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Review

Functional genomics and evolution of tick–*Anaplasma* interactions and vaccine development

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ABSTRACT

The genus *Anaplasma* (Rickettsiales: Anaplasmataceae) includes several tick-transmitted pathogens that impact veterinary and human health. Tick-borne pathogens cycle between tick vectors and vertebrate hosts and their interaction is mediated by molecular mechanisms at the tick–pathogen interface. These mechanisms have evolved characteristics that involve traits from both the tick vector and the pathogen to insure their mutual survival. Herein, we review the information obtained from functional genomics and genetic studies to characterize the tick–*Anaplasma* interface and evolution of *A. marginale* and *A. phagocytophilum*. *Anaplasma* and tick genes and proteins involved in tick–pathogen interactions were characterized. The results of these studies demonstrated that common and *Anaplasma* species-specific molecular mechanism occur by which pathogen and tick cell gene expression mediates or limits *Anaplasma* developmental cycle and trafficking through ticks. These results have advanced our understanding of the biology of tick–*Anaplasma* interactions and have opened new avenues for the development of improved methods for the control of tick infestations and the transmission of tick-borne pathogens.
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1. Introduction

Ticks (Acari: Ixodidae) transmit a wide variety of pathogens to vertebrates including *Anaplasma* spp. (Rickettsiales: Anaplasmataceae) (de la Fuente et al., 2008a). The genus *Anaplasma* contains obligate intracellular organisms found exclusively within membrane-bound inclusions or vacuoles in the cytoplasm of both vertebrate and invertebrate host cells (Dumler et al., 2001; Kocan et al., 2004). The genus *Anaplasma* includes pathogens of ruminants, *A. marginale*, *A. centrale*, *A. bovis*, and *A. ovis*. Also included in this genus are *A. phagocytophilum*, which infects a wide range of hosts including humans and wild and domesticated animals, and *A. platys* which infects dogs.

Anaplasma spp. share many common features (Kocan et al., 2008). Vertebrate hosts and ticks develop persistent infections with *Anaplasma* spp. which, in turn, allows them to serve as a reservoir of infection. *Anaplasma* are transmitted horizontally by ixodid ticks while transovarial transmission does not appear to occur. Transstadial transmission occurs from stage to stage (larvae–nymphs, nymphs–adults and larvae–adults) and therefore every tick generation must obtain infection by feeding on reservoir hosts. The developmental cycles of *A. ovis* and *A. centrale* and most extensively *A. marginale* have been described and shown to be coordinated with the tick feeding cycle (Kocan et al., 1992a,b; Kocan and Stiller, 1992; Ueti et al., 2007). The midgut is the first site of infection in which large membrane bound vacuoles or colonies first contain reticulated forms that divide by binary fission and then subsequently transform into dense forms. After attaching and feeding on a second host, the salivary glands become infected and the pathogen undergoes development similar to the midgut cycle (Kocan et al., 1992a, 2004). In addition to midgut and salivary gland sites, other tick tissues can become infected. While *A. phagocytophilum* has been detected in ticks by molecular technologies and more recently by microscopy (Kocan et al., 2008; Reichard et al., in press), the tick cycle has not been described by use of microscopy and the role of male ticks, similar to that in the transmission of *A. marginale*, has not been reported.

Several major surface proteins (MSPs) have been identified in *Anaplasma* spp., which have been most extensively characterized in *A. marginale* (Palmer et al., 1985; reviewed by de la Fuente et al., 2001a, 2005a; Kocan et al., 2003, 2004, 2008) and *A. phagocytophilum* (Dunning Hotopp et al., 2006; Nelson et al., 2008). Limited information is available for *A. centrale* and *A. ovis* MSPs and reports have not been published for *A. bovis* and *A. platys* (reviewed by Dumler et al., 2001; de la Fuente et al., 2005a). *Anaplasma* MSPs are involved in interactions with both vertebrate and invertebrate hosts (de la Fuente et al., 2001a, 2005a; Kocan et al., 2003, 2004; Brayton et al., 2005; Dunning Hotopp et al., 2006; Nelson et al., 2008), and therefore are likely to evolve more rapidly than other genes because they are subjected to selective pressures exerted by host immune systems.

