Anaplasma marginale outer membrane protein vaccine candidates are conserved in North American and South African strains

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ABSTRACT

Bovine anaplasmosis is a globally economically important tick-borne disease caused by the obligate intraerythrocytic rickettsia, *Anaplasma marginale*. A live *Anaplasma centrale* blood-based vaccine is available, but it does not protect against all *A. marginale* field strains and may also transmit other blood-borne pathogens. Five potential outer membrane protein (OMP) vaccine candidates have been well-characterised in *A. marginale* strains from the USA, however, their levels of conservation in other countries must be ascertained in order to inform their use in a vaccine with regional or global efficacy. This study assessed the amino acid variation in vaccine candidate OMPs in South African strains of *A. marginale*, and also compared the immunogenic properties between South African and US strains. OMP genes *Am779, Am854, omp7, omp8* and *omp9* were amplified and sequenced from a set of genetically diverse South African samples with different *msp1*α-genotypes. OMPs Am854 and Am779 were highly conserved, with 99–100% amino acid identity, while Omp7, Omp8 and Omp9 had 79–100% identity with US strains. As has been shown previously, Omp7–9 possess conserved N- and C- termini, a central variable region, and a highly conserved CD4 T-cell epitope, FLLVDDA(I/V)V, in the N-terminal region. Western blot analysis of recombinant OMPs indicates strong antigenic conservation between South African and US strains of *A. marginale*, suggesting that they are good candidates for use in a novel global vaccine cocktail, although further work on the best formulation and delivery methods will be necessary.

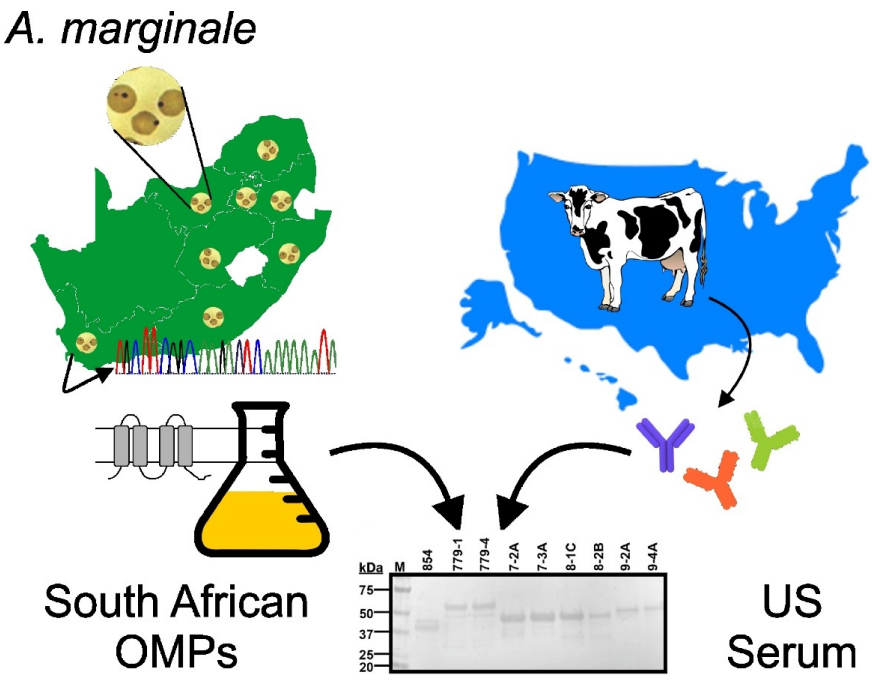
Keywords:

Bovine anaplasmosis, *Anaplasma marginale*, *Anaplasma centrale*, outer membrane proteins, vaccine candidates

Highlights:

- South African *Anaplasma marginale* outer membrane protein (OMP) vaccine candidates were compared to their US counterparts
- There is high identity among US and South African OMPs despite geographic and strain diversity
- OMPs 7, 8 and 9 have a highly conserved CD4 T-cell epitope in the N-terminal region
- US sera recognize South African OMPs in western blot analysis
- OMPs Am779, Am854, Omp7, 8 and 9 are good candidates for a global vaccine cocktail

Graphical abstract



1. Introduction

Anaplasma marginale is an economically important and globally widespread tick-borne, intra-erythrocytic rickettsial pathogen, which causes bovine anaplasmosis (Kocan et al., 2010, 2004). Bovine anaplasmosis is endemic in tropical and subtropical parts of the world and is characterised by fever, weight loss, haemolytic anaemia and even death, which leads to considerable losses in the cattle industry (Kocan et al., 2010). The disease may also be caused by *A. centrale*, a close relative of *A. marginale*, which usually causes inapparent infections (de Waal, 2000; Potgieter and Stoltsz, 2004), although a few cases of anaplasmosis attributed to *A. centrale* have been reported, most recently in Europe (Carelli et al., 2008).

Various control methods for anaplasmosis exist (Kocan et al., 2003; Merck, 2017), including vaccination with the live *A. centrale* blood vaccine, which is still used in essentially the same form as was conceived by Sir Arnold Theiler over 100 years ago (Palmer, 2009; Theiler, 1912). This vaccination strategy takes advantage of the fact that *A. centrale* shares $CD4^+$ T- and B-cell epitopes with *A. marginale* (Brayton et al., 2005; Herndon et al., 2010). While this vaccine can prevent disease after infection with field strains of *A. marginale*, and is used widely in different parts of the world (Bock and de Vos, 2001; Kocan et al., 2010, 2003), due to inherent drawbacks, ranging from partial protection to challenge by heterologous *A. marginale* strains, to the potential to introduce emerging diseases into non-endemic areas, it is not used in some parts of the world such as the USA. Other vaccination methods such as the use of cultured, inactivated or killed *A. marginale* have been developed to prevent bovine anaplasmosis (de la Fuente et al., 2002; Hammac et al., 2013; Kocan et al., 2003;

Pipano, 1995). However, besides partial efficacy, these vaccines have associated safety issues and are not sufficiently well-developed for reproducible, large-scale production, making them unattractive in their current state (de la Fuente et al., 2017; Kocan et al., 2010).

An attractive and practical option to ameliorate the shortcomings of the current blood-based vaccine may lie in the use of subunit or recombinant vaccines (Albarrak et al., 2012; Ducken et al., 2015; Palmer et al., 1999, 1989, 1988, 1986; Santos et al., 2013; Tebele et al., 1991). Polypeptides can be produced in a reproducible manner on a large scale using recombinant DNA technology, thus offering a viable means for producing uniform vaccine stocks (Kocan et al., 2003; Palmer, 1991). Outer membrane protein (OMP) preparations of *A. marginale* have been experimentally shown to induce protection against *A. marginale* infection, by limiting the clinical effects of the pathogen in the majority of cattle tested (Palmer et al., 1999, 1989, 1988, 1986; Tebele et al., 1991).

Using genomic and proteomic methods, vaccine candidate OMPs that could be effective against *A. marginale* in cattle have been identified. There were 62 OMPs predicted in the first sequenced *A. marginale* genome (Brayton et al., 2005), and based on bioinformatic predictions, some of these are conserved between *A. centrale* and *A. marginale* (Herndon et al., 2010). Lopez et al. (Lopez et al., 2005) used 2D electrophoresis, immunoblotting and liquid chromatography tandem mass spectrometry (LC-MS/MS) to show that a subset of 21 of the OMPs identified bioinformatically (Brayton et al., 2005) was immunoreactive with sera from protectively immunized animals. The study revealed that IgG from OMP-vaccinated cattle reacted

with *A. marginale* OMP preparations (Lopez et al., 2005). Subsequently, it was shown (Noh et al., 2010, 2008) that immunisation with cross-linked OMP surface complexes induced protection against *A. marginale* challenge that was similar to the protection induced by immunisation with whole OMP preparations. A subset of OMPs was identified in the cross-linked surface complex that could also be found in the *A. marginale* OMP preparations. These included Msp1a, Msp2, Msp3, Msp4, OpAG2, Omp7, Omp8, Omp9, Am779 and Am854. A proteomic approach was employed to identify OMP immunogens of *A. marginale* that were recognised by IgG2 raised against the Israel vaccine strain of *A. centrale* in calves; this study revealed, amongst others, five OMPs: Omp7, Omp8, Omp9, Am779, and Am854 (Agnes et al., 2011). These have further been shown to be potential vaccine candidates because of the protection they afforded cattle in challenge experiments using the St. Maries strain (Brown et al., 1998; Noh et al., 2008).

