Identification of a gene cluster for cell-surface genes of the SRS superfamily in *Neospora caninum* and characterization of the novel *SRS9* gene

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(*Received 16 March 2011; revised 20 June and 8 July 2011; accepted 8 July 2011; first published online 23 September 2011*)

SUMMARY

Here we present the detection of a gene cluster for *Neospora caninum* surface genes, similar to the *Toxoplasma gondii* SRS9 locus, and the cloning and characterization of the Nc*SRS9* gene. PCR genome walking, using Nc*BSR4* gene as a framework, allows the identification, upstream Nc*BSR4*, of 2 sequences homologous to the *SRS5* and the Ubiquinol-cytochrome C reductase genes and, downstream Nc*BSR4*, of an ORF of 1191 bp coding for a 396-amino acid polypeptide with 59% similarity to the TgSRS9 antigen. A putative 39-residue signal peptide was found at the NH2-terminus followed by a hydrophilic region, and a potential site for a glycosylphosphatidylinositol anchor at the COOH-terminus. A recombinant NcSRS9 protein was produced and was recognized on a Western blot by a low proportion of sera from a panel of naturally infected cows and calves. In addition, Western blot analysis using polyclonal anti-rNcSRS9 revealed stage-specific expression of NcSRS9 in bradyzoites but not in tachyzoites, and immunohistochemistry on brain from a congenitally infected calf showed NcSRS9 recognition in bradyzoites contained in tissue cysts. However, bradyzoite-specific expression of NcSRS9 could not be proven by immunofluorescence on bradyzoites obtained *in vitro* and RT-PCR analysis showed no significant variations of Nc*SRS9* transcripts during *in vitro* tachyzoite-bradyzoite switch, probably due to incomplete maturity of *in vitro* bradyzoites. Initial characterization of NcSRS9 in this study may lead to further studies for a better understanding of *N. caninum* persistence.

Key words: *Neospora caninum*, bradyzoite, genome walking, *SRS9* locus, Nc*SRS9* gene, NcSRS9 protein.

INTRODUCTION

Neospora caninum is an apicomplexan parasite that nowadays continues to be a major abortifacient in cattle worldwide (Dubey and Schares, 2011). Neosporosis is a disease responsible for abortion, stillbirth and transplacental transmission in cattle, in addition to neurological diseases in various animal species (Anderson *et al*. 2000; Dubey, 2003). Dogs, coyotes and Australian dingoes have been reported to serve as definitive hosts for *N. caninum* (Gondim *et al*. 2004; King *et al*. 2010), and recently oocysts have been demonstrated in feces from a naturally infected gray wolf (Dubey and Schares, 2011).

The similarities in the structural and biological characteristics of *N. caninum* and *Toxoplasma gondii* have been thoroughly reviewed (Dubey *et al*. 1988; Lindsay and Dubey, 1989; Hemphill *et al*. 2004). Although the molecular signals that trigger the interconversion between tachyzoites and bradyzoites are not yet fully understood, it is now feasible to study these mechanisms *in vitro* (Risco-Castillo *et al*. 2004; Vonlaufen *et al*. 2004). The cloning and further characterization of stage-regulated genes is critical for uncovering the mechanisms involved in parasite transformation and immune response evasion, 2 crucial events in *N. caninum* pathogenesis.

Besides genomic comparison, the study of stage-regulated proteins can be also achieved via a proteomic approach with other coccidian parasites like *T. gondii*. Thus, several *N. caninum* proteins differentially regulated between tachyzoites and bradyzoites have been recently described by Two-dimensional difference gel electrophoresis (2D-DIGE) and mass spectrometry (Marugan-Hernandez *et al*. 2010). Since the biological function of their orthologue proteins in *T. gondii* is related to the differentiation process these findings open another gateway for research in *N. caninum*.

Neospora participates actively during the adhesion to and invasion of its target cell. The mechanisms

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doi:10.1017/S0031182011001351

https://doi.org/10.1017/S0031182011001351 Published online by Cambridge University Press

employed by the parasite would be partially mediated by the expression of surface molecules displayed sequentially and in a stage-specific fashion (Hemphill *et al*. 2004). Two members of the superfamily of surface antigens related to the SAG1 protein (SRSs), namely NcSAG1 and NcSRS2, have been shown to be tachyzoite-specific and stage-sharing proteins, respectively, and play a role in host cell entry (Hemphill and Gottstein, 1996; Nishikawa *et al*. 2000). However, it is unclear whether SRSs expressed during the bradyzoite stage are involved in the mechanisms of host-parasite interaction and, therefore, in the evasion of the host immune system, as hypothesized for *T. gondii* (Lekutis *et al*. 2001).

Recent studies have identified Nc*SAG4* and Nc*BSR4* as bradyzoite-specific antigens expressed either early or late in the bradyzoite conversion (Fernandez-Garcia *et al*. 2006; Risco-Castillo *et al*. 2007). The existence of an SRS superfamily in *N. caninum* similar to that of *T. gondii* is an unexplored field that should be investigated. This work introduces the first identification of an orthologue of the *T. gondii SRS9* locus (Jung *et al*. 2004) as well as the identification and molecular characterization of the Nc*SRS9* gene, an orthologue of Tg*SRS9* (Cleary *et al*. 2002; Kim and Boothroyd, 2005).

MATERIALS AND METHODS

Parasite production and in vitro *bradyzoite differentiation*

Neospora caninum tachyzoites from Nc-Liv isolate were maintained by serial passage on MARC-145 cell monolayers at a 1:1 host:parasite ratio. Cells were passaged twice weekly and maintained in Dulbecco's modified Eagle medium (DMEM; Cambrex) supplemented with 10% fetal bovine serum, 15 mM HEPES (pH 7·2), 2 mM glutamine, 100 U ml^{-1} penicillin, 100 $\mu g\, ml^{-1}$ streptomycin and 250 ng ml^{-1} fungizone and incubated (37 °C, 5% CO_2).

The *in vitro* induction of stage conversion was carried out in MARC-145 cells infected with tachyzoites at a 2:1 host:parasite ratio and cultured in either 75 cm^2 flasks or on 12 mm coverslips. At 24 h post-infection, the medium was replaced, and 70 μM sodium nitroprusside (Sigma-Aldrich) was added and renewed daily for 7 days, as described previously (Risco-Castillo *et al*. 2004). Untreated infected cell cultures were used as controls. Cells grown on coverslips were fixed every day up to day 3 and after stress on days 5 and 7, whereas the cultures grown in flasks were collected at days 1, 3 and 5 for RNA extraction. Parasites were subsequently purified from the cell monolayer by rupture with a 25-gauge needle followed by centrifugation (1350 ***g***, 4 °C, 15 min). The pellet was resuspended in cold PBS, washed once by centrifugation, and resuspended in 5 ml of PBS. The parasites were then separated from the host cells on PD-10 columns (GE Healthcare). Purified zoites were pelleted and conserved at −80 °C until use.