In this review, we analyzed the tick–pathogen relationships and evolution of *A. marginale* and *A. phagocytophilum* by reviewing the information obtained from functional genomics and genetic studies.

2. *Anaplasma* major surface proteins and their role in host–vector–pathogen interactions

2.1. The MSP1 complex

The *A. marginale* MSP1 complex is composed of a heterodimer of two structurally unrelated polypeptides: MSP1a and MSP1b, which are both encoded by genes that have proved to be stable genetic markers of geographic strains of *A. marginale* throughout the developmental cycle in cattle and ticks (Bowie et al., 2002; Macmillan et al., 2006). MSP1a, encoded by the gene *msp1α*, has only been identified thus far in *A. marginale* despite attempts to clone this gene from *A. centrale*, *A. ovis* and *A. phagocytophilum* (Lew et al., 2002 and unpublished results). MSP1b, encoded by two genes, *msp1β1* and *msp1β2*, has also only been identified in *A. marginale* (Bowie et al., 2002; Macmillan et al., 2006). However, only a single MSP1b protein, MSP1b1, was identified within the MSP1 complex (Macmillan et al., 2006). The MSP1a of *A. marginale* geographic strains differs in molecular weight because of a variable number of tandem 23–31 amino acid repeats, and has been used for identification of geographic strains (Allred et al., 1990; de la Fuente et al., 2001a, 2003a, 2005a, 2007a).

Functionally, MSP1a was shown to be an adhesin for bovine erythrocytes and tick cells (McGarey and Allred, 1994; McGarey et al., 1994; de la Fuente et al., 2001b). MSP1a was also shown to be involved in the transmission of *A. marginale* by *Dermacentor* spp. ticks (de la Fuente et al., 2001c) and to be differentially regulated in tick cells and bovine erythrocytes (Garcia-Garcia et al., 2004a).

MSP1a, although variable in the number of repeated peptides, induces strong T-cell responses and contains a

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conserved B-cell epitope in the repeated peptides that is recognized by immunized and protected cattle (Kocan et al., 2003; de la Fuente et al., 2005a and references therein). Furthermore, vaccination experiments with recombinant MSP1a have resulted in partial protection against clinical anaplasmosis in cattle and reduced infection levels in ticks, thus supporting the inclusion of MSP1a in vaccines for the control of bovine anaplasmosis (Kocan et al., 2003; de la Fuente et al., 2003c, 2005a; Garcia-Garcia et al., 2004c).

2.2. The MSP2 superfamily

The MSP2 protein superfamily contains immunodominant MSPs with orthologues in all *Anaplasma* spp. studied thus far (Dunning Hotopp et al., 2006). The *A. marginale* (strain St. Maries) genome is reported to have 56 genes that belong to this superfamily, including eight *msp2*, eight *msp3*, two *msp3* remnants, one *msp4*, three *opag*, 15 *omp-1*, 12 *orfX* and seven *orfY* (Brayton et al., 2005). The *A. phagocytophylum* (strain HZ) genome has one *msp2*, two *msp2* homologs, one *msp4*, 113 *p44* and three *omp-1*, totaling 121 genes that belong to the MSP2 superfamily (Dunning Hotopp et al., 2006). The best characterized representative of this superfamily, MSP2, is encoded by several genes in *A. marginale*, *A. centrale* and *A. ovis* (Palmer et al., 1998; Shkap et al., 2002a; Brayton et al., 2005), while the corresponding protein in *A. phagocytophilum* is encoded by a single gene, different from the antigenically related P44 protein family (Dunning Hotopp et al., 2006).