The aims of this study were therefore to amplify, sequence and express five OMPs (Am779, Am854, Omp7, Omp8, and Omp9) and their major variants from South African strains of *A. marginale* with differing *msp1α* genotypes, and to determine, by western blotting, whether recombinant OMP proteins bind immune sera derived from: 1) cattle protectively immunised with cross-linked and non-cross-linked OMPs derived from the US *A. marginale* St. Maries strain, 2) cattle protectively immunised with the South African live *A. centrale* blood vaccine and 3) cattle naturally exposed to field strains of *A. marginale* from South Africa.

2. Materials and Methods

2.1 Amplification and sequencing of OMP genes

Am779, *Am854, omp7, omp8* and *omp9* were amplified from 85 selected *A. marginale*-positive field samples previously shown to have diverse *msp1α* genotypes (Hove et al., 2018). In the St. Maries and Florida strains, *omp7, omp8* and *omp9* are encoded as three tandemly arranged genes in an operon with the deduced amino acid sequences having 70–75% identity when comparing the three OMPs (Brayton et al., 2005; Noh et al., 2006). Primer pairs Am779 F and R (*Am779*), Am854 F and R (*Am854*), OMP7 F and R (*omp7*), ALL F and OMP8 R (*omp8*), and ALL F and OMP9 R (*omp9*) (Table 1) were designed to specifically amplify the five OMP genes. Amplification was performed in a 25 µl volume consisting of 1X Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), 0.5 µM primers and 2.5 µl of template DNA (approximately 200 ng). Nuclease free PCR-grade water was used as a negative control. Thermal cycling conditions for *Am779* amplification were 98°C for 10 s, 40 cycles of 98°C for 1 s, 69.5°C for 5 s and 72°C for 18 s, and a final extension at 72°C for 60 s. Thermal cycling conditions for *Am854* amplification were similar to those for *Am779*, the only differences being annealing at 71°C and extension at 72°C for 15 s. Cycling conditions for *omp7*, *omp8*, and *omp9* were similar to those for *Am779* with the exception of the annealing temperature at 68°C.

PCR amplicons were purified using a Qiagen PCR product purification kit (Qiagen, Hilden, Germany), and sent for direct sequencing at Inqaba Biotechnologies (Pretoria, South Africa). DNA sequences were assembled and aligned using CLC Genomics Workbench 8.0.3 (Qiagen, Hilden, Germany) and the AlignX module of Vector NTI

(Thermo Fisher Scientific, Waltham, USA). Assembled sequences were translated into amino acid sequences and aligned with reference sequences from the US strains, St. Maries and Florida (extracted from the genome sequences, accession numbers: St. Maries, CP000030 and Florida, CP001079). Variant groups were determined by alignment of amino acid sequences and construction of phylogenetic trees (Supplementary Fig. 1).

2.2 Accession numbers

All sequences were deposited in GenBank under accession numbers MK164026–MK164088.

2.3 Cloning of OMP genes into pET SUMO

Truncated *Am779*, *Am854*, *omp7*, *omp8*, and *omp9* sequences, excluding the signal peptide, were amplified using nested PCR primers ("PE primers", Table 1) from selected samples. The secondary PCR was carried out in a total volume of 25 µl containing 1X DreamTaq PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), 0.5 µM forward and reverse primers ("PE primers", Table 1) and 2.5 µl of template (primary PCR product diluted 1 in 100 in PCR grade water). The PCR thermal cycling conditions were 95°C for 3 min, 40 cycles of 95°C for 30 s, 64°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 10 min. For each sample, the secondary PCR was carried out in triplicate, with replicates pooled in order to minimize the probability of including *Taq* errors occurring early in any one of the reactions in the PCR products to be cloned. Pooled PCR products were column-purified using the CloneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, USA).

Purified PCR products were ligated into the pET SUMO protein expression vector using the pET SUMO TA Cloning® kit (Thermo Fisher Scientific, Waltham, USA) and transformed into Invitrogen One Shot® Mach1^{TM}-T1^{R} chemically competent *Escherichia coli* cells (Thermo Fisher Scientific, Waltham, USA) following the manufacturer's protocol. Positive transformants were screened using colony PCR using the appropriate forward PE primer (Table 1) and the T7 sequencing/vector reverse primer (5′-TAG TTA TTG CTC AGC GGT GG-3′). Positive colonies were picked into 5 ml Luria-Bertani (LB) broth containing 50 µg/ml kanamycin (Thermo Fisher Scientific, Waltham, USA), and grown for 12–16 h. A 500 µl aliquot of each culture was stored in an equal volume of sterile 50% glycerol. The rest of the culture was centrifuged at 8000 rpm for 30 s to pellet the cells. Plasmid DNA was extracted from the cell pellet and eluted in a final volume of 100 µl of elution buffer (10 mM Tris-HCl, pH 8.5) using the High Pure Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany). Plasmid DNA preparations were sent to Inqaba Biotechnologies (Pretoria, South Africa) for sequencing with SUMO forward (5′-AGA TTC TTG TAC GAC GGT ATT AG-3′) and T7 reverse primers. Sequences were analysed using CLC Genomics Workbench 8.0.3 to select constructs cloned in-frame with no sequence errors.

2.4 Expression and purification of recombinant OMPs

Approximately 10 ng of plasmid DNA from each sample was used to transform Invitrogen *E. coli* BL-21(DE3) One Shot® expression cells, using the heat-shock protocol as specified in the Champion pET SUMO manual (Thermo Fisher Scientific, Waltham, USA). Following this, 500 µl of the overnight culture of transformed BL-21(DE3) cells was used to inoculate 10 ml of fresh LB broth containing 50 µg/ml kanamycin, and grown at 37°C with shaking at 240 rpm for 2 h. After induction of the

culture by addition of isopropylthio-β-galactoside (IPTG) to a final concentration of 1 mM the culture was grown for the optimal expression time of 4–5 h. Thereafter, crude protein extracts from each culture were separated by polyacrylamide gel electrophoresis (PAGE) on a 12% Criterion TGX precast denaturing gel (BioRad, Hercules, USA) in 1X Tris/Glycine SDS electrophoresis buffer, at 100 V for 1 h. Precision Plus Protein Kaleidoscope pre-stained molecular weight marker (BioRad, Hercules, USA) was used. Proteins were visualised using Coomassie Blue SafeStain (Thermo Fisher Scientific, Waltham, USA) according to the supplier's instructions.

Expression of His-tagged recombinant OMPs was confirmed using a standard western blotting protocol. HisDetector Nickel^{2+} Horse Radish Peroxidase (HRP) (KPL Scientific Inc, Gaithersburg, USA) at a final dilution of 1:5000 was used, with the 1-step tetramethylbenzidine (TMB)-blotting solution for western blotting (Thermo Fisher Scientific, Waltham, USA) as substrate. Soluble and insoluble (from inclusion bodies) protein fractions were extracted from the cell pellets using the Novagen BugBuster Protein extraction kit (Merck, Kenilworth, USA), according to the manufacturer's instructions. Crude proteins were purified using Protino Ni-IDA Nickel^{2+} columns (Machery-Nagel, Duren, Germany). Purified extracted proteins of each OMP were separated by PAGE and visualised as previously described. Purified recombinant protein generated for each OMP and control protein samples were quantified using the Bicinchoninic Acid (BCA) method following the protocol in the Pierce BCA kit (Thermo Fisher Scientific, Waltham, USA). Recombinant OMPs were derived mostly from inclusion bodies and were solubilised using urea and refolded by dilution as previously reported (Ducken et al., 2015).

For crude protein lysate controls, a 200 µl volume of each control blood sample [(i) *A. marginale*-infected blood: whole bovine EDTA blood sample with 33% parasitized erythrocytes (9678/2) supplied by the Agricultural Research Council-Onderstepoort Veterinary Research Institute (ARC-OVR), South Africa. Sample 9678/2 was infected with blood originating from an animal at Proefplaas, the UP experimental farm in Pretoria, South Africa, and is designated as Proefplaas strain 1332. (ii) *A. centrale*-infected blood: the bovine blood vaccine produced by Onderstepoort Biological Products (OBP) (Pretoria, South Africa)] was washed at least five times in 1X phosphate buffered saline (PBS) with centrifugation at maximum speed in a benchtop microcentrifuge, then boiled at 95°C in 1X Laemmli sample loading buffer for 2-5 min, prior to BCA quantification. Thereafter, samples were resuspended in 200 µl of 1X PBS. Untransformed *E. coli* BL-21 (DE3) cells were used as a control to check for contaminating *E. coli* proteins. Other controls used were the truncated recombinant *Ehrlichia ruminantium* predicted membrane protein, Erum1040 (Collins et al., 2005) and the recombinant SUMO protein.