PCR amplification and in silico *analysis*

Total DNA from 2×10^7 purified tachyzoites was isolated using the DNeasy Blood & Tissue Kit (Qiagen). Total RNA from tachyzoites and bradyzoites was isolated using the RNeasy Tissue Kit (Qiagen) following the manufacturer's recommendations.

Starting from the Nc*BSR4* gene sequence (NCBI Accession number EF151130), we followed a bidirectional primer walking approach by using the Universal Genome Walker kit (Clontech) according to manufacturer's instructions to determine nucleotide sequences at both ends of this gene. Briefly, 4 *N. caninum* genomic DNA libraries were built using restriction endonucleases included in the Universal Genome Walker kit (Clontech) and gene-specific primers designed using Primer Express software (v.2.0; Applied Biosystems) (Table 1) to amplify either upstream or downstream the Nc*BSR4* sequence (Table 1). Amplification was performed by nested PCR using a DNA polymerase with exonuclease activity (Ecotaq Plus; ECOGEN) and products were sequenced (ABI Prism 3730 genetic analyzer; Applied Biosystems). The reads were manually assembled with the BioEdit sequence alignment editor v. 7.0.9. Primers used for sequencing were designed from the Nc*BSR4* sequence and later on from the preceding sequenced reads (Table 1, Fig. 1).

Identification of conserved domains (CDs) in the obtained *N. caninum* sequence with orthology to *T. gondii* was made with the Basic Local Alignment Search Tool (BLAST) suite of programs accessible via the *Toxoplasma gondii* genome project (http://www.tigr.org/tdb/e2k1/tga1/, http://eupathdb.org/eupathdb/). Also, sequences obtained were compared to annotated ESTs from the *Neospora caninum* genome project (http://www.sanger.ac.uk/sequencing/Neospora/caninum/).

The Expand High Fidelity PCR System (Roche Applied Science) was used to amplify putative open reading frames (ORF) such as the sequence corresponding to Nc*SRS9*. Primers NcSRS9F and NcSRS9R2 were designed from 3′ and 5′ flanking sequences of the putative Nc*SRS9* sequence (Table 1). The PCR conditions were 94 °C for 2 min, 30 cycles at 94 °C for 15 s, 60 °C for 30 s and 72 °C for 1 min, and a final elongation at 72 °C for 7 min. PCR products were analysed by electrophoresis on a 1·5% low-melting agarose gel, excised and purified with the GENECLEAN Turbo nucleic acid purification kit (Q-BIOgene). Purified DNA fragments were sequenced using the same primers. To

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Table 1. Oligonucleotides used for PCR, RT-PCR, sequencing and genome walking

Primer Name	Primer ID	Primer sequence 5′ to 3′[a]	Location (nt)	Direction
GWSRS6.4	R15	ggagaaaagaacgaaagtgaaagag	813–834	Antisense
GWSRS6.3	R14	gtatggtcatcgaggaaaactgtc	915–938	Antisense
SRS6.2B	R13	ccttttctcttgcacacccagacga	1678–1702	Antisense
GWSRS6.2A	R12	ttgaacgtctttgtttcagctcgtag	1709–1734	Antisense
SRS6.1B	R11	acgctgtttttggggaggatatgct	1760–1784	Antisense
GWSRS6.1A	R10	gggaggatatgctacgagtttaactactg	1764–1790	Antisense
R10.2 BSR4	R9	gagttgaacccgaccgtagctcag	2388–2410	Antisense
R9.2 BSR4	R8	aagatcatgtgaaatgctgctgctgt	2416–2441	Antisense
R10.3 BSR4	R7	cgaccgtagctccggctacttagttt	2454–2479	Antisense
R9.3 BSR4	R6	tgctgttgctgtacaggagttgaacc	2480–2505	Antisense
R8 BSR4	R5	gacgtcttaaatatcttcggtggcc	3066–3089	Antisense
R7 BSR4	R4	tgtctgtgctgtttgttctctgatg	3123–3147	Antisense
Sec R2	R3	atgcgtcttgcgaagagttgttca	3291–3944	Antisense
R6BSR4	R2	acacgtttgcacccagaccc	4625–4644	Antisense
R5BSR4	R1	ttggatgtagggctgttttttagg	4664–4687	Antisense
F3.2 BSR4	F1	cacgtcgaccacagcatctatcttttt	9013–9039	Sense
F4.2 BSR4	F2	ctttcgatggaagcgtttttgata	9043–9063	Sense
Sec F1	F3	gctccaagacacatgcccatctt	9679–9701	Sense
Sec F2	F4	ccggcttgcctgttcttat	10327–10345	Sense
F5 BSR4	F5	acaatatccacgatacccccgaat	10963–10986	Sense
F6 BSR4	F6	agaaagtgacaagagctgcaggattga	11024–11050	Sense
F7 BSR4	F7	acagtttcgtttgagttgcgttctc	11616–11639	Sense
F8 BSR4	F8	cgacaaacccagagagtgaaaaggtc	11654–11678	Sense
F9 BSR4	F9	gtgctcgaaaacacagatgagtgcat	12207–12231	Sense
F10 BSR4	F10	aatctcacaccaacatgctcgctttt	12247–12272	Sense
Secig6F	F11	cagcagaatgcgaatcaaaa	12788–12807	Sense
Secig7F	F12	tgttgtcattcaccgtccat	13445–13467	Sense
GWSRS9F1	F13	tttgcggatagaagaagcctct	13677–13698	Sense
GWSRS9F2	F14	acaacagaaaacaacaccctcactctt	14192–14217	Sense
GWSRS9F3	F15	aagatcttcctagttggctgtaaaaacc	14301–14328	Sense
NcSRS9F		taattcgttttccagagtgtgca	10620–10642	Sense
NcSRS9R2		tggtattcccacagaagtcaagg	11848–11870	Antisense
Nc18sF		gatacagaaccaacccaccttcc	189–211[b]	Sense
Nc18sR		agaccgaagtcaaacgcgatc	265–245[b]	Antisense
NcSAG1F		cggtgtcgcaatgtgctctt	721–739[c]	Sense
NcSAG1R		acggtcgtcccagaacaaac	921–901[c]	Antisense
NcSAG4F		gatttcaagaagccgctgga	339–358[d]	Sense
NcSAG4R		tgagaacttgtgtgtcgcctgtt	468–446[d]	Antisense
FoSRS9KpnI		gctggtaccgaaaaactccgtgaa	10748–10771	Sense
ReSRS9SacI		ggcgagctcttaaaacgaagcaaaat	11751–11765	Antisense

[a] Underlined restriction sites for cloning of Nc*BSR4*.
[b] nt position in Nc*18sR*.
[c] nt position in Nc*SAG1*.
[d] nt position in Nc*SAG4*.

confirm fidelity of sequencing, at least 2 sequences from separate PCRs were aligned with 100% consensus.