Antigenic variation of MSP2 occurs during *A. marginale* persistent infections in cattle and ticks (de la Fuente and Kocan, 2001; Brayton et al., 2005). This mechanism of antigenic variation has been posited to allow *A. marginale* to evade the host immune response, thus contributing to the maintenance of persistent infections (Brayton et al., 2005). In *A. phagocytophilum*, diverse *p44* paralogs are expressed in mammals and ticks, a regulatory mechanism that confers environmental adaptations especially during tick transmission (Dunning Hotopp et al., 2006). Multiple antigenic variants of MSP2, MSP3 (*A. marginale*) and P44 (*A. phagocytophilum*) arise during the multiplication of the rickettsia in mammals and ticks as the result of combinatorial gene conversion into expression sites (Brayton et al., 2005; Dunning Hotopp et al., 2006). However, differences between *A. phagocytophilum* and *A. marginale*/*A. centrale*/*A. ovis* MSP2 suggest that the function(s) of MSP2 may vary for *Anaplasma* spp. *A. marginale omp-1* genes are differentially regulated in bovine erythrocytes and tick cells but show a high degree of conservation during pathogen life cycle in the mammalian host and the tick vector (Noh et al., 2006). Although the function of MSP4 is presently unknown, this sequence is genetically stable during the multiplication of *A. marginale* and *A. phagocytophilum* in mammalian and tick cells (Bowie et al., 2002; de la Fuente et al., 2005d). In *A. phagocytophilum*, *msp4* is upregulated in infected *Ixodes scapularis* ISE6 cells when compared to human HL-60 cells (Nelson et al., 2008) and p44 expression is also differentially regulated in mammalian and tick host cells (Wang et al., 2007).

The *A. marginale* MSP2 are capable of inducing a strong T-cell response and contain antigenically variable B-cell epitopes in the hypervariable region that are recognized by the immune system of *A. marginale*-infected cattle, which results in the selection of new variants that most likely allow the pathogen to establish persistent infections (Abbott et al., 2004; de la Fuente et al., 2005a). However, due to recent reports (Abbott et al., 2005) and with consideration of the mechanism of MSP2 antigenic variation, the effect of the immune response against these antigens most likely would not be a neutralizing one, thus pointing toward the need for other protective antigens for the control of bovine anaplasmosis (Kocan et al., 2003; de la Fuente et al., 2005a; Brayton et al., 2006). Recently, Wang et al. (2006) reported the finding of two different epitopes in *A. phagocytophilum* P44 that are neutralizing for rickettsial infection of human HL-60 cells. This result encourages evaluation of engineered synthetic polypeptides containing MSP2/P44 defined regions as candidate immunogens against *Anaplasma* infections.

2.3. MSP5

MSP5 is an immunodominant conserved protein encoded by a single gene, which has been identified in *A. marginale*, *A. centrale*, *A. ovis* and *A. phagocytophilum* (reviewed by de la Fuente et al., 2005a). MSP5 is a SCO1/Senc family protein which may have a conserved but presently unknown function.

2.4. Other surface proteins

Recently, Noh et al. (2008) and Nelson et al. (2008) characterized by proteomics or genomics in *A. marginale* and *A. phagocytophilum*, respectively, other surface proteins differentially regulated during the transition of the pathogen from vertebrate to tick cells. Although the function of these proteins is presently unknown, they may be involved in tick–pathogen and host–pathogen interactions and could be targeted as vaccine candidates for the control of pathogen infection and transmission by ticks.

3. Biogeography and evolution of *Anaplasma* species

3.1. Biogeography of *Anaplasma* species

Anaplasma spp. are transmitted biologically by ticks and are distributed worldwide, infecting a wide range of mammalian hosts (Table 1). Phylogenetic analyses of *Anaplasma* spp. have been done using a variety of gene loci (Dumler et al., 2001; de la Fuente et al., 2005a). However, in this review we have referred exclusively to the analyses using MSPs because they are likely to be more relevant to biogeographic end evolutionary studies due to their role in vector–pathogen interactions.

The geographic strains of *A. marginale* are genetically variable as denoted by analysis of MSP1a and MSP4 sequences (de la Fuente et al., 2005a, 2007a). From these studies, we concluded that MSP1a and MSP4 are not useful markers for the characterization of geographic strains of *A. marginale* (de la Fuente et al., 2003a, 2007a). As discussed

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Table 1
Characteristics of *Anaplasma* species.