2.5 *Anaplasma marginale* and *A. centrale* test serum samples

Anaplasma marginale and *A. centrale* seropositive and seronegative serum samples were used as controls for the western blots. In order to prepare control sera, a total of 17 bovine blood samples were collected from Proefplaas (11 samples), and East Lynne farm in Bergville, KwaZulu-Natal (six samples). Samples were collected in 9 ml Vacuette EDTA and serum tubes. Blood and serum samples were collected from *A. marginale* positive animals at Proefplaas as determined by duplex real-time quantitative PCR (qPCR) (Carelli et al., 2007; Chaisi et al., 2017; Decaro et al., 2008). The Bergville animals selected for sampling were young calves (six to eight months

old) that had not been vaccinated with the live *A. centrale* blood vaccine. After an initial collection (pre-immune) of blood and serum samples from these six animals, they were immediately vaccinated by subcutaneous injection with 5 ml of the live *A. centrale* blood vaccine (OBP, Pretoria, South Africa). After a six-week period for seroconversion, post-immune blood and serum samples were collected.

Genomic DNA was extracted from all ETDA samples using the Qiagen Blood Mini Kit (Qiagen, Hilden, Germany) and tested using the duplex qPCR assay on the Lightcycler (Roche Diagnostics, Mannheim, Germany), which detects *A. marginale* and *A. centrale* as previously described (Chaisi et al., 2017; Decaro et al., 2008). Serological testing of all serum samples was done at the ARC-OVR using the *Anaplasma* antibody test kit (VMRD, Pullman, Washington, USA) (Visser et al., 1992), a competitive inhibition enzyme-linked immunosorbent assay (cELISA) which detects antibodies to the Msp5 protein of *Anaplasma* spp.

Other sera used for western blotting were generated previously (Noh et al., 2013, 2008), as follows: pre- and post-immune sera from cattle (numbers 43071, 43092 and 43100) immunised with non-cross linked OMPs derived from the St. Maries strain of *A. marginale*; pre- and post-immune sera from cattle (numbers C1252, 35100 and 35130) immunised with cross-linked OMPs derived from the St. Maries strain of *A. marginale*.

For western blots, anti-*E. coli* antibodies were adsorbed from all of the pre- and post-immune sera (primary antibodies) as described previously (Ducken et al., 2015). Prior to use in western blots, pre-immune sera were pooled in equal volumes for each serum

and then diluted 1:100 in 1X PBS. Post-immune sera were tested individually, after dilution at 1:100 in 1X PBS.

2.6 Western blotting of recombinant OMPs

Approximately 200 ng of each recombinant OMP and control proteins [crude protein extracts from *E. coli* (BL-21 (DE3)) cells, and recombinant *E. ruminantium* OMP Erum1040 and SUMO proteins] was loaded onto precast Criterion TGX (BioRad, Hercules, USA) denaturing, gradient polyacrylamide gels (4 to 15%) and electrophoresed at 120 V for 50 min. Thereafter, the recombinant OMPs and controls were transferred onto a PVDF membrane, using the Trans-Blot Turbo Transfer System, (BioRad, Hercules, USA) at 110 mA for 90 min. The *A. marginale* (Proefplaas strain 1332 with *msp1*α genotype 3 37) and live *A. centrale* blood vaccine (OBP, Pretoria, South Africa) crude lysate controls were each loaded onto a single gel, transferred to PVDF membranes, and then cut into strips which were used in the western blots. PVDF membranes were blocked overnight at 4°C in 20 ml of 5% non-fat skimmed milk (BioRad, Hercules, USA), with gentle shaking. Test sera (primary antibody) diluted 1:100 (or 1:50 for sera from *A. marginale* naturally infected animals) in 1X PBS were incubated for 1 h at room temperature, with the PVDF membranes containing the recombinant OMPs and control proteins, followed by three washes in 1X PBS-Tween20. The membrane was then incubated for 30 min with polyclonal, HRP-conjugated, rabbit anti-bovine IgG (secondary) antibodies (Dako A/S, Glostrup, Denmark) at a final dilution of 1:2000, in 2.5% non-fat skimmed milk. After three 5 min washes in 1X PBS-Tween20, the PVDF membranes were immersed in TMB substrate, for 5–10 min to facilitate signal detection. Pictures were taken using the G:Box Chemi-XT4 GENESys (Syngene, India) gel documentation system.

3. Results

3.1 Amplification and sequencing of OMP genes

The genes encoding five *A. marginale* OMPs, Am779, Am854, Omp7, Omp8 and Omp9, were amplified from 85 *A. marginale*-positive field samples, containing genetically diverse *A. marginale* strains as previously indicated by *msp1α* genotype analysis (Hove et al., 2018). For each OMP gene, amplicons of the expected size (Table 1) and good quality sequences were obtained from 62 (Am779), 85 (Am854), 38 (Omp7), 58 (Omp8), and 46 (Omp9) of the 85 *A. marginale*-positive field samples examined.

Very few variants of Am779 were detected. Amino acid changes occurred at three positions out of a total of 420 residues (0.55%), resulting in five variants. Four of the five variants had a single amino acid change relative to the St. Maries and Florida sequences. Variants 1 and 4 were the most abundant, with a representation of 59.7% and 30.6%, while the other 3 variants (2, 3 and 5) each had less than 5% abundance (Table 2). By virtue of abundance, variants 1 and 4 were chosen for expression. South African OMP Am779 variants 1 and 4 had 99.9% amino acid sequence identity with the US St. Maries and Florida strain Am779 (Table 3).

Minimal variation was found in the nucleotide sequences of *Am854*. Three synonymous single nucleotide polymorphisms (SNPs) were detected in the *Am854* nucleotide sequences. Thus, no amino acid variants were detected for Am854 over the 220 amino acid residues analysed, and South African OMP Am854 amino acid

sequences were 100% identical to OMP Am854 from the St. Maries and Florida strains of *A. marginale* from the USA (Table 3).

Omp7, Omp8 and Omp9 were found to have, respectively, 86 (of 321; 26.8%), 68 (of 337; 20.2%) and 51 (of 341; 15.0%) positions where amino acids varied. All three OMPs had conserved N- and C- termini and the majority of the variability was found in the central regions. The recently described CD4 T-cell epitope, FLLVDDA(I/V)V (Deringer et al., 2017), was present in all of the South African Omp7, Omp8 and Omp9 sequences (Fig. 1). For each OMP, two variants representing the most genetically distinct variant groups after phylogenetic analysis were chosen for expression (Table 3).

Even though Omp7 had the highest number of amino acid changes relative to its length (26.8%), it had the least variants, with 14 variants in four major variant groups. Omp7 variant group 1 was similar to the St. Maries Omp7 sequence, while variant group 2 was similar to the Florida sequence. Omp7 group 2 and 3 variants represent the two most genetically distinct variant groups of the four major variant groups identified in the field samples examined. Thus, Omp7 variants 2A and 3A were chosen for expression. South African Omp7 variant 2A had 85% and 93% amino acid sequence identity with US St. Maries and Florida strain Omp7, respectively, whereas variant 3A had 79% and 86% identity with the US Omp7 from these strains (Table 3).

Omp8 had the highest number of variants, with 29 variants in two major variant groups. The Florida Omp8 sequence was similar to group 1 and St. Maries Omp8 to group 2. A representative from each major variant group, Omp8 variants 1C and 2B, were

chosen for expression. South African Omp8 variant 1C had 90% and 99% amino acid sequence identity with US St. Maries and Florida Omp8, respectively, while South African variant 2B had 98% and 90% identity with Omp8 from these US strains (Table 3).

Omp9 had the second highest number of variants, with 20 variants in four major groups. Interestingly, Florida and St. Maries Omp9 sequences were both similar to group 1. Omp9 group 2 and 4 variants represent the two most genetically distinct variant groups of the four major variant groups identified in the field samples examined. Therefore, Omp9 variants 2A and 4A were chosen for expression. South African Omp9 variant 2A had 91% amino acid sequence identity with US St. Maries and Florida Omp9, whereas South African variant 4A had 92% identity with Omp9 from these two US strains (Table 3).

