggest that Bb0405 is integrated into the outer membrane of spirochetes [46].

It was repetitively demonstrated, that the expression of BbA64 is tightly regulated with the presence of an alternative sigma factor, RpoS. A mutant of borrelia containing bba64-promoter regulated GFP showed, that the expression of GFP was correlated with the expression of OspC which is also dependent on the RpoS presence [47].

Expression of these seven proteins is positively influenced by temperature and environmental changes induced during the transmission of spirochetes from tick to vertebrate host. ELISA studies also showed immunogenicity of this group of proteins. During the infection, antibodies were produced against all seven proteins. All these data implicate importance of such newly determined outer surface proteins as virulence factors and potential candidates for Lyme disease vaccines [46].

4. Materials and Methods

4.1. Polymerase Chain Reaction (PCR)

DNA from *Borrelia burgdorferi* s.s. isolate B31 was amplified using primers from Generi Biotech (Hradec Králové). Primers were constructed using IDT SciTools Oligo Analyzer 3.1. The reaction components were purchased from Promega. All PCR reactions were run on Eppendorf Mastercycler.

Primer	**T_m**	**Sequence**
pTriEx OspC F (515 N7)	51.9 °C	5'- GAC GAC GAC AAG ATA AAA AAG AAT ACA TTA AGT - 3'
pTriEx OspC R STOP (515 N8)	58 °C	5' - GAG GAG AAG CCC GGT TAA GGT TTT TTT GGA CT - 3'
pTriEx OspC R His (515 N9)	60.6 °C	5' – GAG GAG AAG CCC GGT CCA GGT TTT TTT GGA CT – 3'
pTriEx OspC R His Ex1 (515 P0)	67 °C	5' – GAG GAG AAG CCC GGT CCA GGT TTG ACG ACG ACA TCA GG – 3'
pTriEx OspC R His Ex2 (515 P1)	56.9 °C	5'- GTT TGA CGA CGA CAT CAG GTT TTT TTG GAC TTT C - 3'

Tab. 1: List of applied primers, their sequences and melting temperatures

Product	**T_a**
STREP-Ex-OspC (Forw. N7 + Rev. N8)	56 °C
STREP-Ex-OspC-His (Forw. N7 + Rev. N9)	60 °C
STREP-Ex-OspC-Ex-His (Forw. N7 + Rev. P1)	64 °C
STREP-Ex-OspC-Ex-His (Forw. N7 + Rev. P0)	64 °C

Tab. 2: Overview of annealing temperatures for used primers

Component	Volume
PCR buffer	2 µl
dNTPs (2mM)	2 µl
MgCl2	2 µl
Taq Polymerase	0.1 µl
DNA	0.2 µl
Forward primer	1 µl
Reverse primer	1 µl
H20	11.7 µl

Tab. 3: Reaction mixture for one PCR reaction

Step	PCR program	Temperature	Time
Step 1	denaturation	94°C	4 min.
Step 2	denaturation	94°C	1 min.
Step 3	annealing	**X** °C	1 min.
Step 4	elongation	72°C	1 min.
Step 5	elongation	72°C	10 min.
Step 6	hold	14°C	X min.

Tab. 4: Overview of the PCR program. Steps 2-4 were repeated 30 times.

4.2. Agarose gel electrophoresis

After the PCR, samples were mixed with loading buffer (5X Orange DNA Loading Dye (Fermentas) with 5X Sybr Green (Amresco)). The samples were loaded onto a 1.7% agarose gel. Electrophoresis was performed at 135 V for 40 minutes. The DNA molecules were visualized on UV transilluminator. DNA samples of desired size were cut out of the gel.

4.3. DNA elution from agarose gel

Selected PCR products were used for further experiments. DNA was eluted using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) according to the manufacturer's instructions.

4. 3. 1. Sequencing reactions

Eluted DNA samples were sequenced on ABI Prism 3130 xL (Applied Biosystems) using corresponding primers at 50°C to confirm the desired sequence (see Appendix). Samples with the correct sequence were used for further experiments.

4.4. Transformation of the PCR product

Vector „pTriEx-5 Ek/LIC“ (Novagen) was used for the transformation into NovaBlue GigaSingles Competent Cells (Novagen) according to the manual.

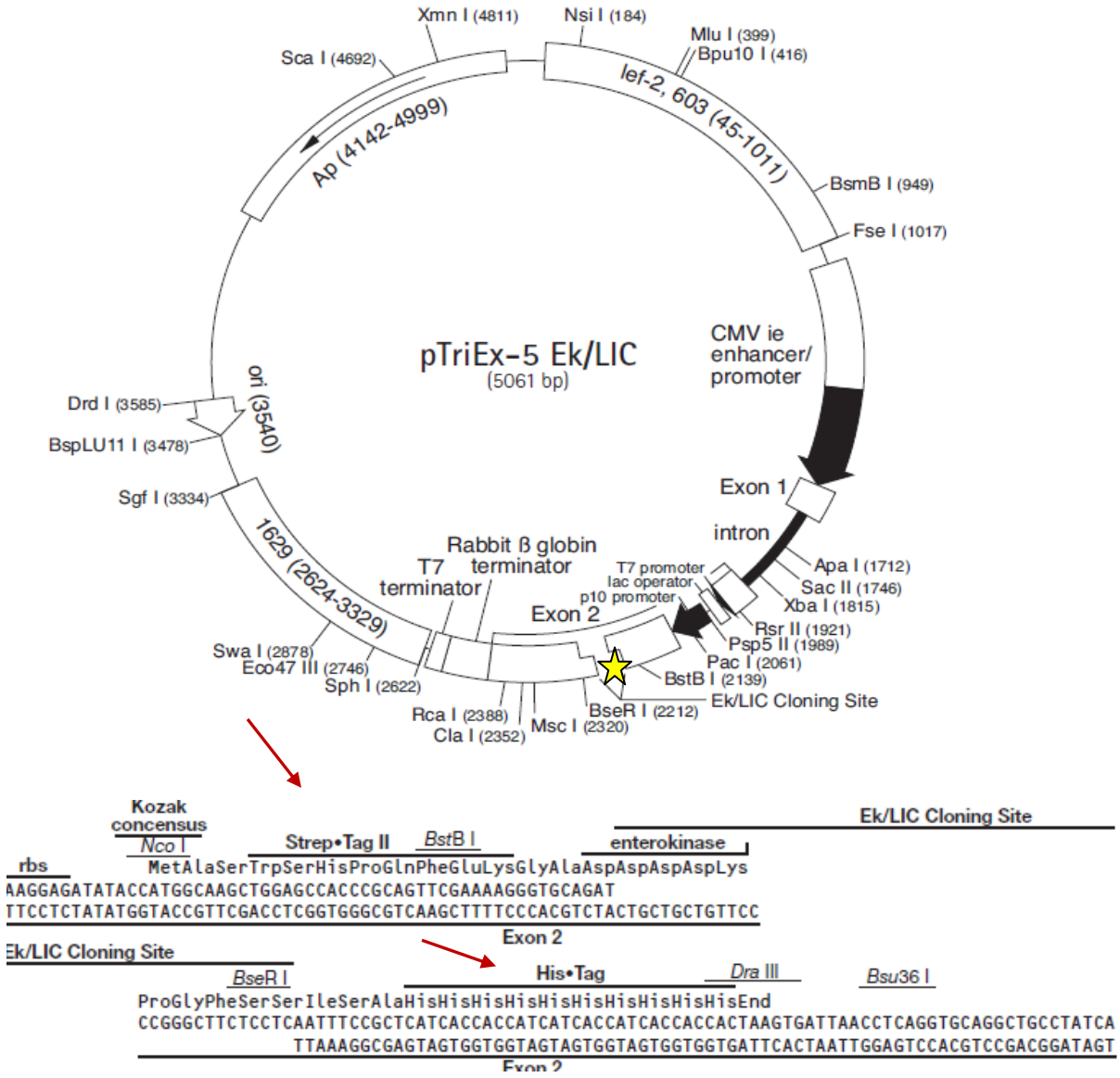

Figure 1: Map of TriEx – 5 Ek/LIC vector with highlighted cloning site and location of STREP-Tag II and His-Tag on the plasmid

Cells were cultured on agar plates (LB agar with Ampicillin - 50 µg/ml or LB agar with Carbenicillin - 50 µg/ml) at 37°C for 24 hours.