Anaplasma spp.	Geographical distribution	Hosts	Host cell tropism	Disease	Main tick vectors (genera)
A. marginale	Tropics and subtropics	Cattle, deer and elk	Erythrocytes	Bovine anaplasmosis	Dermacentor, Boophilus, Rhipicephalus, Hyalomma, Ixodes
A. centrale		Cattle	Erythrocytes	Mild anaplasmosis	
A. ovis	Tropics and subtropics	Sheep, goats, deer, elk, and antelope	Erythrocytes	Ovine anaplasmosis	Dermacentor, Rhipicephalus
A. bovis	North America, Asia, and Africa	Cattle and rabbits	Mononuclear cells	Bovine mononuclear or agranulocytic anaplasmosis	Hyalomma, Rhipicephalus, Amblyomma, Haemaphysalis
A. phagocytophilum	Worldwide	Humans, cattle, sheep, dogs, deer, bison, rodents, cats, horses, llamas, wild boars, rabbits, and birds	Granulocytes	Human, dog and equine granulocytic anaplasmosis Tick fever	Ixodes
A. platys	Worldwide	Dogs	Platelets	Infectious cyclic thrombocytopenia	Rhipicephalus, Dermacentor, and Hyalomma

previously (de la Fuente et al., 2005a), the genetic heterogeneity observed among strains of *A. marginale* within endemic regions such as Oregon (Palmer et al., 2001), Oklahoma (de la Fuente et al., 2003a) and Kansas (Palmer et al., 2004) in the United States, Mexico (de la Fuente et al., 2002a, 2007a; Jiménez Ocampo et al., 2008; Almazán et al., 2008), Argentina (de la Fuente et al., 2002a; Ruybal et al., 2009), Israel (Shkap et al., 2002b; de la Fuente et al., 2005a), Minas Gerais and Parana in Brazil (de la Fuente et al., 2004a, 2007a), Castilla-La Mancha in Spain (de la Fuente et al., 2004b, 2005e), and Sicily in Italy (de la Fuente et al., 2005b,c) could be explained by cattle movement and maintenance of different genotypes by independent transmission events, due to infection exclusion of *A. marginale* in cattle and ticks which commonly results in the establishment of only one genotype per animal (de la Fuente et al., 2002b, 2003d; Palmer et al., 2004).

Due to the high degree of sequence variation within endemic areas, MSP1a sequences failed to provide phylogeographic information on a global scale (de la Fuente et al., 2005a). These studies also suggest that multiple introductions of *A. marginale* strains from different geographic locations occurred in these regions. Furthermore, the sequences of Australian strains were the only ones to cluster together, supporting a genetically more homogeneous *A. marginale* population in this region with fewer cattle introductions (de la Fuente et al., 2005a).

An updated analysis of MSP1a repeat sequences corroborated the genetic heterogeneity of geographic strains of *A. marginale* worldwide (de la Fuente et al., 2007a). The phylogenetic analysis of MSP1a repeat sequences did not result in clusters according to the geographic origin of *A. marginale* strains (Fig. 1). The only support (>70%) was provided for clusters containing repeats found exclusively in European (Italian) or American (Mexican and Brazilian) strains (Fig. 1). Most of the repeat sequences (45/50) were present in strains from a single geographic region, while 5 sequences were present in strains from two (repeats Q, Γ, Φ and F) or three (repeat M) of the geographic regions analyzed (Fig. 1).

The phylogenetic analysis of MSP4 sequences of *A. marginale*, *A. centrale*, *A. ovis* and *A. phagocytophilum* strains derived from infected mammals and ticks in countries from America, Europe and Asia did not provide phylogeographic information but the analysis did differentiate between *Anaplasma* spp. (de la Fuente et al., 2005a; unpublished results updated after the inclusion of the Chinese (DQ336607) and Zulia, Venezuela (AY737009) *A. marginale* strain MSP4 sequences). These results supported those obtained with MSP1a and indicated that while MSP4 is not a good genetic marker for global phylogeographic analysis of *A. marginale* strains, it may still be useful for strain comparison in some regions (de la Fuente et al., 2001d, 2002a, 2003a).