Deduction of some physico-chemical properties and functional motifs from the putative NcSRS9 protein sequence were computed using ExPASy proteomics tools (http://au.expasy.org/). Hydrophobic domains were identified using both the Kyte–Doolittle hydropathy scale (Kyte and Doolittle, 1982) and the TMHMM predictor (http://www.cbs.dtu.dk/). The N-terminal signal peptide was identified with the SignalP v3.0, while the C-terminal glycosylphosphatidylinositol anchor (GPI) was sought by http://mendel.imp.univie.ac.at/sat/gpi/gpi_server.html. To analyse its putative pattern and profile of the amino acid sequence, we used the CYSPRED prediction method (http://www.predictprotein.org) and the Motif Scan predictor (http://myhits.isb-sib.ch/cgi-bin/motif_scan).

Quantitative RT-PCR

Specific primers were designed for Nc*SRS9* (NcBSR4F3 and NcBSR4R3), and we used the primers described by Fernandez-Garcia *et al.* (2006) for the Nc*SAG1*, Nc*SAG4* and 18S ribosomal RNA (Nc*18sR*) genes (Table 1). Complementary DNA was synthesized from 200 ng of total parasite RNA using Superscript II RNase H minus

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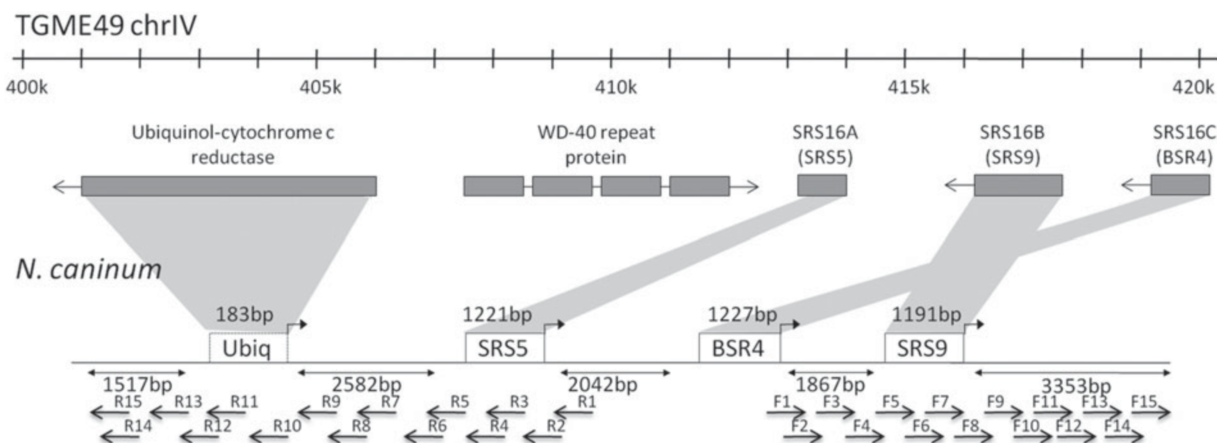

Fig. 1. Synteny representation between *Neospora caninum* and *Toxoplasma gondii*. Putative genes are depicted in grey (*T. gondii*) or white (*N. caninum*) boxes. Double arrowed lines indicate the size (bp) of intergenic regions, and the orientation of the *N. caninum* genes is given by arrows in the upper right side of the boxes. Single arrowed lines with numbers indicate primer location during genome walking, and either forward (F1-F15) or reverse (R1-R15) was used with a complementary adapter, according to manufacturer's recommendations. The distribution of *T. gondii* genes was generated with ToxoDB v.6.0 (http://toxodb.org/toxo/).

Reverse Transcriptase (2 U, Invitrogen), random hexamers (12·5 nM, Applied Biosystems) and an RNase inhibitor (40 U, Ambion) in 20 μl of reaction mixture according to the manufacturer's instructions.

Quantitative PCR was performed with the ABI Prism 7300 Sequence Detector (Applied Biosystems) using the DNA binding dye SYBR Green I (Platinum SYBR Green qPCR SuperMix UDG, Invitrogen) (Fernández-García *et al*. 2006). Three independent experiments were performed with 1:10 diluted cDNA obtained from 3 different *in vitro* productions of *Neospora* bradyzoites. All of the samples were processed in triplicate, and the absence of DNA was confirmed by direct PCR of the total RNA from each sample. For amplification, data acquisition and data analysis, we used the Sequence Detection System Software v.1.6. (Applied Biosystems). The mean Ct value between replicate samples did not exceed a standard deviation of 0·5. The relative quantification of Nc*SRS9*, Nc*SAG1* and Nc*SAG4* transcription was carried out using the comparative Ct method, using the 18S ribosomal RNA as the normalizer and *N. caninum* tachyzoites as the baseline reference. The results are expressed as the x-fold induction calculated by the $2^{-\Delta\Delta Ct}$ formula.

Plasmid construction and NcSRS9 expression and purification

The expression vector system pET-45b(+) (Novagen) was used to produce a truncated form of the putative NcSRS9 (amino acids 40 to 375, devoid of the putative signal peptide and GPI anchor sequences) as a recombinant protein. A Nc*SRS9* gene fragment corresponding to nucleotides 118 to 1125 was amplified from the genomic DNA of *N. caninum* by PCR with oligonucleotides FoNcSRS9KpnI and ReNcSRS9SacI (Table 1). The PCR was performed on 100 ng of genomic DNA using the Expand High Fidelity PCR System. The amplified DNA fragment was purified, digested with *Kpn*I and *Sac*I restriction enzymes, and cloned into the plasmid previously linearized with the same endonucleases.