After the incubation colonies were submitted to Colony PCR (primers 515 N7 (Forw) and 515 N9 (Rev), annealing at 60°C). PCR products were then separated via agarose gel

electrophoresis (1.7%, 135 V, 40 min), viewed on UV transilluminator, cut off the gel, eluted and sequenced.

4.5. Transformation into expression cells

Colonies containing the correct insert were grown in LB medium and plasmids were isolated using GeneJET Plasmid Miniprep Kit (Fermentas). Next the purified plasmids were transformed into competent cells BL21 (Invitrogen) and Rosetta (Novagen) according to the standard protocol (pTriEx-5 Ek/LIC, Novagen).

Cells were cultured for 36 hours at 37°C (LB agar plates with Ampicillin - 50 µg/ml, LB agar plates with Carbenicillin - 50 µg/ml).

PCR of selected colonies was performed (Forw primer 515 N7 and Rev primer 515 N9, annealing at 60°C) and the presence of correct insert was verified by sequencing.

4.6. Pilot expression

Colonies containing the positive insert were grown at 37°C in TB medium for 12 hours . IPTG (Fermentas) was added to the final concentration of 1 mM. Cultures were collected immediately and after 1, 2, 3 and 4 hours.

4.7. SDS-PAGE

Pellets were mixed with 100 µl of 4x Loading Buffer with 0.8 M DTT (Fermentas). Samples were investigated by SDS-PAGE (15% separating gel, 5% stacking gel, PageRuler™ Unstained Protein Ladder (Fermentas)). Electrophoresis was performed at 125V for 90 minutes. Gel was stained using PageBlue™ Protein Staining Solution (Fermentas).

	15% separation gel	5% stacking gel
30% acrylamide/bisacrylamide mixture	2,5 ml	0,25 ml
4x Separation Buffer	1,25 ml	_
4x Stacking Buffer	_	0,25 ml
10% APS	0,05 ml	0,02 ml
TEMED	0,002 ml	0,002 ml
Distilled water	1,2 ml	1,48 ml

Tab. 5: Preparation of acrylamide gels for SDS-PAGE analysis

4.7.1. Ag – staining

If the Coomassie staining did not give satisfactory results, SDS-PAGE gel was furthermore stained with silver (Silver Staining Kit, Amersham Biosciences) to reveal protein which was not visible by PageBlue™ Protein staining. The procedure was performed according to the manufacturer's instructions.

4.8. Immunoblotting

After the SDS-PAGE proteins were electroblotted onto a PVDF membrane using Trans-Blot® Semi Dry Electrophoretic Transfer Cell (Bio-Rad). Blotting was performed according to the standard protocol for Western blot (20 V, 1 hour). At first membrane with transferred proteins was washed in distilled water and stained with Ponceau S for 5 minutes.

Afterwards the membrane was washed repeatedly in PBS buffer (pH 7.4) and incubated in blocking solution (5% non-fat dry milk in PBS) for 2 hours at room temperature. A mixture of primary monoclonal antibodies was used: BBM 45 directed against OspC protein and BBM 20, BBM 23, 5H4 M and 4C12/C2 against *Borrelia* (provided by Dr. Ian Livey, Baxter, Austria). Membrane was incubated overnight at 4°C in solution of antibodies (each diluted 1:2000 in 5% non-fat dry milk). Afterwards it was washed with PBS + 0.05% Tween-20 solution and incubated in secondary antibody (SwAM/Px, Vector) diluted 1:1000 in 5% non-fat dry milk for one hour at RT. Finally, the membrane was washed using PBS + 0.05% Tween-20 solution and developed using H_2O_2 / DAB solution.

4.8.1. SYPRO Ruby staining

Separated proteins were blotted onto a nitrocellulose membrane using Trans-Blot® Semi Dry Electrophoretic Transfer Cell (Bio-Rad) under 20 V for 1 hour. The membrane was stained using SYPRO ® Ruby protein blot stain (Bio-Rad) according to the manual. Membrane was afterwards visualized on UV-Transilluminator and was further used.

4.9. Affinity chromatography

4.9. 1. NiMAC

NTA agarose resin (Qiagen) was charged according to the manual with Ni_2SO_4 solution. Cytosolic extract was prepared using BugBuster™ Protein Extraction Reagent (Novagen)

according to the given instructions. Purification of the protein was performed using BioLogic LP System (Bio-Rad).
Manual and gradient programs with Wash buffer (20 mM imidazole with PBS) and Elution buffer (200 mM imidazole with PBS) were applied to get elution of the protein. Eluates were precipitated in 4x the sample volume of ice-cold acetone overnight. After centrifugation the dry pellets were kept for SDS-PAGE.

4.9.2. CoMAC

NTA agarose resin was charged with Co^{2+} using Co_2SO_4. Lysate was prepared as in 2.9.1. Gradient program under the same conditions as in NiMAC was used. Eluates were precipitated overnight in 4x sample volume of ice-cold acetone and afterwards centrifuged. Pellets were saved for further analysis by SDS-PAGE.

4.9.3. StrepTactin chromatography

Cytosolic extract was prepared similarly to samples for Co and Ni chromatography (using BugBuster™ Protein Extraction Reagent, Novagen). StrepTactin Sepharose column was prepared and affinity chromatography was performed according to the given protocol (Strep Tactin Sepharose High Performance, GE Healthcare). Eluates were precipitated using ice-cold acetone (4x sample volume) and pellets were subjected to SDS-PAGE analysis.

4. 10. Mass spectrometry

Very strict criteria were used for protein identification (Table 6). Purified proteins were trypsin-digested and the resulting peptides were subjected to LC-MS analysis. Peptides were separated by HPLC (Dynex) on C18 modified silica using acetonitrile gradient (5-80%) as mobile phase and ionized in ESI source. They were analyzed in FT- MS (Thermo Fischer) mass spectrometer. The resulting data were compared to Swiss-Prot and *Borrelia* non-redundant protein databases using MASCOT. The analyses were performed at NCGG, Indiana University, Bloomington, IN, USA.

peptide mass	+/- 0.03 Da
charges	+1,2,3,4
ion score	≥ 30
expect	≤ 0.8
precursor intensity	> 10000
min. rank	1
min. mass	300 Da
min. length	0 AA

Tab. 6: Criteria used for peptide identification

5. Results:

5. 1. Agarose gel electrophoresis

DNA from B31 strain of *Borrelia burgdorferi* was amplified via PCR using designed primers. From the first PCR only STREP-Ex-OspC-His product at two different annealing temperatures was obtained (Figure 2). For further experiments product formed at higher annealing temperature (60°C) was used. It is clearly seen (Figure 2) that STREP-Ex-OspC was not formed. This was due to wrong annealing temperature; therefore, different conditions were chosen for next PCR.