3.2 Western blotting of recombinant OMPs

Pilot expression experiments demonstrated that a four hour induction time was suitable for all proteins (Supplementary Fig. 2), and purified expressed protein products were examined for purity and appropriate size of the expressed product (Supplementary Table 1 and Supplementary Fig. 3). Expression of the OMP variants was confirmed by detection of the His-tag at the N-terminus using HRP-labelled antibody (Supplementary Fig. 4). Recombinant Am854 migrated higher than the expected molecular weight (Table 3 and Figs. 2–5). All five recombinant OMPs and their variants were recognised by anti-*A. marginale* and *A. centrale* bovine sera from both the USA and South Africa (Table 4 and Figs. 2–5). Generally strong signals corresponding to the molecular weight of the recombinant proteins were detected for

all sera tested. Some additional faint bands were recognized by the antisera in most of the blots, which could be ascribed to protein degradation products.

Sera derived from cattle immunized with cross-linked or non-cross-linked OMPs recognised multiple bands in the *A. marginale* or *A. centrale* crude lysate controls. The *A. marginale* Proefplaas 1332 strain (Am) infected blood crude lysate control was recognised by the test sera, with several protein bands being detected (Figs. 2–5). It is likely the immunodominant proteins Msp2 and Msp3 (approximately 36 and 74 kDa, respectively) were among the proteins detected by the sera. The live *A. centrale* blood vaccine crude lysate control was also recognised by all of the sera, with multiple strong bands (see AcV lanes in Figs. 2–5). No signal was detected for the *E. ruminantium* and SUMO recombinant proteins. Slight signals were detected in the *E. coli* negative control lanes. None of the pre-immune sera (Figs. 2D, 3D and 5D) produced signal for either the recombinant OMPs or the controls, although faint bands were detected in some of the *E. coli* negative control lanes.

4. Discussion

Determining the correct immunogens for inclusion in a recombinant vaccine cocktail that offers lasting and broad immune protection against infection with *A. marginale* is challenging. In South Africa, high genetic diversity of *A. marginale* has been demonstrated for field strains (Hove et al., 2018; Mtshali et al., 2007; Mutshembele et al., 2014) and by extrapolation, immunogenically significant genes would also be expected to be highly variable. Three of the five OMPs in this study revealed high

numbers of genetic variants as expected from the high diversity in *msp1*α genotypes in the samples used to generate the sequences.

As was seen previously (Ducken et al., 2015), we detected a single variant of Am854 which was recognised by sera from animals immunised with both cross-linked and non-cross-linked *A. marginale* OMPs, and also by sera from *A. centrale* vaccinates, making it a promising vaccine candidate. It will be necessary to overcome the obstacle of low antibody production when vaccinating with individual recombinant proteins, as compared with much higher titres for an individual protein in OMP vaccinates, before this immunogen can be used in a vaccine. Antibody titres may be influenced by many complex factors such as choice of adjuvant and folding of antigens during recombinant protein vaccine formulation (Ducken et al., 2015). It is possible that if several vaccine candidates are included as part of a recombinant protein vaccine cocktail, the components of the cocktail may act as adjuvants for each protein, but this remains to be tested.

There were three amino acid positions that varied in Am779 sequences; however, these changes were not predicted to significantly affect the structure of the protein. Overall, the three amino acid substitutions appeared to have minimal effects on the antigenicity of the OMP, since we were able to show that both major variants of Am779 were recognised by *A. marginale* and *A. centrale* antisera. This supports the selection of Am779 as an immunogen for inclusion in a recombinant vaccine cocktail. This view is supported by Albarrak et al., who showed that Am779 was able to prime the immune system; a trait which is highly desirable in a good vaccine candidate (Albarrak et al., 2012).

Despite the high levels of amino acid variation we found for the closely related antigens, Omp7, Omp8, and Omp9 (i.e. up to 29 genetic variants for the *omp8* gene), the two major variants we selected and expressed for each OMP were detected by all post-immune sera, suggesting that the variations we observed in these three OMPs may not be significant enough to alter the immunologically important regions in these antigens. The recently described CD4 T-cell epitope FLLVDDA(I/V)V, previously reported in both *A. marginale* and *A. centrale* to be conserved between strains from North America, Brazil, Australia and Ghana for Omp7, Omp8 and Omp9 (Deringer et al., 2017), was detected just upstream of the central variable region in our sequences. This contrasts somewhat with *A. marginale* Msp2 (Abbott et al., 2004), which has T-cell epitopes uniformly distributed between conserved regions and the hypervariable region (HVR). The high levels of conservation of these three OMPs in *A. marginale* strains from South Africa and US strains further suggests that these three OMPs are good vaccine candidates as they contain the known CD4 T-cell epitope and react with sera from geographically distant regions of the world, for both *A. marginale* and *A. centrale*. This is crucial for vaccine-induced immunity against *A. marginale* in which outer membrane protein-vaccinated cattle produce CD4 T-cells, which activate macrophages and stimulate B-cell antibody production via interferon gamma (INF-γ) generation (Palmer et al., 1999).

Sera from *A. centrale* vaccinates were shown to strongly bind all recombinant OMPs and like all the sera from the *A. marginale* vaccinates (from both South Africa and the USA), also gave signal for *A. centrale* crude lysate. These data could suggest that the OMPs may be important in the protective immune response, given that antigens

shared between the two organisms result in the cross-protection afforded by the *A. centrale* vaccine against infections by field strains of *A. marginale*. Interestingly, *A. marginale omp7, omp8* and *omp9* have close relatives in *A. centrale*, although these are collapsed into one coding DNA sequence in *A. centrale* (Herndon et al., 2010). *A. marginale* Am854 and Am779 have sequence identity of between 81 and 84% with *A. centrale* sequences (Herndon et al., 2010). These data emphasize the strong antigenic relationship between *A. marginale* and *A. centrale*, a finding that was exploited by Theiler to develop the live *A. centrale* blood vaccine still used with little modification to this day (Palmer, 2009; Potgieter, 1979; Theiler, 1912). The potential risks associated with vaccination using the live *A. centrale* blood vaccine have led to some countries not using the vaccine. Our results provide evidence to support the continued use of this vaccine in countries that allow its distribution. Despite the strong immunological relationships demonstrated *in vitro* between *A. centrale* and *A. marginale*, this may not translate to immune protection by these vaccine candidates *in vivo.* This remains the greatest hurdle to producing recombinant vaccines, as protection against *A. marginale* has not yet been achieved with recombinant vaccines, as recently demonstrated for vaccine candidate Am854 (Ducken et al., 2015). This is likely the result of the failure of subunit vaccines to recapitulate a complete, long-lasting immune response as results from infection by the whole pathogen; this may point to either improper antigen configuration resulting in poor epitope presentation or an omission of other important subdominant antigens necessary to elicit the protective immune response.

The detection of *A. marginale* recombinant immunogens by both anti-*A. centrale* and anti-*A. marginale* sera from the USA and South Africa, point to significant and complex immunological and antigenic relationships between these two pathogens and may

therefore be preliminary evidence in support of the inclusion of the recombinant OMPs Am779, Am854, Omp7, Omp8 and Omp9, in a global rather than region-specific recombinant vaccine against anaplasmosis.

5. Conclusion

The data generated in this study, supported by previous studies, reveal that *A. marginale* OMPs Am779, Am854, Omp7, Omp8 and Omp9 are good vaccine candidates for inclusion in vaccines against the rickettsia, *A. marginale*. Additionally, data presented in this study reveal sequence conservation and antigenic similarities between South African and US strains of *A. marginale,* making a case for a global recombinant vaccine against bovine anaplasmosis. However, further work into vaccine composition and efficient delivery mechanisms still needs to be explored before recombinant proteins become viable as working vaccines.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics statement

Permission to perform the research under Section 20 of the Animal Diseases Act, 1984 (Act number 34 of 1984) was granted by the Department of Agriculture Fisheries and Forestry, reference number 12/11/1/1/6. Approval was also granted by the University of Pretoria's Animal Ethics Committee, project number V067/13.

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Table 1: Primers for amplification of *A. marginale* OMP genes.