The cloned sequence was expressed in Rosetta (DE3) pLysS *Escherichia coli* cells (Novagen) as a polyhistidine (His_6) fusion protein, as previously described (Risco-Castillo *et al*. 2007). The insoluble recombinant NcSRS9 (rNcSRS9) inside inclusion bodies was denatured by incubating overnight at room temperature with a binding buffer containing 8 M urea and 40 mM imidazole in a phosphate buffer (pH 7·4). rNcSRS9 was purified by immobilized metal ion-affinity chromatography (IMAC) using a HisTrap™ HP column placed onto an ÄktaPrime purification system (GE Healthcare). The column was washed with dH_2O and equilibrated with binding buffer, as described above. The column was then loaded with the His_6-tagged rNcSRS9 protein, and the protein was re-natured on-column (Zhao *et al*. 2005) and finally eluted using an imidazole ascending gradient of up to 500 mM.

The protein concentration was estimated using Coomassie blue staining with serial dilutions of BSA as a standard. The concentration was measured using Quantity One software (v.7.2; Bio-Rad). The results were confirmed through an absorbance assay, which uses the molar extinction coefficient of rNcSRS9 calculated from its amino acid composition (Gill and von Hippel, 1989).

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Mass spectrometry analysis

The protein band corresponding to rNcSRS9 was manually excised from prepared Coomassie-stained 1-D gels. Stained gel pieces were processed as described elsewhere (Risco-Castillo *et al*. 2007). All of the extracts were pooled, and the volume was reduced by SpeedVac. The list of monoisotopic peptide masses was compared with the peptide mass databases T/TrEMBL and ToxoDB 5.0 using the MASCOT algorithm (Matrix Science) to determine similarity with the TgSRS9 protein.

Polyclonal rabbit anti-rNcSRS9 serum production

The polyclonal rabbit anti-rNcSRS9 serum was produced by immunization of 2 New Zealand rabbits following standard procedures employing a custom antibody service (Proteogenix SA, France).

SDS-PAGE and Western blot analysis

Western blot analysis was carried out in order to investigate the immunoreactivity of rNcSRS9 and rNcBSR4. Ten μg of each of the purified recombinant proteins rNcSRS9 and rNcBSR4 were resolved in 15% SDS-PAGE and transferred to nitrocellulose membranes (Aguado-Martínez *et al*. 2008). Membranes were incubated with a panel of 8 serum samples from naturally infected cows and 11 pre-colostral serum samples from congenitally infected calves. All cows and calves had *N. caninum* seropositive titres higher than 1:250 by the Indirect Fluorescence Antibody Test (IFAT) and all of them recognized at least the immunodominant antigen p17 (Alvarez-Garcia *et al*. 2002). A seronegative cow and calf were also included as negative controls.

A second Western blot analysis was performed in order to study stage-specific expression of native NcSRS9 protein by using tachyzoites and a mixture of tachyzoites and bradyzoites, and the polyclonal rabbit anti-rNcSRS9 antibody. Total extracts of the Nc-Liv isolate equivalent to 2×10^6 tachyzoites and 10^6 of a mix of tachyzoites and bradyzoites per membrane were dissolved in denaturing conditions, sonicated in a water bath for 15 min and boiled for 5 min. The samples were then resolved in 15% SDS-PAGE and transferred into nitrocellulose membranes by standard methods. Western blot was performed by blocking the membranes with 5% skimmed milk in TBS-Tween overnight at 4 °C and probing at 37 °C for 1 h with the polyclonal rabbit anti-rNcSRS9 serum (dilutions 1:100, 1:200 and 1:400). The polyclonal rabbit anti-*N. caninum* tachyzoite serum (dilution 1:4000) (Alvarez-Garcia *et al*. 2007) and the polyclonal rabbit anti-rNcSAG4 serum (dilution 1:2000) (Fernández-García *et al*. 2006) were used as controls of tachyzoite and bradyzoite recognition, respectively. Membranes were then washed 5 times with TBS-Tween at 5-min intervals and incubated at 37 °C for 1 h with ECL anti-rabbit IgG secondary antibody. Detection of the specific bands was performed by chemiluminescence using the Immobilon Western Chemiluminescent HRP Substrate (Millipore, UK).

Immunofluorescence

Fixed coverslips were double-labelled (Risco-Castillo *et al*. 2004) with the monoclonal mouse anti-NcSAG1 antibody (anti-SAG1; 1:200) (Bjorkman and Hemphill, 1998) and polyclonal rabbit anti-rNcSRS9 serum (anti-SRS9; 1:50; 1:100 and 1:200). Other coverslips were also double-labelled with the polyclonal rabbit anti-rNcSAG4 (anti-SAG4) (Fernández-García *et al*. 2006) and anti-SRS9 or with the polyclonal rabbit anti-BAG1 serum (anti-BAG1; 1:50 dilution) (McAllister *et al*. 1996) and anti-SRS9. To confirm the tachyzoite-to-bradyzoite conversion, other coverslips were double-labelled with anti-BAG1 together with anti-SAG1. Goat anti-rabbit and goat anti-mouse secondary antibodies conjugated to Alexa 488 (green) or Alexa 594 (red) (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. Parasite and host cell nuclei were labelled with 4,6-diamidino-2-phenylindole (DAPI). Photomicrographs were taken with a 100X oil-immersion objective on a fluorescence-inverted microscope (Nikon Eclipse TE200) connected to a digital camera. Duplicates from the stage-conversion assay were tested each day.

Immunohistochemistry

The immune reactivity of anti-SRS9 and anti-SAG4 (Fernández-García *et al*. 2006) against mature bradyzoites produced *in vivo* was evaluated. Brain slices containing *N. caninum* tissue cysts were sampled from a congenitally *Neospora*-infected calf in which infection had been previously confirmed by the IFAT (*N. caninum*-antibody titre of 1:800), detection of *N. caninum*-associated histopathological lesions (Regidor-Cerrillo *et al*. 2008) and by isolation of the parasite from the brain (Nc-Spain7 isolate) (Regidor-Cerrillo *et al*. 2008). Moreover, several tissue cysts in the brain were also previously detected by immunohistochemistry with antibodies reacting against other bradyzoite-specific protein, anti-BSR4 (Risco-Castillo *et al*. 2007). Both polyclonal rabbit antibodies were used at 1:100 dilution, and the streptavidin-biotin-peroxidase technique was performed as previously described (Pereira-Bueno *et al*. 2003).

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NcSRS9    MARRGSMQHRRGGLKSKAGKLMVVCMGGVLLIASGQAGAEKLREGLQHRGLQQQASTTVT 60
TgSRS9    MERTGNMQQRLGGFKSQVRKLMAVCIGGILLLSSGQGLADSLHEGLQSLSLKSQVGVSSK 60
          * * *.**:* **:**:. ***.**:**:**::***. *:.*:****   .*:.*...: .