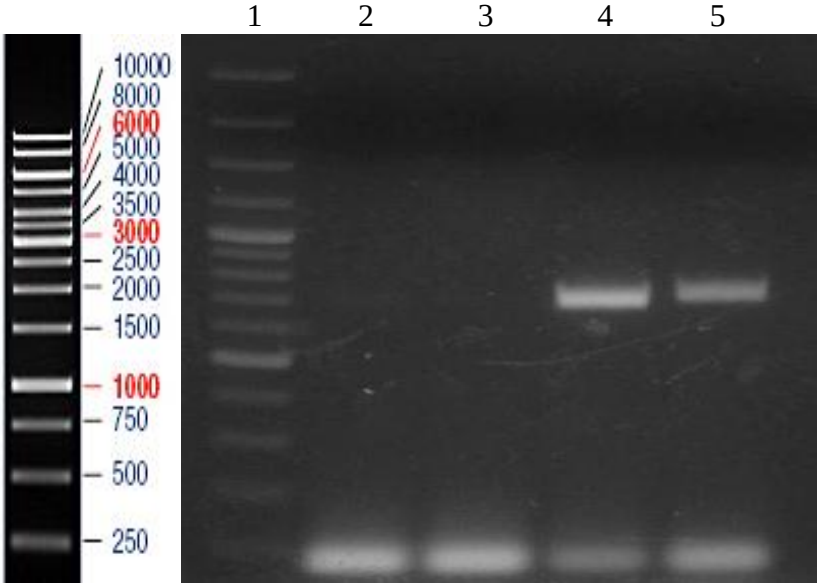

Figure 2: Results from the first agarose gel electrophoresis of DNA segments amplified by PCR. Lane 1 = O'GeneRuler^{TM} 1kb DNA ladder (Fermentas), Lanes 2, 3 = "STREP-Ex-

OspC" (annealing at 58°C and 60°C, respectively – no product); Lanes 4, 5 = "STREP-Ex-OspC-His" (annealing at 58°C and 60°C, respectively – two products).

Second PCR was performed to obtain STREP-Ex-OspC-His and STREP-Ex-OspC-Ex-His products. For the first one, two distinct conditions were used – annealing at 54°C and 56°C, respectively. In both cases, the desired product was formed (Figure 3) although not in big amount.

To obtain STREP-Ex-OspC-Ex-His product, DNA was first amplified using 515 N7 (Forw.) and 515 P1 (Rev.) primers (see Figure 3). Such product was subjected to additional PCR with 515 N7 as Forw. and 515 P0 as Rev. primers to get enterokinase cleavage site on both sides of OspC sequence. Two different annealing temperatures (62 and 64°C) were used. The product was obtained in same amount and size in both conditions (Figure 4).

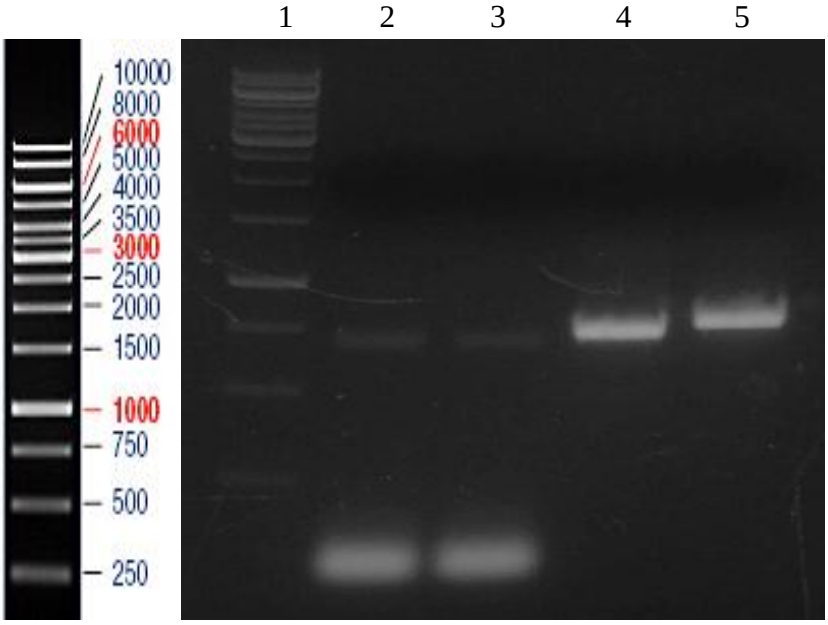

Figure 3: Second agarose gel electrophoresis. Lane 1 = O'GeneRuler^{TM} 1kb DNA ladder (Fermentas), Lanes 2-3 = "STREP-Ex-OspC" (annealing at 54°C and 56°C, respectively – two products); Lanes 4-5 = "STREP-Ex-OspC-Ex-His" (annealing at 62°C and 64°C, respectively – two products).

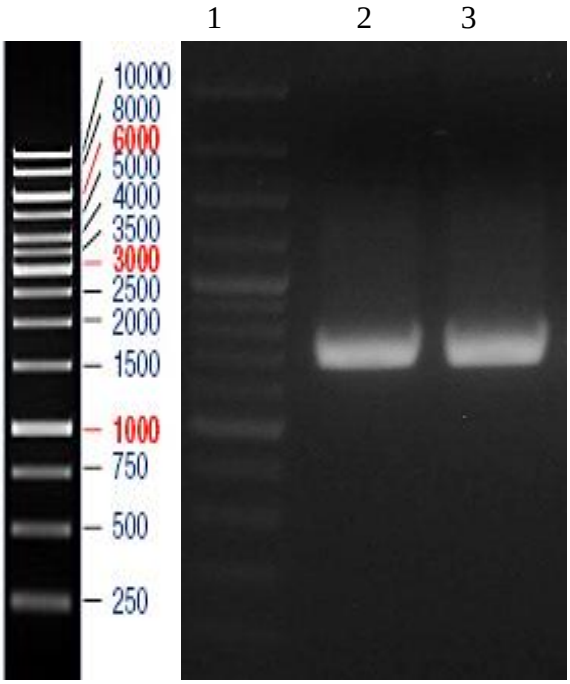

Figure 4: Results from agarose gel electrophoresis of STREP-Ex-OspC-Ex-His construct DNA amplified by PCR. Lane 1 = O'GeneRuler^{TM} 1kb DNA ladder (Fermentas), Lanes 2, 3 = "STREP-Ex-OspC-Ex-His" (annealing temperatures 62℃ and 64°C).

5. 2. Transformation of competent cells

A representative sample of 20 colonies (10 grown on agar plate with Carbenicillin (50 µl/ml) and 10 on agar plates with Ampicillin (50 µl/ml), respectively) from each "STREP-Ex-OspC", "STREP-Ex-OspC-His" and "STREP-Ex-OspC-Ex-His" was selected. DNA from these colonies was further amplified by PCR with corresponding primers. Results from agarose gel electrophoresis showed that only one colony contained the construct – STREP-Ex-OspC-His (Figure 5). Transformation of the two other constructs was not successful (data not shown).