Primer name	Sequence (5′–3′)	Annealing temperature	Amplicon size
Am779 F Am 779 R	ATG GTA CAT AAA GGT TCT CTG GTG GCT C GTT TGT GGC TTT CAC GCT CCT GAG	69.5°C	1 593 bp
Am779 F PE[a] Am779 R PE	ATG AGC TAT GCT TTT GTC ACC GGG CGA GTG C TTA CCT CAG TAC GTG CTC ACC ATC AAA CCC C	64.0°C	1 265 bp
Am854 F Am854 R	GCT GCA TCG TTG GTT AGC TCT GTG GCC GAA GAA TCC TGT GCC ACT TC	71.0°C	711 bp
Am854 F PE Am854 R PE	ATG TCT GCC GTG ACA GGT TGT GGT CTT TTC AGC TTA TCT TCA GGC GCC GCT TCT TCG	64.0°C	586 bp
OMP7 F[b] OMP7 R	TCT TTT CTG TTG GGT GCG GTT GTA GAC ACG CGG CAC TGC TCT TTA TAC TC	68.0°C	1 100 bp
OMP7 F PE OMP7 R PE	ATG AGC TTT GGT GGT GAC GAT ACC GAC TTA TTG TGG GGA GAG CTC GTA ACT C	64.0°C	935 bp
ALL F OMP8 R	GGT CTT TTC TGT TGA GCG CGG TTG CGC GCG CTC TGA TAT TTT CCC TT	68.0°C	1 133 bp
OMP8 F PE OMP8 R PE	ATG AGC GAC TTT TAC TTA GGA TTT GGG CTT GCC TTA TTC TTC AGG CGC CGC TTC TTC GGA	64.0°C	950 bp
ALL F OMP9 R	GGT CTT TTC TGT TGA GCG CGG TTG GTG CCT TGA CAT CTT CCC TCT CAA C	68.0°C	1 142 bp
OMP9 F PE OMP9 R PE	ATG TCT GCA GGG TTT GGT GGT GAT GAT ACT GAC TTA TCC ATC GAC AAA AAC CCT AGC CCG	64.0°C	980 bp

[a] PE indicates secondary (nested) PCR primers used to generate amplicons for cloning into the pET SUMO expression vector.
[b] Primer sequence previously published (Junior et al., 2010).

Table 2: Amino acid variation in Am779 in 62 South African field samples.

Locus variant	Amino acid at position			Number of variants detected	Percent representation
	67	113	442		
1	R	T	A	37	59.7%
2	R	T	V	2	3.2%
3	R	A	A	3	4.8%
4	H	T	A	19	30.6%
5	H	T	V	1	1.6%

Table 3: Samples chosen for amplification and expression of OMP variants.

OMP [a]	Expected size (kDa) [b]	OMP variant	Sample number [c]	Origin of sample	*Msp1α* genotype [d]	Identity [e]
Am779 (421, 46.3)	59.3	1	C14	Mpumalanga	42 43 25 31 13 27 27	StM – 99.9% FL – 99.9%
		4	127	Western Cape	34 13 37 3 4 37	StM – 99.9% FL – 99.9%
Am854 (195, 21.4)	34.4	–	136	Mpumalanga	42 43 25 31 34 13 13 37 84 UP19 UP19 78 31 31 31 34 13 3 36 38	StM – 100% FL – 100%
Omp7 (311, 34.2)	47.2	2A	112	Western Cape	154	StM – 85% FL – 93%
		3A	NW C28	North West	34 36 36 38	StM – 79% FL – 86%
Omp8 (316, 34.8)	47.8	1C	LPC 46	Limpopo	41 4 37 13 155 37 4 38 41 13 4 37	StM – 90% FL – 99%
		2B	84	Mpumalanga	34 3 3 3 36 38 34 13 13 37 34 36 36 3 36 38 3 37 37 34 3 27 3 UP1 34 UP2 36 38 34 3 34 3 38	StM – 98% FL – 90%
Omp9 (326, 35.9)	48.9	2A	GP-K C6A	Gauteng	34 13 13 13 37 27 13 18 27 13 3 36 38	StM – 91% FL – 91%
		4A	GP-K C12	Gauteng	34 13 4 4 13 4 27 4 13 4 4 4 37	StM – 92% FL – 92%

[a] Length in amino acids and size of the truncated, recombinant OMP in kDa are indicated in parentheses below each OMP name.
[b] Indicates molecular weight of truncated, recombinant OMP + SUMO (13 kDa) fusion product.
[c] Collection of the samples and *msp1α* genotype analysis have been reported previously [31].
[d] Number of *msp1α* genotypes gives an indication of the number of *A. marginale* strains present in the sample.
[e] Amino acid identity to US *A. marginale* strains, StM = St. Maries strain, FL = Florida strain.

Table 4: Recognition of South African recombinant *A. marginale* OMP variants by South African and US antisera using western blot analysis.

Origin & source of serum	Serum ID	South African origin recombinant antigen								
		Am854	Am779 Var 1	Am779 Var 4	Omp7 Var 1	Omp7 Var 2	Omp8 Var 1	Omp8 Var 2	Omp9 Var 1	Omp9 Var 2
South Africa: *A. centrale* bovine vaccinates [a, b, c]	2503	+	+	+	+	+	+	+	+	+
	2505	+	+	+	+	+	+	+	+	+
	2523	+	+	+	+	+	+	+	+	+
Pooled pre-immune sera[d]		-	-	-	-	-	-	-	-	-
South Africa: *A. marginale* naturally infected cattle[a, b, c]	1303	+	+	+	+	+	+	+	+	+
	1313	+	+	+	+	+	+	+	+	+
	1334	+	+	+	+	+	+	+	+	+
USA: non-cross-linked OMP bovine vaccinates[a, b, c]	43071	+	+	+	+	+	+	+	+	+
	43092	+	+	+	+	+	+	+	+	+
	43100	+	+	+	+	+	+	+	+	+
Pooled pre-immune sera[d]		-	-	-	-	-	-	-	-	-
USA: cross-linked OMP bovine vaccinates[a, b, c]	35100	+	+	+	+	+	+	+	+	+
	35130	+	+	+	+	+	+	+	+	+
	C1252	+	+	+	+	+	+	+	+	+
Pooled pre-immune sera[d]		-	-	-	-	-	-	-	-	-

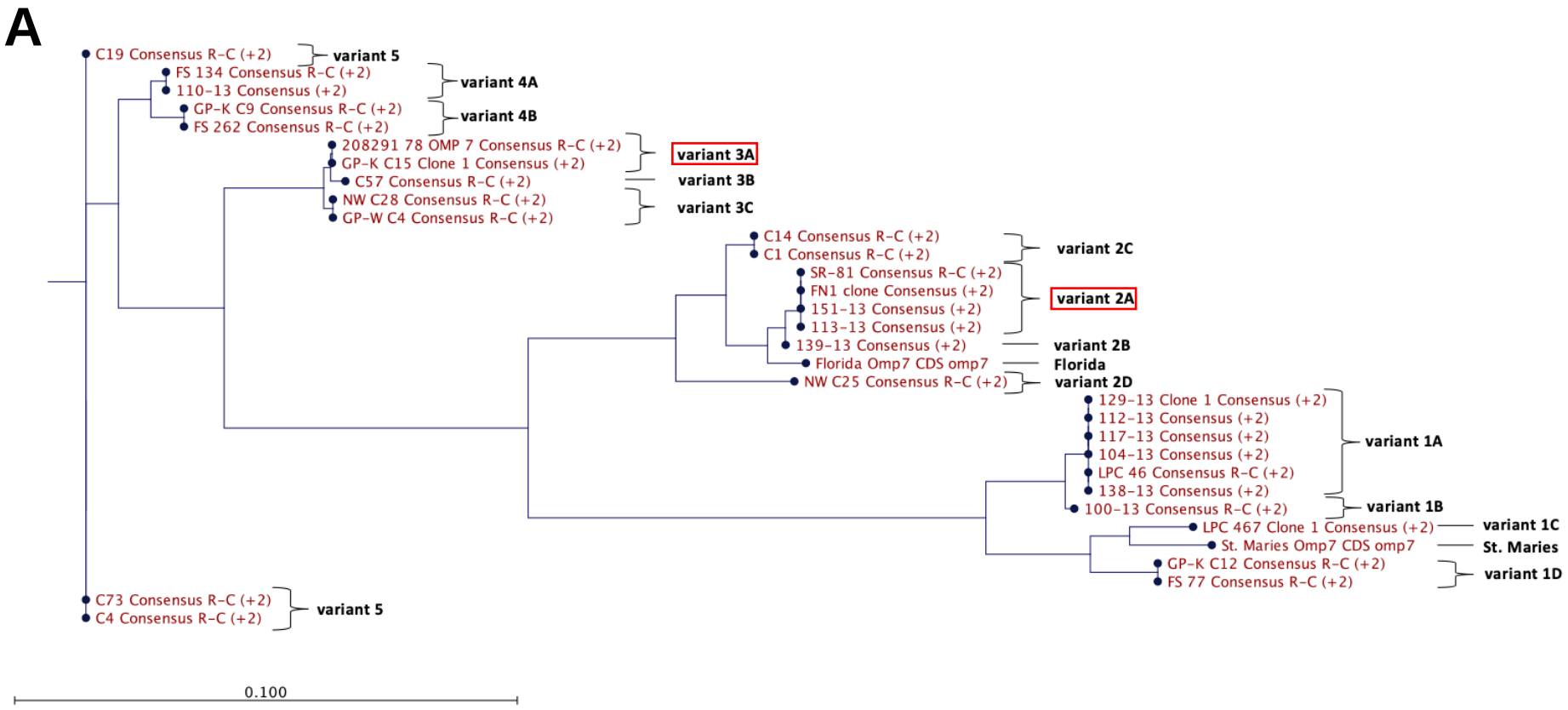
[a] Positive for the following controls: *A. marginale* Proefplaas 1332 strain, infected blood crude lysate & *A. centrale* live blood vaccine crude lysate.
[b] Negative for the following controls: *E. ruminantium* and SUMO recombinant proteins.
[c] Showed non-specific bands for the following control: BL-21(DE3) *E. coli* untransformed cell lysate.
[d] Negative for all above controls.
+ – Positive (gave a band of the expected size on the western blot).
- – Negative (no band on the western blot).