NcSRS9    PTINGATATCTFPTA-VGQQTVAASSGLKLSKESLTAVLQCSGEQGATISTIPPNLGANV 119
TgSRS9    VDVTGTHAKCEFSTAGAGSSTAVSKQSLTLSKGSLTATLECTAKDGGTISSIPNPLAGNV 120
            :.*: *.* *.** .*..*..:...*.*** ****.*.*:.::*.***:**  *..**

NcSRS9    CVTNYNGKESDKSCRIEG-STDGAETALKELLGTTRDITWTKKTVNEISKASTPTDWSLE 178
TgSRS9    CVTKQKQSETPCNFGENGGAAASVQVPLKDVLGTTSAVQWMNTTAEQKAIQSQ--KWTLK 178
          ***: : .*:  .    :* :: ..:..**::****  : * :.*.:: :  *   .*:*:

NcSRS9    LLDSDLPLSEKAFFVGCQQTTGTDKRARQTKSCTVTVNVDARDSAVGANNVVTCAYGKNS 238
TgSRS9    LNEEDLPISDKTFDVGCTYTT-TGKAAANPAVCQLTVNVKARASSVADENVVTCSYGAES 237
          * :.***:*:*:* ***  ** *.* * :.  * :****.** *:*. :*****:** :*

NcSRS9    NPEPLKVVMTTEKNTLTLHCGSEGSVNPASYETQFCDPQGKDMKNCVRKNYVDILPTFLT 298
TgSRS9    NSEALKVDMTTTRNTVTIDCGSEGTLNPAADSAQYCNPQNKDLDDCKKK-FVDILPNFVI 296
          *.*.*** *** :**:*:.*****::***: .:*.*:**.**:..* :* :*****.*:

NcSRS9    SWWTKEHEGSTVKLTIPETDFPESEQQFRLSCVLKDRDATNPESEKVKEDSDGAQAAATA 358
TgSRS9    SWWKTPTPGNASILTIPAADFPESEQQFRLGCVPNKAPQSDSQDTRVDNQSQVQSRTSAP 356
          ***..   *.:  **** :***********.** :.   ::.:. :*.::*:  . :::.

                           ↓
NcSRS9    SRCNVVVVVVKSANFASFASP----AAEIAAAAAVTGLFASSL 396
TgSRS9    TTCSVLVTVKAASSASYASPSLQTLIAVSGAAVVSGFVVASL 398
          : *.*:*.**:*. **:***       ::.**.*:*:..:**
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Fig. 2. Deduced polypeptide sequence of NcSRS9 and alignment produced by ClustalW with the homologous protein in *Toxoplasma gondii* (TgSRS9). The amino acid sequence of NcSRS9 contains a polypeptide of 396 amino acids. The putative signal peptide at the amino end is in bold and italics, while the arrow indicates the potential GPI anchor cleavage at the carboxyl end. Boxes indicate conserved cysteines, and the putative SAG domains are shaded.

RESULTS

Identification of the NcSRS9 locus and isolation of the NcSRS9 gene

After amplification and sequencing analysis of the polynucleotide sequences obtained both upstream and downstream from the Nc*BSR4* gene, a nucleotide sequence with a total of 15 183 bases was obtained. These sequences were blasted against already annotated *T. gondii* and *N. caninum* sequences using the BLASTN and BLASTX algorithms (ToxoDB database, TIGR database), and 3 different sequences flanking Nc*BSR4* were identified. These sequences were termed Ubiquinil-cytochrome C reductase, *SRS5* and *SRS9*, given their homology to sequences previously reported in the *T. gondii SRS9* locus and in a locus located on chromosome IV of *N. caninum* (Fig. 1). The sequence found 2042 bp upstream of Nc*BSR4* was formed by 1221 nt and had up to 75% similarity to the Tg*SRS5* pseudogene. This sequence also included a termination codon and a likely frameshift followed upstream by a 3876 bp intergenic space and a 183 nt sequence with 100% similarity to the *T. gondii* Ubiquinol-cytochrome C reductase. Both sequences had homologues that read consecutively in annotated sequences from chromosome IV of *T. gondii* and *N. caninum* (BLASTX, ToxoDB) and were also found contiguous after comparisons with contigs deposited in the public database at the Sanger Centre (TGG_856).

We also isolated the *N. caninum* Nc*SRS9*, an orthologue of *T. gondii* Tg*SRS9* (Howe and Sibley, 1999). Analysis of a nucleotide sequence 1867 bp downstream from Nc*BSR4* confirmed the presence of an 1191 nt ORF with no introns that translated into a sequence of 396 amino acids (Fig. 2) with a predicted mass of 41 957·4 Da. The similarity of this encoded protein to TgSRS9 is 59%, sharing up to 222 residues (TBLASTX, NCBI), and analysis using re-annotated *T. gondii* databases showed up to 63% similarity between Nc*SRS9* and sequences flanking the 3′ end of the Tg*BSR4* gene on the *T. gondii* SRS9 locus.

Furthermore, DNA from 3 *N. caninum* isolates (Nc-1, Nc-Liv, and Nc-Spain1H) was amplified using NcSRS9F and NcSRS9R2 oligonucleotides (Table 1) and sequenced to confirm the composition of the complete NcSRS9 ORF. The absence of polymorphisms in Nc*SRS9* was confirmed among these isolates. Due to the fact that the *T. gondii* genome is completely sequenced and that no other sequence showed higher similarity, the *N. caninum* gene identified is the most likely orthologue of the Tg*SRS9* gene.

Predicted NcSRS9 protein sequence analysis

Two hydrophobic domains were identified at the N- and C- terminal ends of the NcSRS9 protein