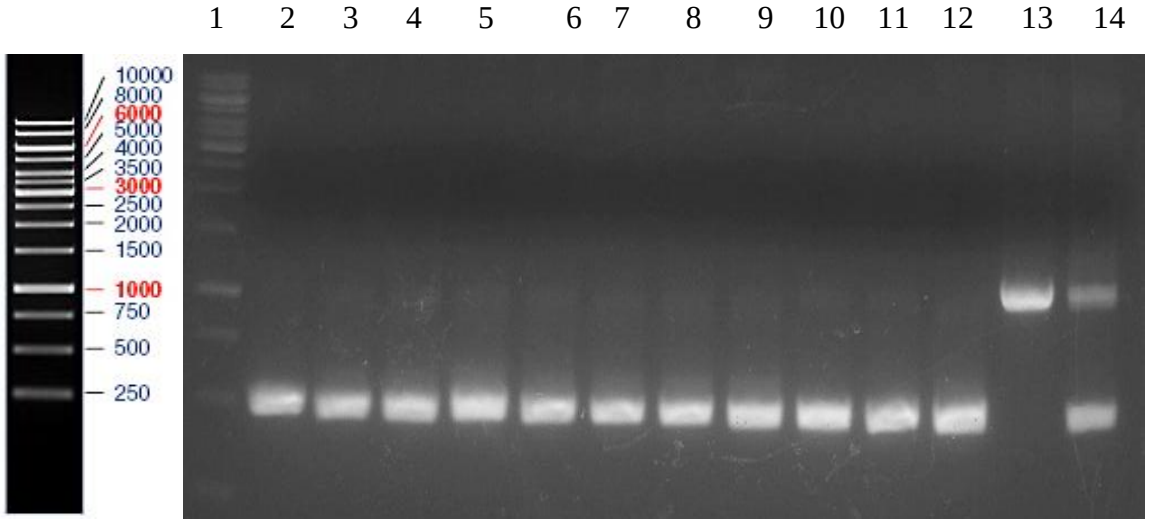

Figure 5: Agarose gel electrophoresis of Colony PCR reactions of transformed colonies. Lane 1 = O'GeneRuler^{TM} 1kb DNA ladder (Fermentas); Lane 2 – 12 = "STREP-Ex-OspC" transformants; Lane 13, 14 = "STREP-Ex-OspC-His" transformants with the construct (colonies grown on agar plate with carbenicillin)

5. 3. Transformation of expression cells

Sample of culture containing the insert was used for the transformation into the expression cells – BL21 (Invitrogen) and Rosetta (Novagen). The cells were cultivated (LB agar plates with Carbenicilin - 50 µl/ml) at 37°C for 36 hours. Transformation into Rosetta cells did not give positive results since the cells did not grow (data not shown). BL21 colonies were subjected to PCR with corresponding primers. Results from the agarose gel electrophoresis are given below (Figure 6). It was proved that the cultured expression cells contained the desired construct.

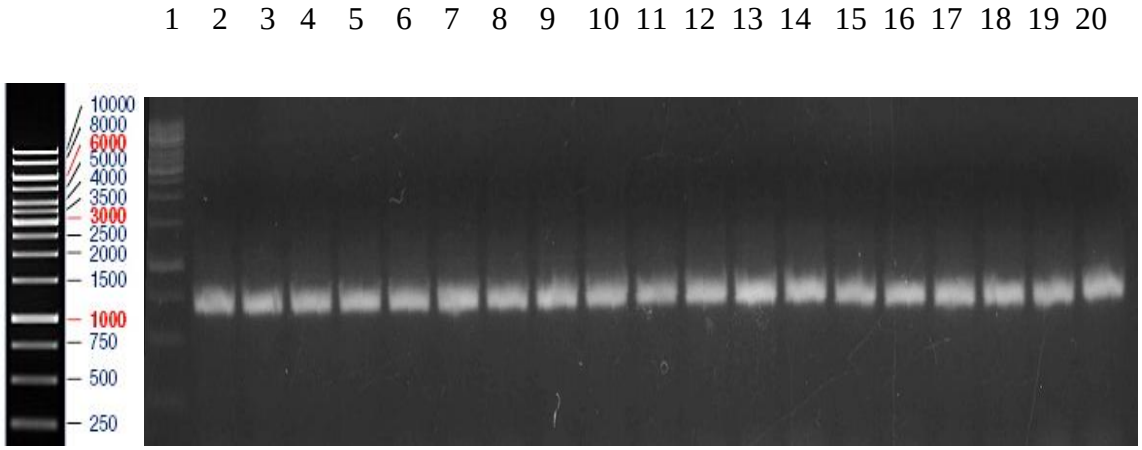

Figure 6: Agarose gel electrophoresis of DNA from selected BL21 colonies amplified by Colony PCR. Lane 1 = DNA ladder; Lanes 2 – 20 = Colonies of transformed BL21

5. 4. Pilot expression

IPTG was added to grown cultures of BL21 cells to induce the expression of rOspC. Bacteria were harvested after 0 (control), 1, 2, 3 and 4 hours. Subsequent SDS-PAGE analysis (15% separating gel, 5% stacking gel, PageRuler™ Unstained Protein Ladder (Fermentas)) showed that rOspC was expressed in all cases in the same amount except sample after 3 hours of induction (Figure 7). This was caused by loading lower amount of sample onto the gel.

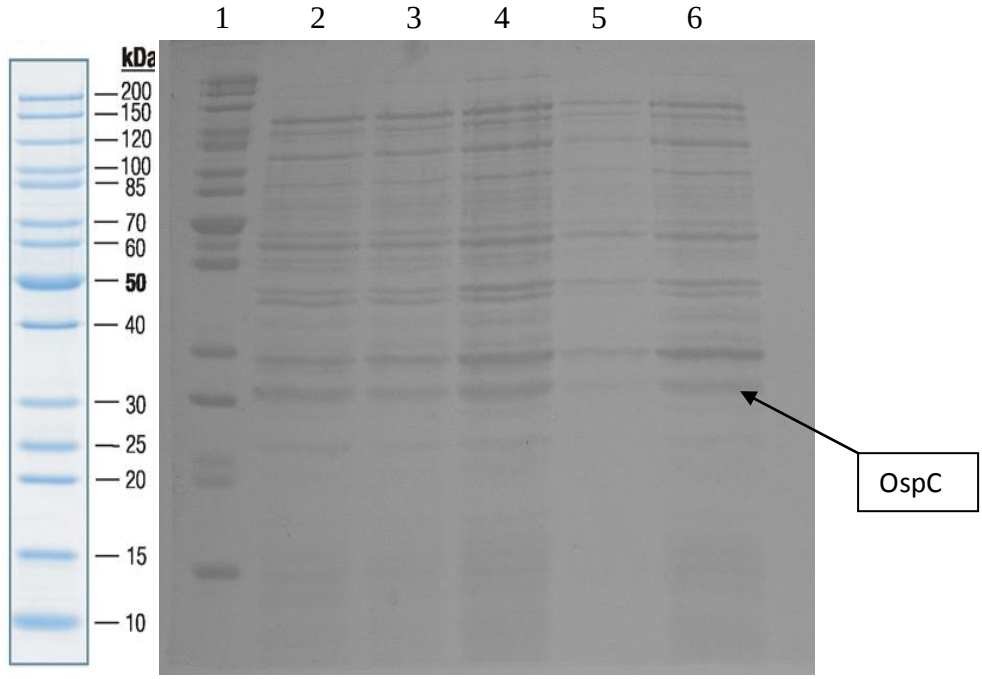

Figure 7: SDS-PAGE of cultured BL21 after induction with ITPG. Stained with PageBlue™ Protein Staining Solution (Fermentas). Lane 1 = PageRuler™ Unstained Protein Ladder (Fermentas); Lane 2 = control (0 h induction); Lane 3 = 1 h induction; Lane 4 = 2 h induction; Lane 5 = 3 h induction; Lane 6 = 4 h induction

5. 5. Immunoblotting

Detection of rOspC via immunoblotting did not give positive results (data not shown). Ponceau S stained the protein bands, however, none of the primary antibodies recognized the protein. The method was repeated multiple times with the same outcome. This is probably caused by variable epitopes found in OspC.