The genetic variation of *A. ovis* MSP4 has been analyzed in the United States for sheep, bighorn sheep and mule deer strains, in Spain for European roe deer strains (de la Fuente et al., 2008b) and in Sicily, Italy and Hungary for sheep strains (de la Fuente et al., 2002a, 2005b, 2006a; Yabsley et al., 2005; Hornok et al., 2007). The results of these studies demonstrated that, while *A. ovis msp4* genotypes may vary among geographic regions, the variation observed thus far is less than that observed in *A. marginale* and *A. phagocytophilum* (de la Fuente et al., 2007b). This finding may have resulted from restricted movement of infected hosts. Additionally, the limited host range of *A. ovis* as compared with *A. phagocytophilum* may have also contributed to the lack of genetic diversity of this rickettsia.

Phylogenetic analyses of MSP2 and MSP5 sequences differentiate between *Anaplasma* spp. but do not provide phylogeographic information (de la Fuente et al., 2005a,d). Phylogeographic studies with other MSPs and for other *Anaplasma* species have not been published, most likely due to the lack of sequence information.

3.2. Comparative genomics

Recent genome-based analyses support the hypothesis that *Anaplasma* spp. are closely related to *Ehrlichia* spp., but differences in the genome organization and function of these rickettsia were noted (Dunning Hotopp et al., 2006; Frutos et al., 2006). The phylogenetic analysis of *Anaplasma* and *Ehrlichia* MSPs supports this observation (Dumler et al., 2001; de la Fuente et al., 2005d).

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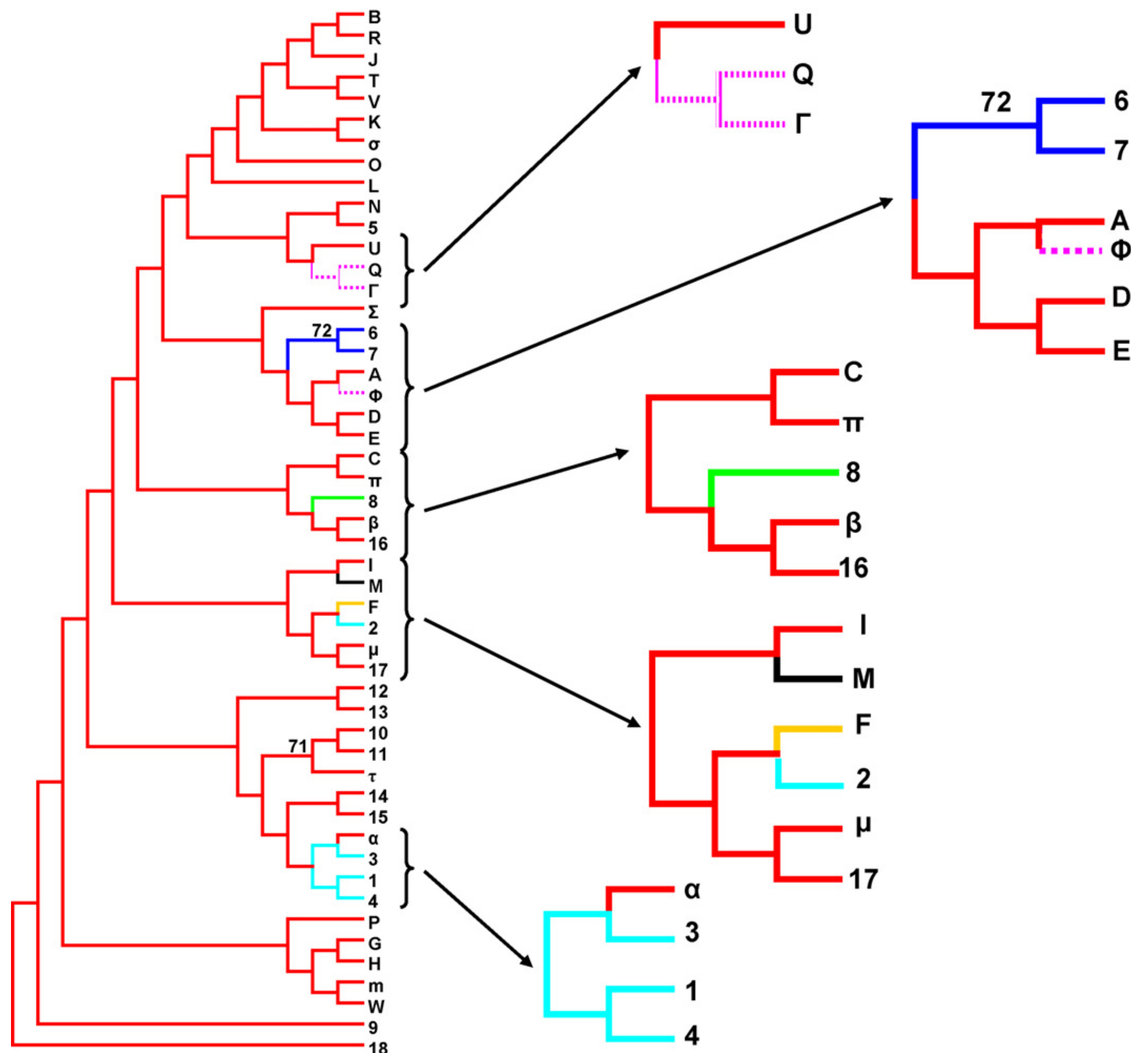