Supplementary Table 1: Estimated sizes in kiloDaltons (kDa) of the truncated recombinant OMPs.

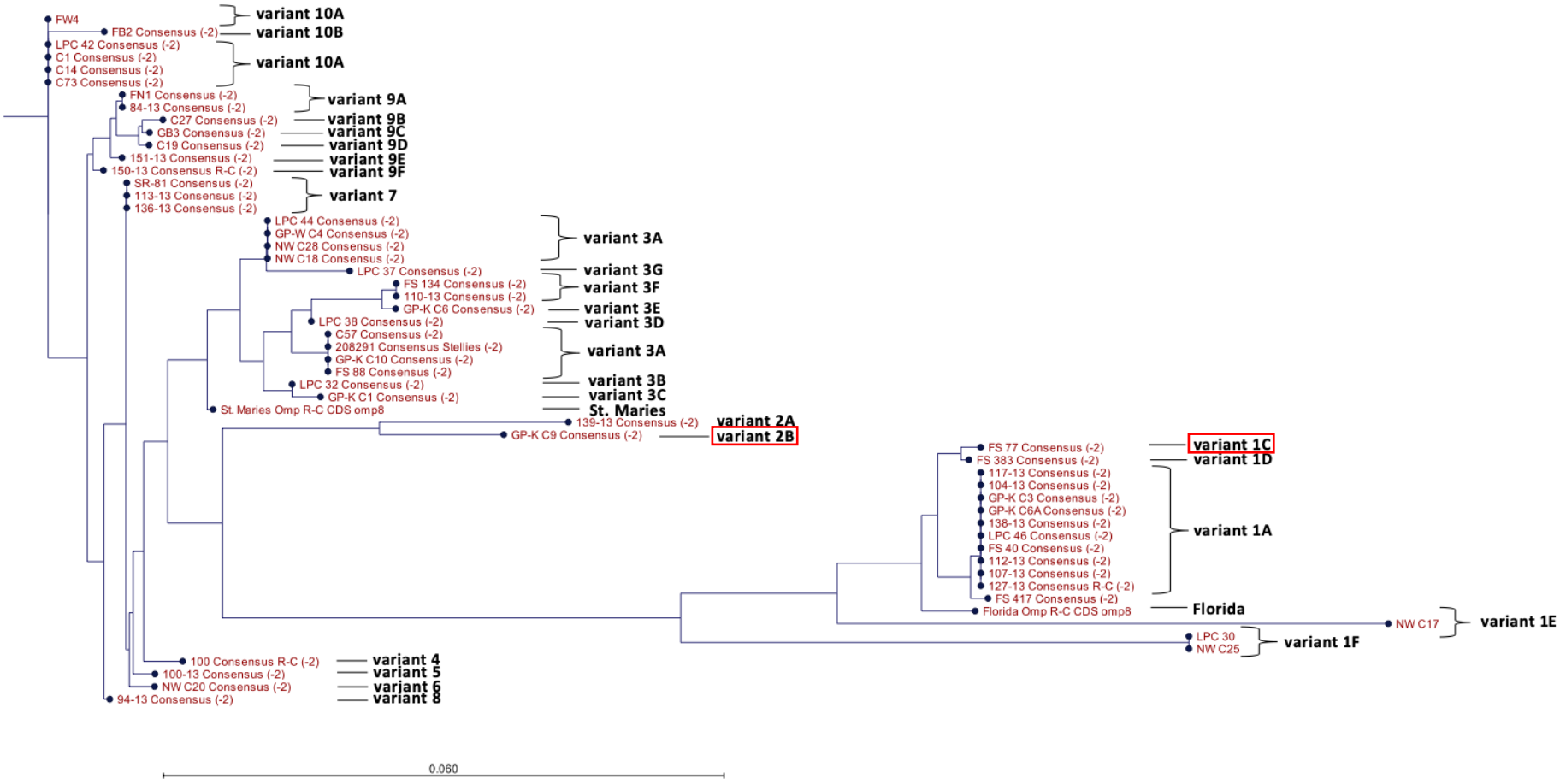
OMP name	PCR product size	Protein Size (kDa)	+ N-terminal SUMO protein and 6x His Tag (13 kDa)*
Am779	1 265 bp	46.3	59.3
Am854	586 bp	21.5	34.8
Omp7	935 bp	34.2	47.2
Omp8	950 bp	34.8	47.8
Omp9	980 bp	35.9	48.9

* Formulas used for calculations:

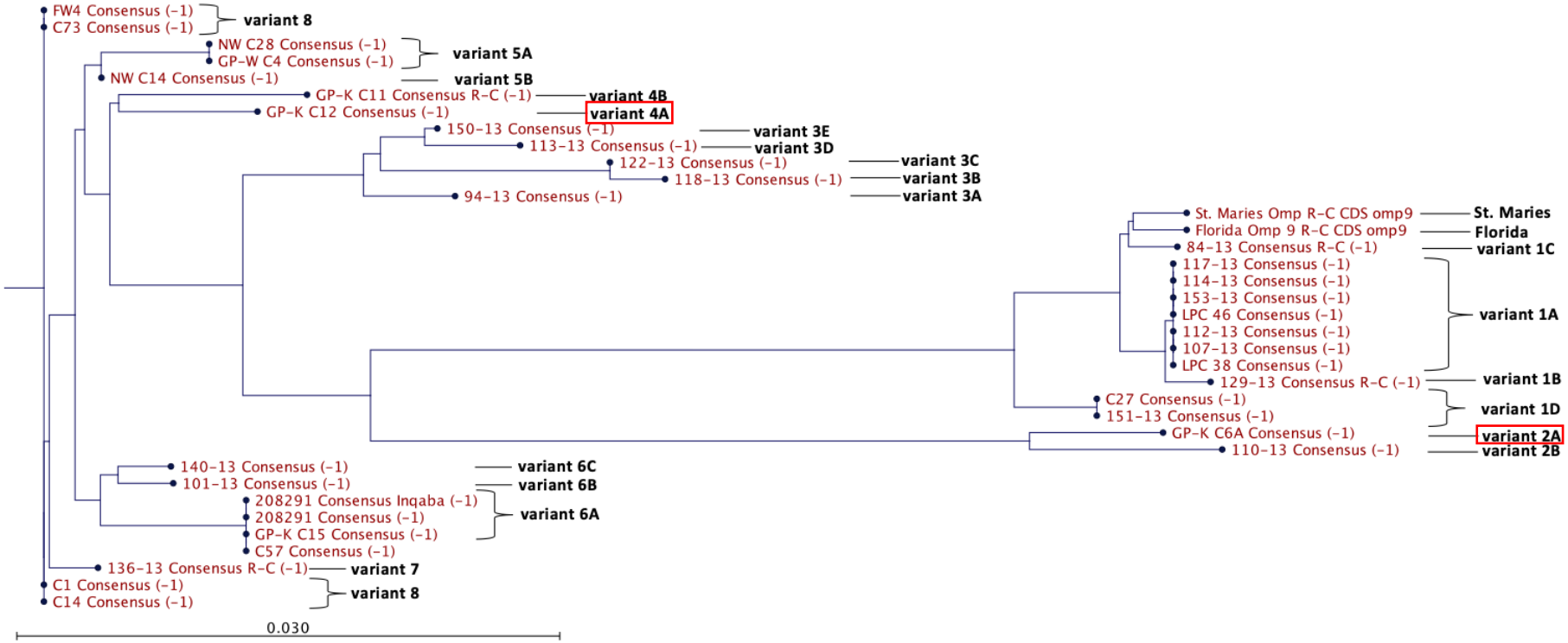
1. Amino acids encoded = Length of DNA sequence (in bases) / 3
2. Predicted size of protein = number of amino acids × 0.11 kDa (the average molecular weight of an amino acid)