5. 6. SDS-PAGE

To verify the presence of OspC in cytosol and inclusion bodies, SDS-PAGE of both samples was performed. Analysis showed that OspC is found in cytoplasm and it was also detected in inclusion bodies (see Figure 8). Therefore both, cytosolic extract and inclusion bodies, were used for purification by chromatography.

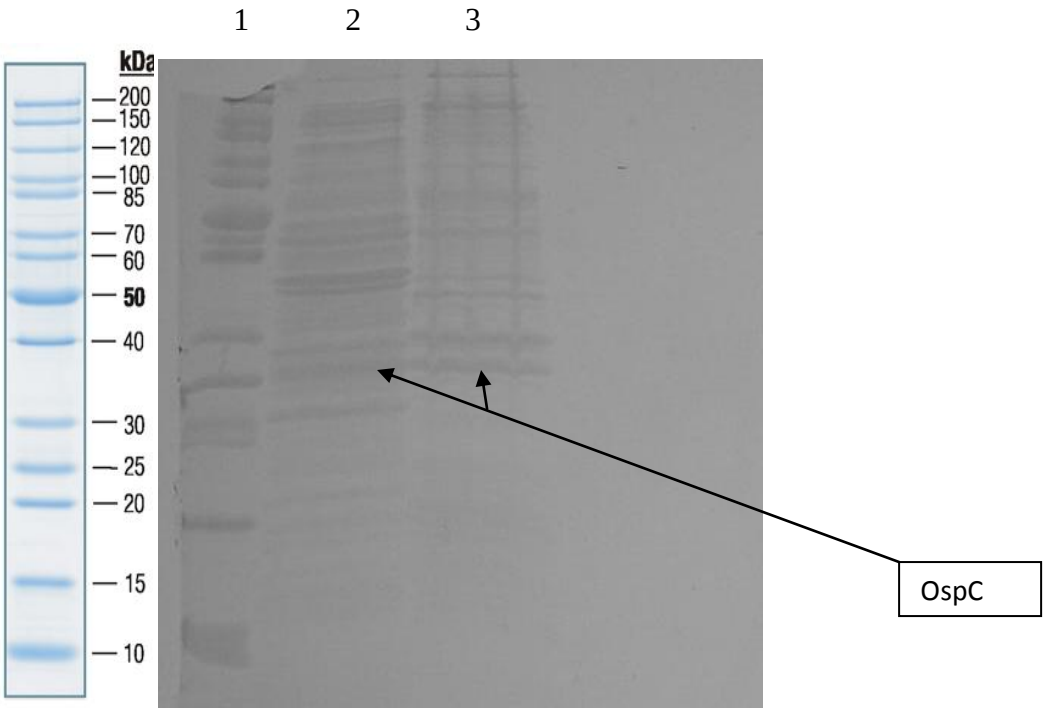

Figure 8: SDS-PAGE of cytosolic extract and inclusion bodies from the cultured cells. Stained with PageBlue™ Protein Staining Solution (Fermentas). Lane 1 = PageRuler^{TM} Unstained Protein Ladder (Fermentas); Lane 2 = cytosolic extract; Lane 3 = inclusion bodies

5. 7. Affinity chromatography

5. 7.1. NiMAC:

NiMAC purification of the inclusion bodies did not give any purified protein (data not shown). The experiment was repeated with the same result. Therefore, for next purification methods, only cytosolic extract which is more feasible was used.

Eluates from the NiMAC of cytosolic extract together with flow-through and EDTA fractions were investigated by SDS-PAGE analysis (15% separating gel, 5% stacking gel). Fraction of purified protein was detected in eluates 5 and 6 (Figure 9) in the range of 175 - 200 mM imidazole. Nevertheless the protein amount was not very satisfactory.

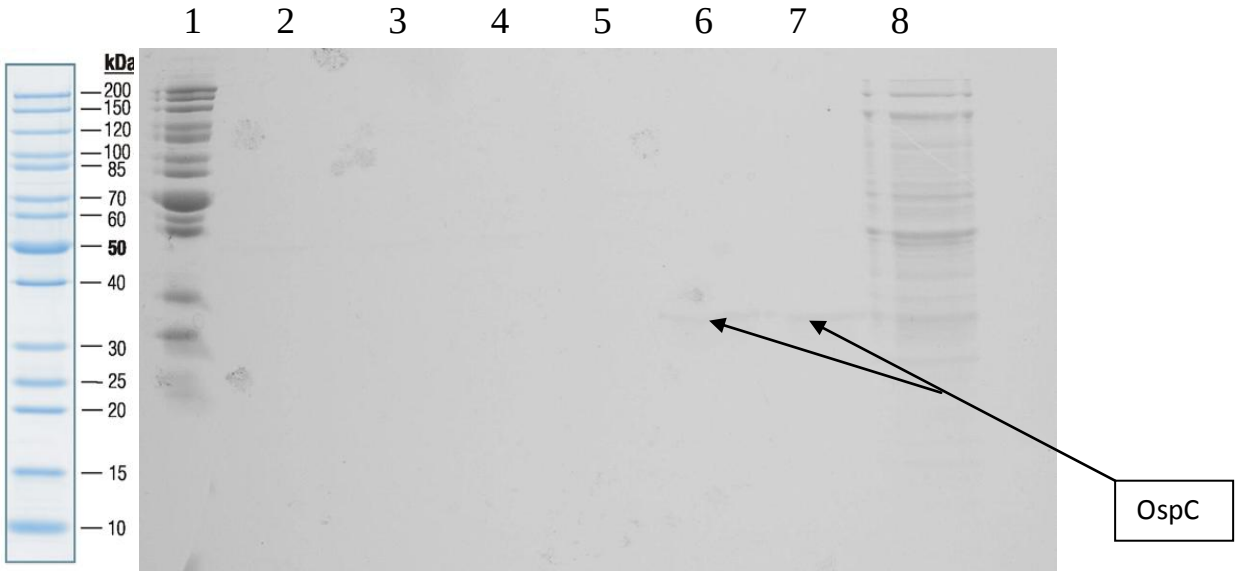

Figure 9: SDS-PAGE analysis of eluates from NiMAC. Stained with PageBlue™ Protein Staining Solution (Fermentas). Lane 1 = PageRuler^{TM} Unstained Protein Ladder (Fermentas); Lane 2 = El. 1 (20 – 50 mM imid.); Lane 3 = El. 2 (50 – 90 mM imid.); Lane 4 = El. 3 (90 – 135 mM imid.); Lane 5 = El. 4 (135 – 175 mM imid.); Lane 6 = El. 5 (175 – 200 mM imid.); Lane 7 = El. 6 (200 mM imid.); Lane 8 = flow-through.