Fig. 1. Phylogenetic analysis of MSP1a repeat amino acid sequences. A condensed strict consensus of 442 maximum parsimony trees was constructed using MEGA version 3.0 (Kumar et al., 2004). Numbers above branches indicate percent support (>70%) for 1000 bootstrap replicates with 10 random additions of input taxa. The geographical distribution on MSP1a repeats was mapped onto the tree (red, America; dark blue, Europe; green, Australia; light blue, Israel; dotted purple, America and Europe; yellow, America and Israel; black, America, Europe and Israel) and these areas were magnified. MSP1a repeat sequences were described in de la Fuente et al. (2007a). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The comparative genomic analysis revealed differences between *A. marginale* and *A. phagocytophilum* with important evolutionary implications (Dunning Hotopp et al., 2006). For example, genes for cell wall biosynthesis are present in *A. marginale* but absent in *A. phagocytophilum*, which may represents an evolutionary adaptation to allow *A. phagocytophilum* to infect vertebrate immune cells without the activation of leukocytes that occurs after binding of peptidoglycan to Toll-like receptor 2. Dunning Hotopp et al. (2006) also identified one ortholog cluster containing conserved hypothetical proteins with homology to the patatin family of phospholipases that is present in animal rickettsia such as *A. marginale* but has been lost from *A. phagocytophilum* and other human pathogens, possibly being related to establishment of infection in humans.

The analysis of several genomes from *A. marginale* tick-transmissible (Virginia, Genbank accession number ABOR00000000; Mississippi, ABOP00000000; Puerto Rico, ABOQ00000000; St. Maries, CP000030) and non-transmissible (Florida, CP001079) strains evidenced differences in the sequence between strains (Dark et al., 2009). Additionally, comparison of genomes from *A. marginale* strains with other intracellular bacteria strains (*Mycobacterium tuberculosis*, *Bacillus anthracis*, *Nessieria meningiditis*, *Chlamydophila pneumoniae*) suggested that intracellular bacteria have variable single nucleotide polymorphism (SNP) retention rates and may have closed-core genomes in response to the host organism environment and/or reductive evolution (Dark et al., 2009).

3.3. Glycosylations of rickettsial MSPs

The glycosylation of MSPs may be a common feature of *Anaplasma* and *Ehrlichia* spp. (de la Fuente et al., 2004c; Kuyler Doyle et al., 2006). Glycosylation of MSPs may have important biological functions during rickettsial adhesion that have been retained during the evolution of rickettsiae

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and other vector-borne pathogens (Dinglasan and Jacobs-Lorena, 2005). In *A. marginale*, both native and recombinant *Escherichia coli*-expressed MSP1a were found to be glycosylated, a feature of the repeated N-terminal peptides which appears to contribute to the adhesive properties and thus the function of the protein (de la Fuente et al., 2003b; Garcia-Garcia et al., 2004b). The study of the structure, function and evolution of *Anaplasma* protein glycans opens an interesting venue for future research and perhaps vaccine development.