A
C19 Consensus R-C (+2)
FS 134 Consensus R-C (+2)
110-13 Consensus (+2)
GP-K C9 Consensus R-C (+2)
FS 262 Consensus R-C (+2)
208291 78 OMP 7 Consensus R-C (+2)
GP-K C15 Clone 1 Consensus (+2)
C57 Consensus R-C (+2)
NW C28 Consensus R-C (+2)
GP-W C4 Consensus R-C (+2)
C14 Consensus R-C (+2)
C1 Consensus R-C (+2)
SR-81 Consensus R-C (+2)
FN1 clone Consensus (+2)
151-13 Consensus (+2)
113-13 Consensus (+2)
139-13 Consensus (+2)
Florida Omp7 CDS omp7
NW C25 Consensus R-C (+2)
129-13 Clone 1 Consensus (+2)
112-13 Consensus (+2)
117-13 Consensus (+2)
104-13 Consensus (+2)
LPC 46 Consensus R-C (+2)
138-13 Consensus (+2)
100-13 Consensus R-C (+2)
LPC 467 Clone 1 Consensus (+2)
St. Maries Omp7 CDS omp7
GP-K C12 Consensus R-C (+2)
FS 77 Consensus R-C (+2)
C73 Consensus R-C (+2)
C4 Consensus R-C (+2)
variant 5
variant 4A
variant 4B
variant 3A
variant 3B
variant 3C
variant 2C
variant 2A
variant 2B
Florida
variant 2D
variant 1A
variant 1B
variant 1C
St. Maries
variant 1D
variant 5
0.100

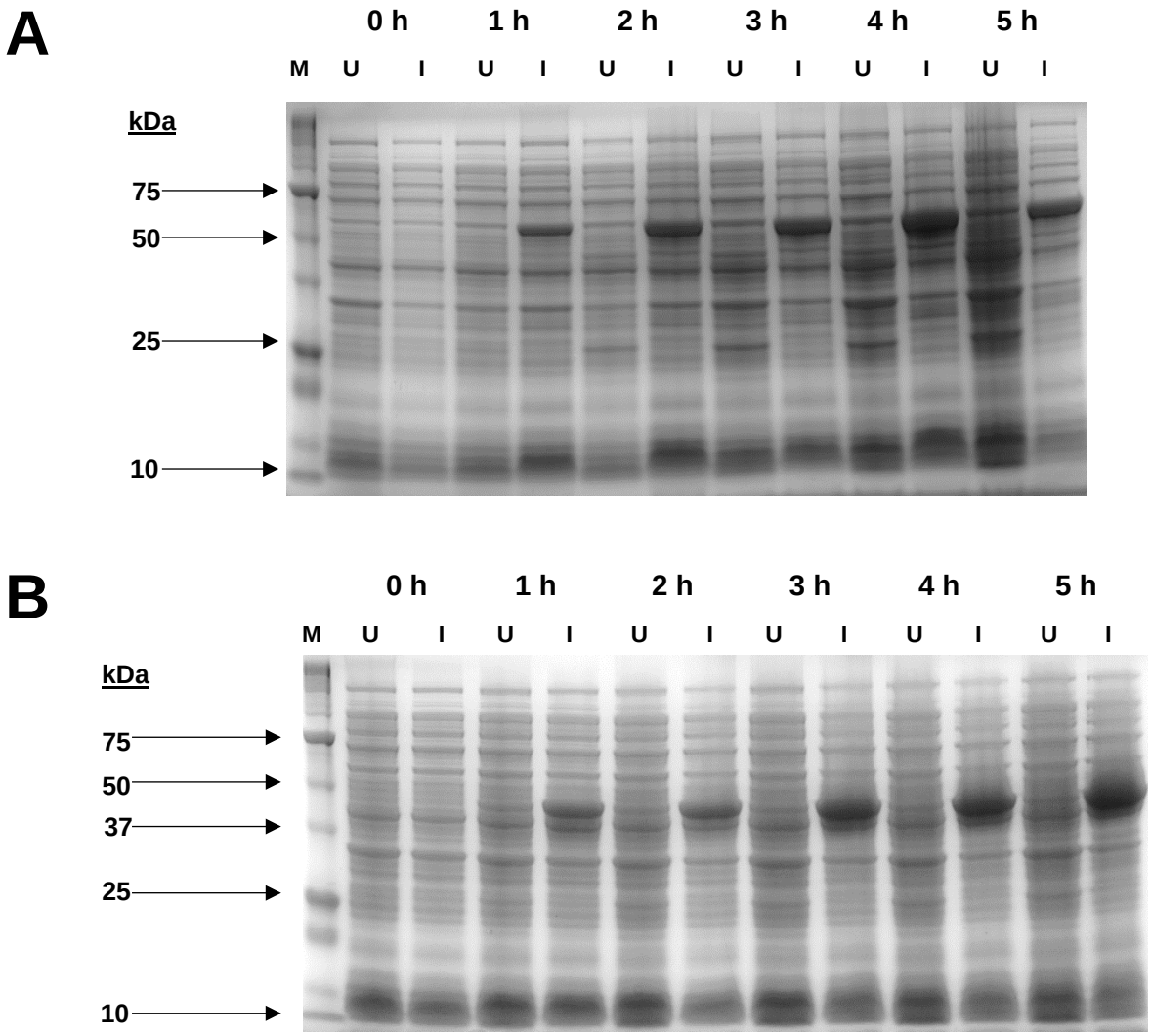
B


FW4
FB2 Consensus (-2)
LPC 42 Consensus (-2)
C1 Consensus (-2)
C14 Consensus (-2)
C73 Consensus (-2)
FN1 Consensus (-2)
84-13 Consensus (-2)
C27 Consensus (-2)
GB3 Consensus (-2)
C19 Consensus (-2)
151-13 Consensus (-2)
150-13 Consensus R-C (-2)
SR-81 Consensus (-2)
113-13 Consensus (-2)
136-13 Consensus (-2)
LPC 44 Consensus (-2)
GP-W C4 Consensus (-2)
NW C28 Consensus (-2)
NW C18 Consensus (-2)
LPC 37 Consensus (-2)
FS 134 Consensus (-2)
110-13 Consensus (-2)
GP-K C6 Consensus (-2)
LPC 38 Consensus (-2)
C57 Consensus (-2)
208291 Consensus Stellies (-2)
GP-K C10 Consensus (-2)
FS 88 Consensus (-2)
LPC 32 Consensus (-2)
GP-K C1 Consensus (-2)
St. Maries Omp R-C CDS omp8
139-13 Consensus (-2)
GP-K C9 Consensus (-2)
100 Consensus R-C (-2)
100-13 Consensus (-2)
NW C20 Consensus (-2)
94-13 Consensus (-2)
FS 77 Consensus (-2)
FS 383 Consensus (-2)
117-13 Consensus (-2)
104-13 Consensus (-2)
GP-K C3 Consensus (-2)
GP-K C6A Consensus (-2)
138-13 Consensus (-2)
LPC 46 Consensus (-2)
FS 40 Consensus (-2)
112-13 Consensus (-2)
107-13 Consensus (-2)
127-13 Consensus R-C (-2)
FS 417 Consensus (-2)
Florida Omp R-C CDS omp8
NW C17
LPC 30
NW C25
variant 10A
variant 10B
variant 10A
variant 9A
variant 9B
variant 9C
variant 9D
variant 9E
variant 9F
variant 7
variant 3A
variant 3G
variant 3F
variant 3E
variant 3D
variant 3A
variant 3B
variant 3C
St. Maries
variant 2A
variant 2B
variant 4
variant 5
variant 6
variant 8
variant 1C
variant 1D
variant 1A
Florida
variant 1E
variant 1F
0.060