5. 7.2. CoMAC:

Eluates from CoMAC together with flow-through and EDTA fractions were subjected to SDS-PAGE. This analysis did not give sufficient results (see Fig. 10). Protein bands were not visible by Coomassie staining and the protein amount was low. Therefore the gel was visualized with silver staining. However, staining with silver solution did not reveal any purified protein (data not shown).

Samples were investigated also by blotting onto a nitrocellulose membrane. Blotted membrane was visualized using SYPRO Ruby stain (Figure 11). A small fraction of purified protein can be seen in eluate 3.

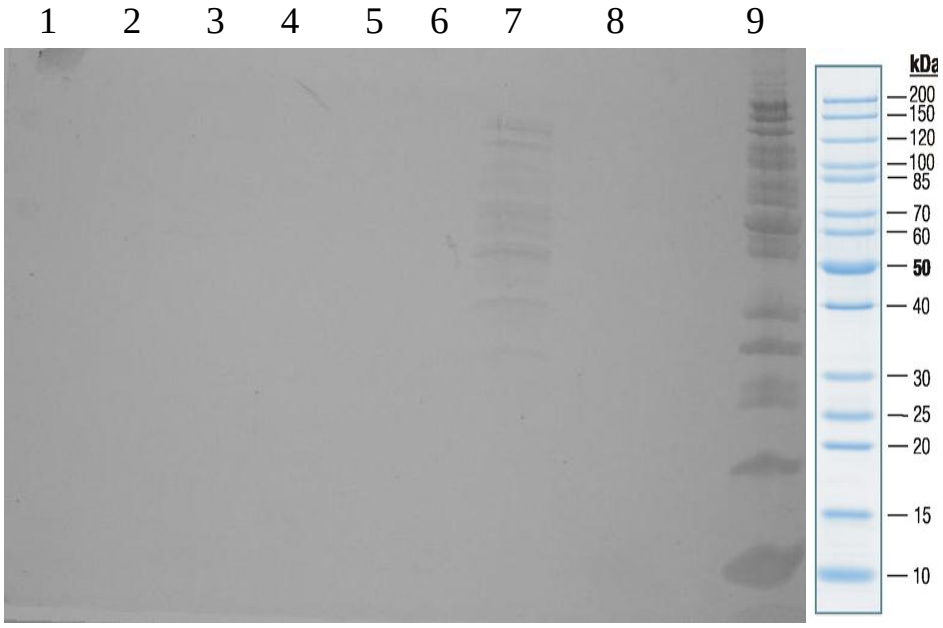

Figure 10: SDS-PAGE results of the eluates from CoMAC (cytosolic extract). Stained with PageBlue™ Protein Staining Solution (Fermentas). Lane 1= El. 1 (20 - 25 mM imidazol); Lane 2 = El. 2 (25 - 65 mM imid.); Lane 3 = El. 3 (65 - 105 mM imid.); Lane 4 = El. 4 (105 - 150 mM imid.); Lane 5 = El. 5 (150 – 190 mM imid.); Lane 6 = El. 6 (190 – 200 mM imid.); Lane 7 = flow-through; Lane 8 = EDTA fraction; Lane 9 = PageRuler^{TM} Unstained Protein Ladder (Fermentas).

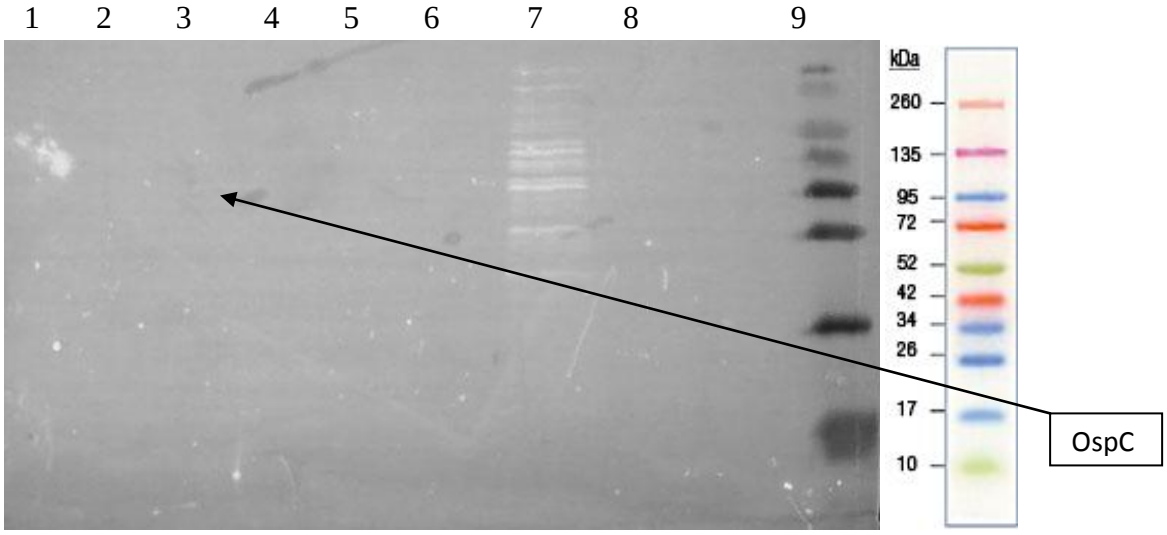

Figure 11: Nitrocellulose membrane stained with SYPRO ® Ruby protein blot stain (Bio-Rad). Lanes 1-6 = eluates 1-6; Lane 7 = flow-through; Lane 8 = EDTA fraction; Lane 9 = Spectra^{TM} Multicolor Borad Range Protein Ladder (Fermentas). Small protein band can be found in Lane 3.

Since data from SYPRO Ruby detection suggested the presence of eluted protein, CoMAC was repeated. In second experiment, the purified protein was obtained in significantly higher amount (Figure 12) also when compared to NiMAC purification.