4. Vector–pathogen relationships

4.1. *Anaplasma MSPs and vector–pathogen interactions*

The evolutionary history of vector–pathogen interactions could be reflected in the sequence variation of the *Anaplasma* MSPs. Previous studies demonstrated that *A. marginale msp1α*, but not *msp4* is under positive selection pressure (de la Fuente et al., 2003a). Initial analysis of *A. marginale msp1α* and *Dermacentor variabilis* 16S rDNA sequences from various areas of the United States suggested tick-pathogen co-evolution (de la Fuente et al., 2001d), a result that is consistent with the biological function of MSP1a in the transmission of *A. marginale* by ticks (de la Fuente et al., 2001c). However, the genetic diversity of MSP1a sequences complicates the study of tick–pathogen co-evolution. Analysis of MSP1a repeats provided evidence of the presence of common sequences in strains from different geographic regions (Fig. 1), a finding consistent with the existence of the same vector tick species in these regions (Table 1). For example, *Rhipicephalus* (*Boophilus*) spp. and *Dermacentor* spp. are vectors of *A. marginale* in South and Central America, Australia, Africa and Asia and in North America and Europe, respectively (reviewed by Kocan et al., 2004). Furthermore, as suggested by sequence analysis of MSP1a repeats (Fig. 1), tick–pathogen interactions and co-evolution could influence the presence of unique MSP1a repeats in strains of *A. marginale* from particular geographic regions. However, mechanical transmission of *A. marginale* strains not transmissible by ticks could also play an important role in the evolution of *A. marginale* (reviewed by Kocan et al., 2004).

Biting flies, including species of Tabanidae and Culicidae, have been long recognized as mechanical vectors of *A. marginale* (Ewing, 1981; de la Fuente et al., 2005e; Hornok et al., 2008). Some *A. marginale* strains are apparently not transmissible by ticks and rely on mechanical transmission for completion of the life cycle in nature (de la Fuente et al., 2001c, 2003b). These facts pose the question of what evolutionary adaptations may have occurred to insure an efficient mechanical transmission of non-tick-transmissible *A. marginale* strains. Recently, Scoles et al. (2005a) concluded that biological transmission by ticks is more efficient than mechanical transmission of *A. marginale*. In this study, two strains of *A. marginale* were compared, the Florida strain which is not transmissible by ticks and the St. Maries strain that is transmissible by *Dermacentor* spp. ticks. The St. Maries strain was more efficiently transmitted by *D. andersoni* ticks than by *Stomoxys calcitrans* stable flies. However, evidence was provided that the Florida strain of *A. marginale* was more efficiently retained in fly mouthparts than the St. Maries strain. These differences suggest that the non-tick-transmissible strains of *A. marginale* may have evolved mechanisms to improve rickettsial survival and transmission by biting flies in regions where tick vectors are absent or inefficient for transmission of *A. marginale* (Ewing, 1981; Foil, 1989; Coronado, 2001; Kocan et al., 2003). Some of these evolutionary adaptations may be reflected in the sequence and characteristics of MSP1a.

The adhesion domain of *A. marginale* MSP1a was identified on the extracellular N-terminal region of the protein that contains the repeated peptides (de la Fuente et al., 2003b). The adhesive capacity of individual MSP1a repeated peptides for tick cell extract (TCE) demonstrated that peptides containing acidic amino acids D or E at position 20 bound to TCE, while peptides with a G as the 20th amino acid were not adhesive to TCE (de la Fuente et al., 2003b). The comparative analysis of the tandem repeated MSP1a peptides of several geographic strains of *A. marginale* revealed a complex relationship between the *msp1α* genotype and the tick-transmissible phenotype of the strain and suggested that both the sequence and conformation of this portion and/or as yet unidentified characteristics of MSP1a could influence the adhesive properties of the protein (Fig. 2). Interestingly, the Israeli and Australian strains of *A. marginale*, which have MSP1a that are composed exclusively of TCE non-adhesive peptide sequences, are transmitted by ticks (Bock et al., 1999; V. Shkap, personal communication) (Fig. 2). These results suggest that other amino acids such as L at position 16, which have not been tested for adhesion to TCE, may contribute to the adhesive properties of MSP1a. Alternatively, other yet to be discovered proteins may participate together with MSP1a in the adhesion to and infection of tick cells by *A. marginale*. Unfortunately, information about the adhesive properties of MSP1a repeated peptides and the tick-transmissible phenotype of *A. marginale* strains is limited and more research is needed to fully address evolutionary hypotheses concerning the relationship of MSP1a repeats sequences and transmission of *A. marginale* strains.