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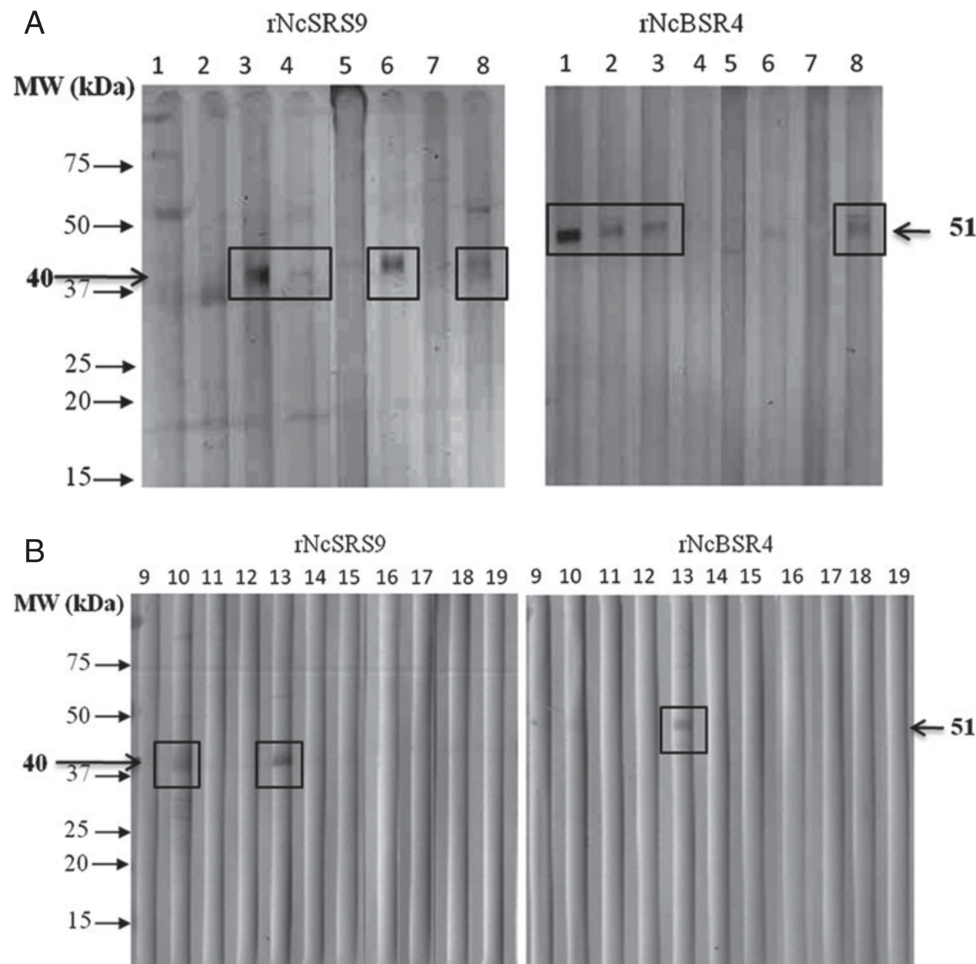

Fig. 3. Western blot analysis of recombinant NcSRS9 protein. The membrane was probed with a set of *Neospora caninum* IFAT-positive sera from naturally infected cows (lanes 1–8; panel A) and pre-colostral sera from congenitally infected calves (lanes 9–19; panel B). *N. caninum* IFAT-negative controls were included in both panels (lane 5 from panel A; lane 19 from panel B). Samples were also probed against anti-rNcBSR4 serum to confirm the development of an immune response against antigens produced during the bradyzoite stage. Arrows show apparent molecular weights of both recombinant proteins.

using the Kyte–Doolittle hydrophobicity scale. A 39-amino acid putative signal peptide was found at the N-terminus, and a potential GPI anchor was found 22 amino acids upstream of the C-terminus, in accordance with the ω and $\omega+2$ rule (Gerber *et al.* 1992). Furthermore, the CYSPRED prediction method found 12 conserved cysteine residues of the SRS antigen family (Fig. 2), and the Motif Scan engine found 2 putative SAG domains between amino acids 25 and 178 and between amino acids 187 and 328.

Production and immunoreactivity of the NcSRS9 recombinant protein (rNcSRS9)

A recombinant version of the putative mature NcSRS9 protein (amino acids 40–375; rNcSRS9) was produced in the prokaryotic expression vector system pET-45b(+) and expressed as a His_6-tag fusion protein of the expected size (40 kDa approximately) (data not shown). Recombinant NcSRS9 accumulated as inclusion bodies that were then purified by IMAC. The obtained protein, rNcSRS9, was confirmed by mass spectrometric analysis as the expected protein after the nucleic acid translation of the corresponding gene. Furthermore, purified rNcSRS9 was run in SDS–PAGE under reducing conditions and incubated against the polyclonal rabbit anti-rNcSRS9. As expected, the protein reacted specifically (data not shown). To confirm the immunoreactivity of rNcSRS9, a Western blot assay using 8 serum samples from *N. caninum*-naturally infected cows (Fig. 3A) and 11 serum samples from *N. caninum*-congenitally infected pre-colostral calves (Fig. 3B) showed that only 4 naturally infected cows and 2 congenitally infected calves reacted against rNcSRS9 (apparent molecular weight of 40 kDa) and other 4 cows and 1 calf reacted against rNcBSR4 (51 kDa) (Fig. 3).

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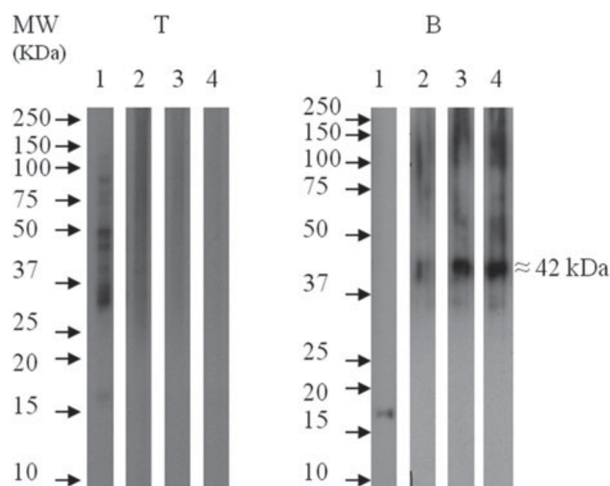

Fig. 4. Western blot analysis of total extracts of *Neospora caninum* tachyzoites (T) and bradyzoites (B) produced *in vitro*. The membranes were probed with the rabbit anti-rNcSRS9 serum at dilutions 1:400, 1:200 and 1:100 (lanes 2, 3 and 4, respectively). Polyclonal rabbit antisera against *Neospora caninum* tachyzoites (lane 1 in tachyzoite membrane) or against NcSAG4 (lane 1 in bradyzoite membrane) were used as controls for tachyzoites and bradyzoites, respectively.

NcSRS9 expression

Transformation of tachyzoites into bradyzoites along the time-course assay was confirmed by BAG1 detection by IFAT (data not shown). However, double immunofluorescence after 5 and 7 days of stress with anti-SRS9 and anti-SAG1, or anti-BAG1 failed to show significant NcSRS9 labelling in BAG1-positive vacuoles or SAG1-negative vacuoles (data not shown). A single immunofluorescence assay with anti-SRS9 was also unable to produce a good labelling of *in vitro* cultures containing bradyzoites.