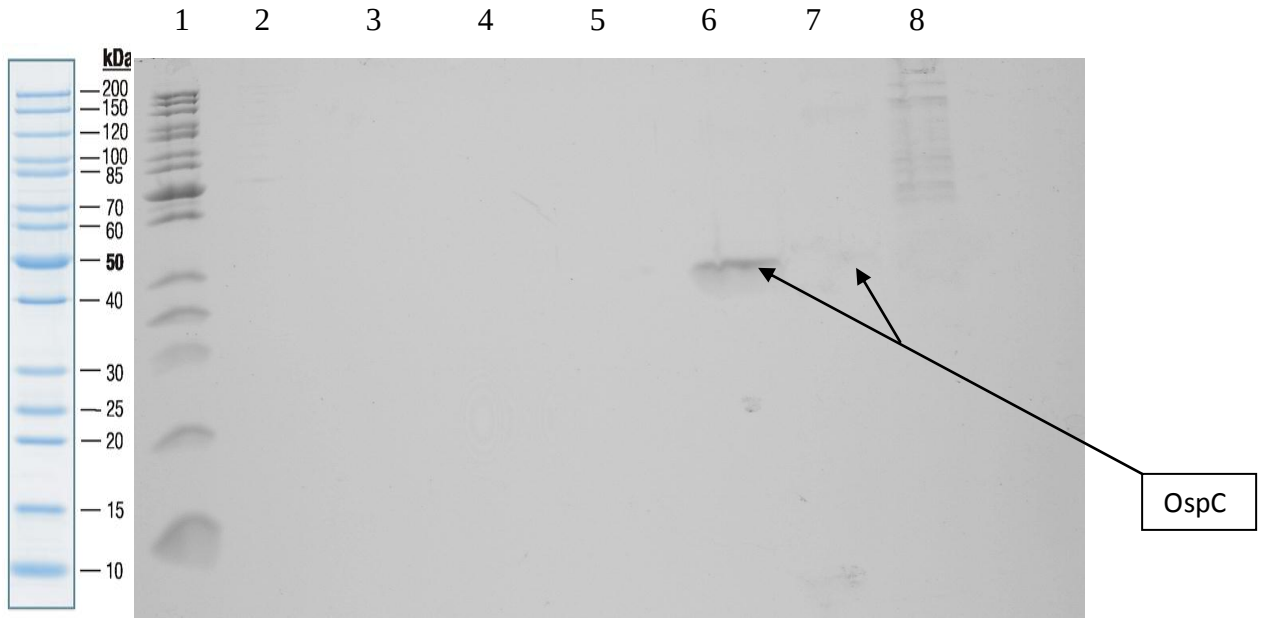

Figure 12: SDS-PAGE gel of CoMAC (cytosolic extract). Stained with PageBlue™ Protein Staining Solution (Fermentas). Lane 1 = PageRuler^{TM} Unstained Protein Ladder (Fermentas); Lane 2 = El. 1 (20 – 40 mM imid.); Lane 3 = El. 2 (40 – 60 mM imid.); Lane 4 = El. 3 (60 – 100 mM imid.); Lane 5 = El. 4 (100 – 140 mM imid.); Lane 6 = El. 5 (140 – 180 mM imid.); Lane 7 = El. 6 (180 – 200 mM imid.); Lane 8 = flow through. In Lanes 6 and 7 a fraction of purified protein is seen.

5. 7. 3. StrepTactin chromatography

The last purification method was Strep-Tactin chromatography (StrepTactin Sepharose High Performance, GE Healthcare). The protocol for Strep-Tactin purification is much faster compared to metal affinity chromatography. The quantity of pure protein was incomparable with CoMAC and NiMAC (Figure 13).

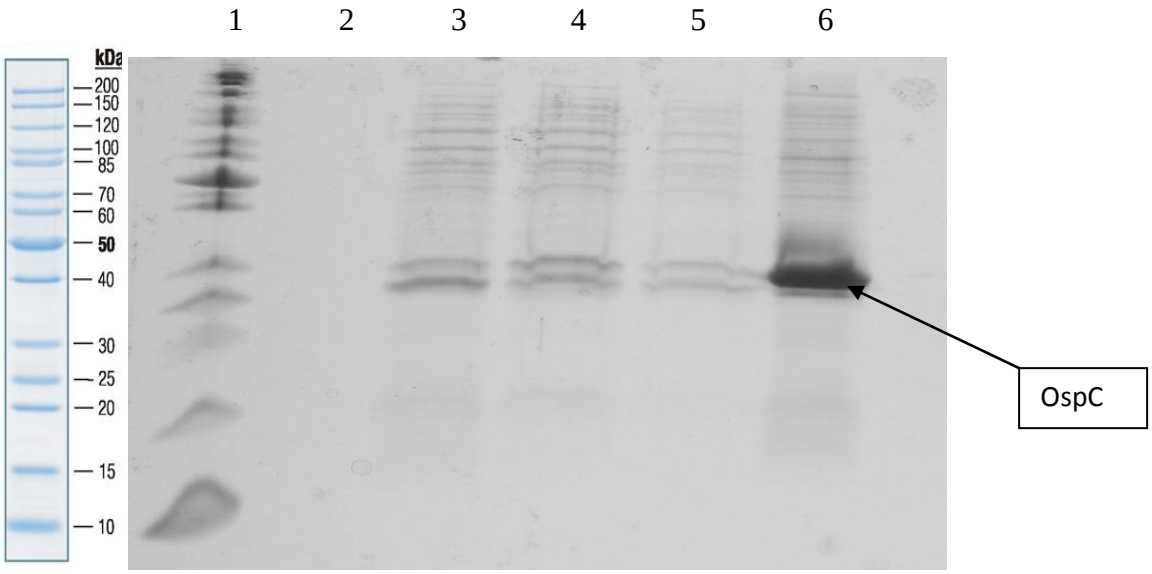

Figure 13: SDS-PAGE of eluates from Strep-Tactin chromatography. Stained with PageBlue™ Protein Staining Solution (Fermentas). Lane 1 = PageRuler^{TM} Unstained Protein Ladder (Fermentas); Lanes 2 – 6 = eluates 1 – 5. Purified protein is clearly seen in Lane 6.

5. 8. Mass spectrometry

The affinity-purified proteins from all three purif. methods were analyzed via mass spectrometry. Each purified protein was confirmed as OspC. Coverage of OspC sequence was different in every sample. Biggest number of peptides was detected in the sample from Strep-Tactin chromatography and smallest in the NiMAC sample. Total coverage was 57.065%. All MS-identified peptides were 100% identical to the OspC protein amino acid sequence from B31.

Purification Method	**Score**	**Determined Queries**	**Coverage**
NiMAC	1356	20	30.435%
CoMAC	6384	108	45.109%
Strep-Tactin	18755	334	55.978%

Tab. 6: Results from MS analysis

1 mkkntlsail mtlflfiscn nsgkdg **ntsa nsadesvkgp nlteiskkit dsnavllavk**

61 **eveallssid eiaak**aigkk ihqnngldte nnhngsllag ayaistlik**q kldglknegl**

121 **k**ekidaakkc setftnklke k**htdlgkegv tdadak**eail ktngtktk**ga eelgklfesv**

181 **evlsk**aakem lansvk**elts pvvaespk**kp

Figure 14: OspC sequence. Underlined segment represents the signal peptide which was not used in recombinant. Bold amino acids were determined by mass spectrometry.

6. Discussion

Main goals of my work included construction of corresponding primers for amplification of ospC gene using PCR. Further steps involved transformation of bacterial cells and preparation of expression system for rOspC. Next objective was to perform pilot expression of the recombinant protein and purify it using several different affinity chromatography techniques. Sequences of all products were confirmed by DNA sequencing and composition of the purified protein was checked via liquid chromatography coupled mass spectrometry.

6.1 Polymerase Chain Reaction

Three different products were produced: "STREP-Ex-OspC", "STREP-Ex-OspC-His" and "STREP-Ex-OspC-Ex-His". The signal peptide sequence of OspC was not used in any case.

"STREP-Ex-OspC" was formed using two annealing temperatures – 54 and 56°C. In both cases the product was obtained in very low concentration. This could be caused by improper interactions of primers with template DNA. Another possibility is to lower the annealing time to 30-45 seconds. For cloning, the product formed at higher annealing temperature (56°C) was used.

Second PCR product, "STREP-Ex-OspC-His" was prepared in desired amount at both 62 and 64°C. For further steps product formed at 64°C was used due to its higher stability.

In last case, the product was prepared stepwise to get desired sequence. First, a DNA segment of OspC having enterokinase recognition site on one side was prepared by PCR (annealing at 64°C). This product was amplified using additional reverse primer to get enterokinase sequence also on the other terminus of OspC (PCR conditions were same as in first reaction).

6.2 Transformation

All three PCR products were ligated into the pTriEx-5 Ek/LIC vector. It is an universal vector which could be used in different expression systems. It is used for expression in *E. coli* as well as in vertebrate and insect cells. In our case, cells of *E. coli* (BL21, Invitrogen; Rosetta, Novagen) were used.