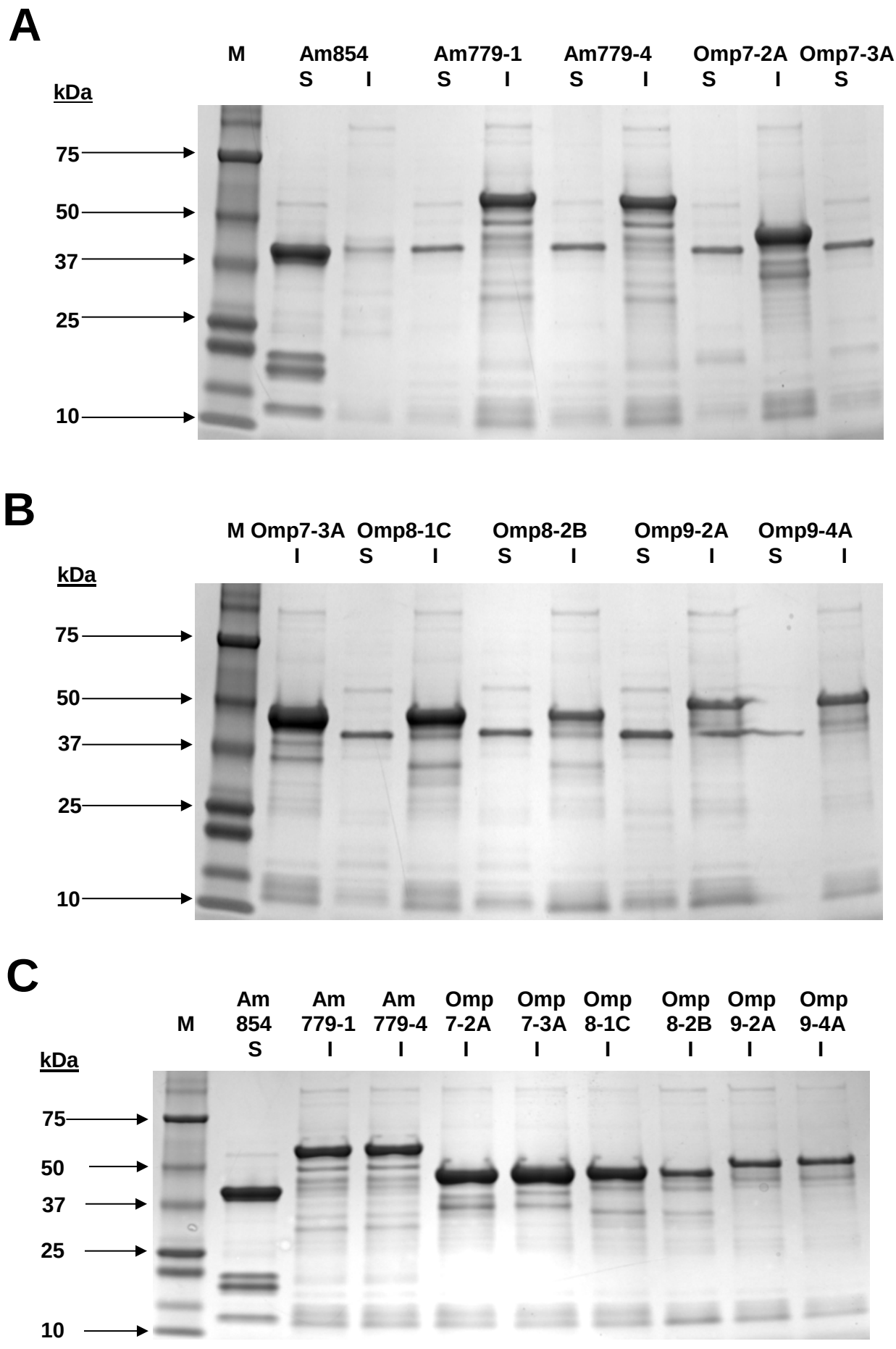
C



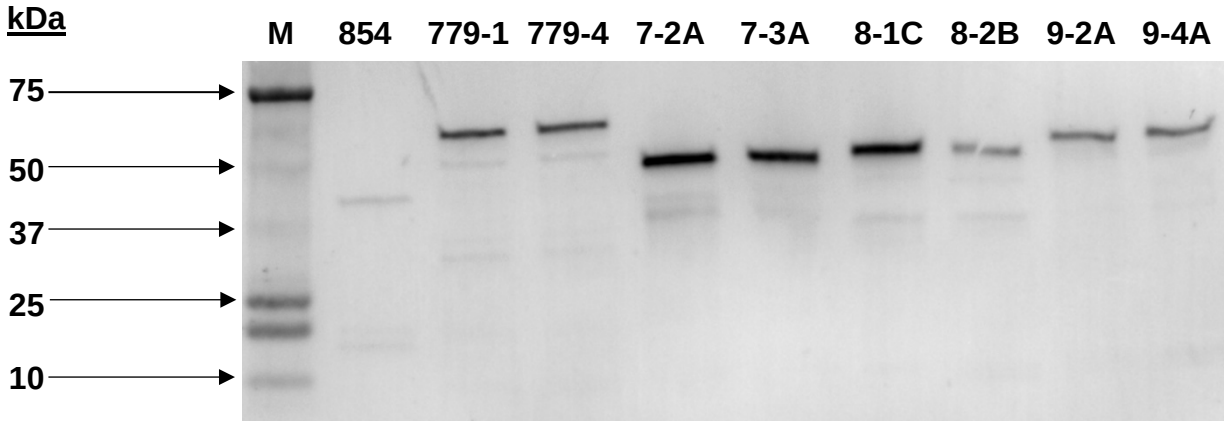
Supplementary Figure 1. Phylogenetic trees showing relationships between variants of Omp7 (**A**), Omp8 (**B**), and Omp9 (**C**) based on deduced amino acid sequences. Trees were generated using the Neighbour Joining method implemented in CLC Genomics Workbench 8.0.3. The numbers on each of the scale bars in each tree represent percentage genetic variation between sequences, given as a decimal. Samples representing variants selected for recombinant OMP expression are shown in red boxes.



Supplementary Figure 2. A: Am779-1 (variant 1 from positive transformant 127-6) pilot protein expression in uninduced (U) and induced (I) cultures over a 5-hour growth period. M = Precision Plus Protein Kaleidoscope pre-stained size standard (BioRad). **B:** Omp7-2A (variant 2A from positive transformant NW C28-1) pilot protein expression in uninduced (U) and induced (I) cultures over a 5-hour growth period. M = Precision Plus Protein Kaleidoscope pre-stained size standard (BioRad).


A
M
Am854
S
I
Am779-1
S
I
Am779-4
S
I
Omp7-2A
S
I
Omp7-3A
S
kDa
75
50
37
25
10
B
M
Omp7-3A
I
Omp8-1C
S
I
Omp8-2B
S
I
Omp9-2A
S
I
Omp9-4A
S
I
kDa
75
50
37
25
10
C
M
Am
854
S
Am
779-1
I
Am
779-4
I
Omp
7-2A
I
Omp
7-3A
I
Omp
8-1C
I
Omp
8-2B
I
Omp
9-2A
I
Omp
9-4A
I
kDa
75
50
37
25
10

Supplementary Figure 3. Expressed proteins extracted from the soluble (S) and insoluble (I) fractions of transformed *E. coli* BL-21(DE3) liquid cultures. **A:** Crude protein extracts from expressed OMPS Am854 (S and I), Am779-1 (S and I), Am779-4 (S and I), Omp7-2A (S and I), Omp7-3A (S); **B:** Crude protein extracts from expressed OMPS Omp7-3A (I), Omp8-1C (S and I), Omp8-2B (S and I), Omp9-2A (S and I), Omp9-4A (S and I). OMP Am854 was shown to be expressed mostly in the extract from the soluble fraction, whereas OMPs Am779, 7, 8, and 9 were all shown to be expressed in the largest quantities in the extract from the insoluble fraction. **C:** Purified recombinant OMP fractions that showed highest quantities of recombinant protein. The column-purified proteins have fewer background protein bands compared to those observed in the crude protein extracts, although background protein banding is not completely absent, indicating that there may still be carryover *E. coli* proteins in the column-purified OMP preparations. M = Precision Plus Protein Kaleidoscope pre-stained size standard (BioRad); S = soluble fraction; I = insoluble fraction.



Supplementary Figure 4. Colorimetric detection of recombinant His-tagged OMPs using HRP-labeled His-detector Nickel^{2+} conjugate. A blue colour developed on the areas of the membrane that corresponded to the molecular weights of the transferred His-tagged OMPs. M = Precision Plus Protein Kaleidoscope pre-stained size standard (BioRad). 779-1 = Am779 variant 1, 779-4 = Am779 variant 4, 7-2A = Omp7 variant 2A, 7-3A = Omp7 variant 3A, 8-1C = Omp8 variant 1C, 8-2B = Omp8 variant 2B, 9-2A = Omp9 variant 2A, 9-4A = Omp9 variant 4A.