Besides immunofluorescence, NcSRS9 expression was studied by Western blot and immunohistochemistry using the generated anti-SRS9. Using Western blot, a band with the expected size for native NcSRS9 (42 kDa) was observed reacting specifically in the sample containing bradyzoites induced *in vitro* but not in the sample containing only tachyzoites (Fig. 4). Moreover, mature bradyzoites on paraffin sections of brain tissue obtained from a congenitally infected calf showed that all bradyzoites contained in a tissue cyst were labelled with both anti-SRS9 and anti-SAG4 (Fig. 5).

Real-time quantitative RT-PCR was used to compare the mRNA levels of Nc*SRS9*, Nc*SAG1* and Nc*SAG4* genes in *N. caninum* parasites obtained during the *in vitro* stage-conversion assay. When compared with the reference (non-stressed *N. caninum* cell culture) and normalized to 18S ribosomal RNA, the Nc*SAG1* mRNA transcription levels showed minor variations. However, while the levels of Nc*SAG4*, an early expressed bradyzoite-specific gene, reached a mean value of 254·8-fold induction on day 5 after stress, no significant variations of Nc*SRS9* transcription levels were detected during the time-course assay (Fig. 6).

DISCUSSION

The ability of bradyzoites to differentially express a broad range of surface antigens as a strategy for host immune evasion has been observed in most apicomplexan parasites (Ajioka and Soldati, 2007). Nevertheless, characterization of the *N. caninum* SRS superfamily specifically expressed at the bradyzoite stage is still lacking. In this work, we applied the genome walking method using the Nc*BSR4* gene as a framework to elucidate the presence of other bradyzoite up-regulated genes flanking Nc*BSR4*. Our results introduce preliminary data regarding the *N. caninum SRS9* locus, which displays significant homology to the *T. gondii SRS9* locus with some differences (Jung *et al*. 2004). Our efforts led us to locate 3 genes (putative Ubiquinol-cytochrome C reductase, Nc*BSR4* and Nc*SRS9*) and 1 putative pseudogene (*SRS5*) on the same locus based on those observed through *Neospora* genome annotation made by the Welcome Trust Sanger Institute (data available on http://www.sanger.ac.uk/sequencing/Neospora/caninum/).

The Ubiquinol-cytochrome C reductase is an enzyme and a component of the cytochrome bc complex associated with the mitochondrial system of electron transport and ATP synthesis. This enzyme had been previously identified in *Plasmodium falciparum* (Krungkrai *et al*. 1997) and *T. gondii* (Morales-Sainz *et al*. 2008), and orthologues of other components of this complex in *N. caninum* have been described recently (Kang *et al*. 2008). This enzyme could be related to the metabolic pathways of *N. caninum*, and our findings could lead to further studies that identify the former as a target for the potential development of treatments to prevent drug resistance (McFadden *et al*. 2000).

We also identified a putative *SRS5* pseudogene, described for the first time in *N. caninum*. This sequence would be an orthologue of Tg*SRS5*, whose sequence was also obtained by the genome walking method (Lekutis *et al*. 2001). Additionally, the presence of a stop codon and a frameshift with a distribution similar to that of *T. gondii* would confirm our results. While the SRS superfamily in *T. gondii* would include more than 160 genes (Jung *et al*. 2004), little is known regarding the clusters that contain these genes in *N. caninum*. In that sense, this report offers the opportunity to determine whether *SRS9* and other *SRS* loci in *Neospora* are anciently derived or if they have expanded recently.

In addition, this paper reports the cloning, expression, and molecular and immunological characterization of Nc*SRS9*, a novel surface gene of *N. caninum*, homologous to *T. gondii* Tg*SRS9*.

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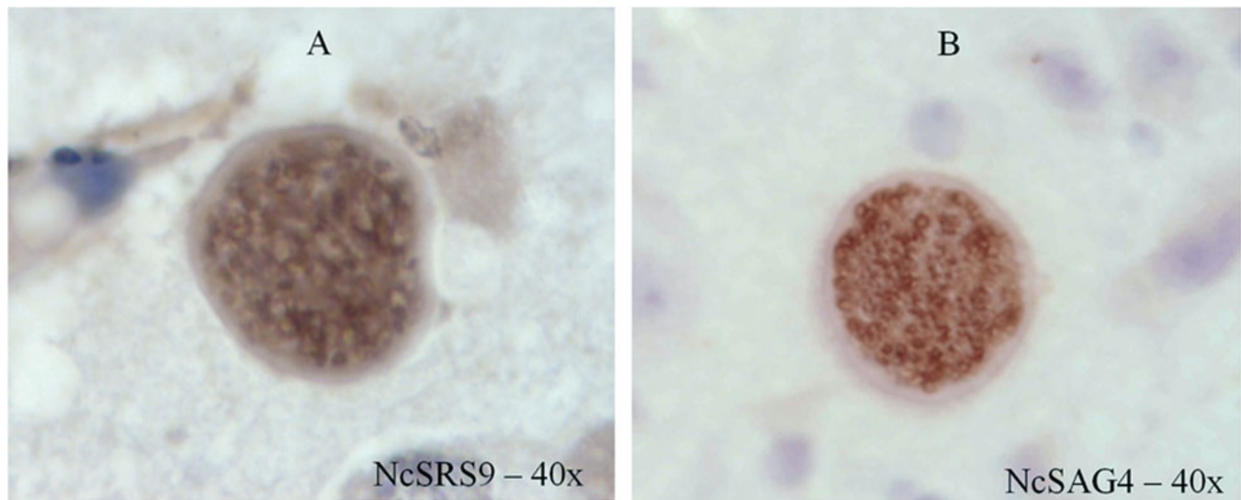

Fig. 5. A *Neospora caninum* cyst on the brain of a naturally infected calf. Labelling of bradyzoites with anti-rabbit rNcSRS9 (panel A) or anti-rabbit rNcSAG4 (panel B) by immunohistochemistry is located on the parasite's surface (40X).