In the case of "STREP-Ex-OspC" construct, product having an enterokinase region on one end and a stop codon on the second end was ligated into the vector. This product had therefore only STREP-tag which is located upstream the vector cloning site. However, ligation of this construct into the vector was not successful.

"STREP-Ex-OspC-His" was designed to contain two distinct tags. STREP tag is included at N-terminal sequence in the cloning vector. His tag is on the other hand located on C-terminal. Such constituted sequence codes for recombinant OspC which could be purified by two different chromatographic methods. This presents a very potent tool for protein purification. Agarose gel electrophoresis revealed that only this was the only positive insert and was further used for transformation into expression cells.

Last option of rOspC preparation was to use enterokinase recognition region on both sides of OspC sequence. This precaution allows cleavage of both tags to get the pure OspC protein. Resulting "STREP-Ex-OspC-Ex-His" has the same advantages in purification modes like the previous product. Nevertheless, cloning of this construct was not effective.

E. coli strains BL21 and Rosetta were chosen for expression of the recombinant protein. Ek/LIC vector regulates the expression in these cells by T7 *lac* promoter which is located upstream the Strep tag. Successful transformation into the expression cells was achieved only in BL21. This strain of *E. coli* was previously proved to be suitable for rOspC expression [48].

6.3 SDS-PAGE

Before the purification, both the cytosolic extract and inclusion bodies were investigated via SDS-PAGE. Results showed that rOspC was present in the cytosolic extract and it was also visible in inclusion bodies. These data are in accordance with the assumption that rOspC which is expressed in BL21 is mainly found in soluble form in cytoplasm [49].

6.4 Immunoblotting

Immunoblotting using mixture of monoclonal antibodies against OspC did not yield positive results. Since SDS-PAGE and membrane staining with Ponceau S and also SYPRO Ruby revealed protein bands the problem lies in the selection of primary antibodies. It was previously shown that these antibodies do not recognize OspC from B31 isolate, only the OspC from CB53 strain of *B. burgdorferi* s. l. (personal communications, Ján Štěrba). This phenomenon is very likely caused by immunodominant epitopes in OspC which are mainly located in hypervariable regions and which can vary between the different strains [50]. Thus the monoclonal antibody recognizing only one specific epitope of the protein from CB53 may not recognize the similar epitope in B31 strain OspC.

6.5 Affinity chromatography

For the purification of rOspC, affinity chromatography was used. Since the resulting protein carried two tags, Strep and His, two kinds of purification techniques were utilized.

First type of chromatography was Metal Affinity Chromatography (MAC). This method made use of the His tag affinity to nickel and cobalt ions. Since SDS-PAGE showed rOspC in cytosolic extract as well as in inclusion bodies, both samples were used for the purification. However, the purification from inclusion bodies turned out to be very inconvenient and gave no results. Therefore only the cytosolic extract was used for further experiments.

NTA resin was charged first with Ni^{2+} and the chromatography was performed using manual and gradient program with Wash and Elution buffer. Pure rOspC was eluted in the range of 174 – 200 mM imidazole. Protein was analyzed via SDS-PAGE. The size of the purified protein was between 26 – 27 kDa which is in accordance with the theoretical size (OspC = 22,3 kDa; STREP tag = 2 kDa; His tag = 2 kDa → rOspC = 26,3 kDa). Amount of rOspC obtained was very low.
Next, CoMAC under the same conditions was performed. SDS-PAGE gel stained with

PageBlue™ Protein Staining Solution did not reveal any protein band. To check whether the rOspC was purified even in low concentration the gel was stained with silver. Unfortunately this method did not work as well. Samples were therefore subjected to SDS-PAGE and blotted onto a nitrocellulose membrane. Membrane was stained with SYPRO ® Ruby protein blot stain (Bio-Rad) which developed a small protein band of approximately 27 kDa. This protein fraction was eluted at 65 – 105 mM imidazole. This is significantly lower imidazole concentration compared to values from NiMAC.
For more satisfactory results second CoMAC experiment was performed. This gave a purified rOspC which eluted at 140 – 200 mM imidazole. Concentration of the protein was higher than in the sample from the first CoMAC assay and also from NiMAC.
A reason why the elution of pure protein was not very satisfactory in MAC is that the imidazole concentration was low. Recent experiments showed that the pure rOspC is eluted around at least 250 mM imidazole [48]. Another possibility how to increase the yield is the use of different resin. It was shown that in case of His tagged OspC use of Ni^{2+} charged HiTrap™ Chelating Column (Amersham Biosciences) gave the best results [48].

Another possibility how to purify rOspC was to utilize STREP tag affinity to Strep-Tactin Sepharose. This method was three times faster than MAC. The purification was accomplished yielding the highest amount of pure protein. Size of the protein was between 26 and 27 kDa.

From the given data, it is clearly seen, that for the purification of rOspC Strep-Tactin affinity chromatography is most suitable. It is less time consuming and the protein obtained is of a good purity and high concentration.

For future purification experiments it would be convenient to combine CoMAC and Strep-Tactin purification.

6.4 Mass spectrometry

Purity of the proteins from all three chromatographies was confirmed by means of LC-MS. As it was assumed, best result was achieved in the sample after Strep-Tactin affinity chromatography. The number of determined peptides was 334. For samples from NiMAC and CoMAC it was 20 and 108, respectively. These findings support the advantages of Strep tag related purification methods.

7. Conclusion

The gene of OspC from B31 *B. burgdorferi* was successfully inserted into the vector and cloned in bacterial cells. The desired rOspC was expressed by BL21 and used for the affinity chromatography purification. The protein was purified by all three chromatography methods with the best yield from Strep-Tactin chromatography. Protein samples were confirmed to be rOspC by HPLC-MS analysis.

8. Appendix

Protein	Coordinates	Nucleotide sequence
OspC	16903 - - 17535	5‘ – ATG AAA AAG AAT ACA TTA AGT GCA ATA TTA ATG ACT TTA TTT TTA TTT ATA TCT TGT AAT AAT TCA GGG AAA GAT GGG AAT ACA TCT GCA AAT TCT GCT GAT GAG TCT GTT AAA GGG CCT AAT CTT ACA GAA ATA AGT AAA AAA ATT ACG GAT TCT AAT GCG GTT TTA CTT GCT GTG AAA GAG GTT GAA GCG TTG CTG TCA TCT ATA GAT GAA ATT GCT GCT AAA GCT ATT GGT AAA AAA ATA CAC CAA AAT AAT GGT TTG GAT ACC GAA AAT AAT CAC AAT GGA TCA TTG TTA GCG GGA GCT TAT GCA ATA TCA ACC CTA ATA AAA CAA AAA T TA GAT GGA TTG AAA AAT GAA GGA TTA AAG GAA AAA ATT GAT GCG GCT AAG AAA TGT TCT GAA ACA TTT ACT AAT AAA TTA AAA GAA AAA CAC ACA GAT CTT GGT AAA GAA GGT GTT ACT GAT GCT GAT GCA AAA GAA GCC ATT TTA AAA ACA AAT GGT ACT AAA ACT AAA GGT GCT GAA GAA CTT GGA AAA TTA TTT GAA TCA GTA GAG GTC TTG TCA AAA GCA GCT AAA GAG ATG CTT GCT AAT TCA GTT AAA GAG CTT ACA AGC CCT GTT GTG GCA GAA AGT CCA AAA AAA CCT TAA – 3‘

Tab. 7: OspC sequence from *Borrelia burgdorferi* B31 (retrieved from NCBI database)

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