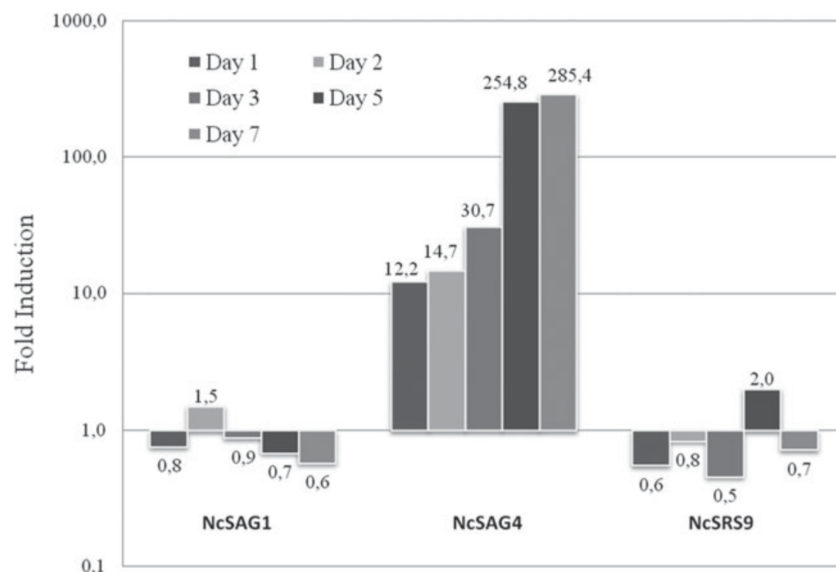

Fig. 6. Transcription rates of the Nc*SRS9*, Nc*SAG1* and Nc*SAG4* genes in *Neospora caninum* parasites along the tachyzoite-to-bradyzoite conversion assay *in vitro*. Transcription rates were determined by real-time RT-PCR and calculated as the fold induction when compared with control tachyzoites.

The identification of a putative N-terminal signal peptide and GPI anchor at the C-termini in the protein encoded by Nc*SRS9* correlated to that expected for a protein tethered to the cytoplasmic membrane and exposed to the surface of the parasite (Cross, 1990; Jung *et al*. 2004; Knoll and Boothroyd, 1998). This feature is similar to the previously described NcSAG4 and NcBSR4 bradyzoite-specific surface proteins (Fernandez-Garcia *et al*. 2006; Risco-Castillo *et al*. 2007).

The presence of 12 cysteine residues in the NcSRS9 antigen has also been observed in other SRS antigens of *T. gondii* and *N. caninun* (Howe *et al*. 1998; Risco-Castillo *et al*. 2007). Their conservation may indicate that they are involved in the intramolecular disulfide bonding of SRS antigens and therefore suggests that they would show a similar folding pattern (Howe and Sibley, 1999). This result further suggests that they may participate in the adhesion of the zoite to some host cell receptors and therefore in an interaction with the host immune response (Ajioka and Soldati, 2007).

The Western blot performed with rNcSRS9 and the serum from naturally infected cows and calves showed that only a small proportion of *N. caninum*-infected animals reacted against rNcSRS9 as well as against rNc*BSR4*. This moderate to low immunogenicity of both surface proteins could be explained by a possible late bradyzoite up-regulated expression during chronic infection reducing antigenic exposure to the host immune response. This hypothesis is in accordance with previous

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findings with NcBSR4 and TgSRS9 (Risco-Castillo *et al*. 2007; Kim and Boothroyd, 2005; Kim *et al*. 2007) and also with the lack of NcSRS9 recognition on *in vitro* bradyzoite-infected cell cultures by immunofluorescence.

The expression and localization of NcSRS9 in the parasite was studied by immunofluorescence, Western blot and inmunohistochemical assays on *in vitro* (Risco-Castillo *et al*. 2004) and *in vivo* bradyzoites using the polyclonal rabbit antiserum raised against rNcSRS9. Whereas no bradyzoite-stage-specific expression of NcSRS9 could be observed by immunofluorescence, probably due to the absence of mature bradyzoites and/or cysts, the immunohistochemical technique revealed NcSRS9 expression in mature tissue cysts, and Western blot showed the recognition of a specific band of NcSRS9 expected molecular weight only on the tachyzoite/bradyzoite extract and not on the tachyzoite extract. The fact that positive results were obtained by Western blot and not by immunofluorescence can be explained by the significantly higher sensitivity of Western blot, since even low expression levels of NcSRS9 can be revealed by this technique.

No significant increase of Nc*SRS9* transcription levels during bradyzoite transformation *in vitro* was observed. As it has been shown by immunofluorescence, the lack of *in vitro* mature bradyzoites might explain this result if an up-regulation of this gene takes place in late or mature bradyzoites. This is in accordance with previous studies where only early-expressed bradyzoite genes, like NcSAG4, showed a significant increase of transcription levels with up to 200-fold up-regulation (Fernández-García *et al*. 2006), whereas other late-expressed genes, such as NcBSR4, showed significantly lower transcription levels of up to 20-fold up-regulation (Risco-Castillo *et al*. 2007).

The transcription factors described in apicomplexan parasites might specifically regulate the expression of developmental stage-specific target genes and mould gene expression in an early or late fashion (Yang and Parmley, 1997; Balaji *et al*. 2005). Even if the bradyzoite-specific expression of SRS antigens would avoid the induction of a potentially protective immune response, it is important to recall that SRS antigens expressed in bradyzoites are the first to be exposed to the intestinal epithelium of the definitive host during oral infection. In *T. gondii*, it has been observed that a lack of SRS9 expression does not affect parasite infectivity in mice but does compromise its ability to persist in the brain (Kim and Boothroyd, 2005). Furthermore, TgSRS9 seems to play an important role in reactivation of *T. gondii* in intestinal tissues from chronically infected mice (Kim *et al*. 2007). In that sense, further investigation into an immunological role of NcSRS9 other than the reinforcement of immune response evasion is required to unveil its role in the mechanisms by which *Neospora* is able to evade the host immune response and undergo persistent infection.

ACKNOWLEDGEMENTS

We thank Louis M. Weiss (Albert Einstein College of Medicine, New York) and Andrew Hemphill (Institute of Parasitology, University of Bern) for the polyclonal rabbit anti-recombinant BAG1 (*T. gondii*) serum and the mAb mouse anti-NcSAG1, respectively. We also thank the genomics and proteomics units from Complutense University for sequencing assistance, Angel Zaballos Sanz (CNB) for his advice and helpful discussion regarding the cloning system, José Manuel Bautista (Department of Biochemistry II, UCM) for assistance with protein purification, Antonio Rodriguez Bertos for his help with immunohistochemistry, and Maria Díaz for excellent technical support. This work was financed by grants from the Spanish government (AGL-2004-02529/GAN and AGL-2007-60132/GAN) and was part of the EU COST-Actions 854 and 857. Verónica Risco-Castillo was supported by a grant financed by the Agencia Española de Cooperación Internacional (AECI).

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https://doi.org/10.1017/S0031182011001351 Published online by Cambridge University Press