4.2. *Characterization of the molecular events at the tick–pathogen interface*

Tick–pathogen co-evolution also involves genetic traits of the vector. Recent reports have confirmed the presence of tick receptors for tick-borne pathogens. Pal et al. (2004) identified a tick receptor (TROSPA) for the OspA bacterial ligand that is required for *B. burgdorferi* colonization of *I. scapularis*. Furthermore, genetic factors have been associated with intraspecific variation in vector competence for a variety of vector-borne pathogens (Woodring et al., 1996), including *A. marginale* (Scoles et al., 2005b; Futse et al., 2003) and *A. phagocytophilum* (Teglas and Foley, 2006). Scoles et al. (2005b) demonstrated significant variation in *D. andersoni* midgut susceptibility to *A. marginale*. Futse et al. (2003) took a different approaches to study tick–*A. marginale* evolutionary adaptations. These

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A. marginale strain	Structure of MSP1a tandem repeats								Tick-transmissible
Florida	A	B	B	B	B	B	B	B	No
California	B	B	C						No
Okeechobee, FL	L	B	C	B	C				No
Illinois	M	N	B	M	H				No
Brazil	B	B	Q	σ	μ				No
Idaho	D	D	D	D	D	E			Yes
Virginia	A	B							Yes
Wetumka, OK	K	C	H						Yes
Stillwater 68, OK	K	B	M	F	H				Yes
St. Maries, ID	J	B	B						Yes
Mississippi	D	D	D	D	E				Yes
Oregon	A	F	H						Yes
U.S. bison (buffalo)	K	B	M	F	W				Yes
Puerto Rico	E	Φ	Φ	Φ	Φ	Φ			Yes
Israel tailed 1FM3	1	F	M	3					Yes
Israel non-tailed	1	4							Yes
Australia F12	8								Yes

Fig. 2. Functional organization of MSP1a tandem repeats of *A. marginale* geographic strains. The organization of MSP1a repeated peptides in *A. marginale* strains for which the tick-transmission phenotype is known was updated after de la Fuente et al. (2003b). The TCE-adhesive peptides are shown shaded in grey. MSP1a repeat sequences were described in de la Fuente et al. (2007a).

authors demonstrated that *Rhipicephalus* (*Boophilus*) *microplus*, which has been eradicated from the continental United States for over 60 years, was able to transmit the St. Maries strain of *A. marginale* from Idaho, and *D. variabilis* transmitted an *A. marginale* strain from Puerto Rico. Although the Puerto Rico strain replicated at higher levels in the salivary glands of *B. microplus*, a finding that is consistent with vector–pathogen adaptive co-evolution, ticks and *A. marginale* strains retained competence for tick transmission in the absence of vector–pathogen interaction (Futse et al., 2003). In recent studies, Ueti et al. (2009) identified morphologic and copy-number differences in the tick salivary gland colonization between the *A. marginale* St. Maries and the *A. centrale* Israel vaccine strains. These pathogens differ in replication and transmission efficiency by ticks which results in higher infectivity of the *A. marginale* St. Maries strain. They found that *D. andersoni* adult male ticks infected with the *A. marginale* St. Maries strain showed 10-fold higher salivary gland titer and a significantly greater percentage of infected ticks secreted *A. marginale* into the saliva and at a significantly higher level when compared to the *A. centrale* Israel vaccine strain. These results are likely to reflect species-specific differences in vector–pathogen interactions that affect pathogen replication and secretion pathways in the tick. These results illustrate the complexity of tick–pathogen co-evolution relationships and suggest that genetic loci of both the vector and the rickettsia are affected.

Recently, we showed that *A. marginale* and *A. phagocytophilum* modulate gene expression in infected ticks and tick cells (de la Fuente et al., 2007c and unpublished results). The global tick cell response to infection with *A. marginale* was characterized by suppression subtractive hybridization (SSH) in *B. microplus* male salivary glands and IDE8 cultured tick cells (Fig. 3). *A. marginale* infection affected molecular functions such as protein binding and metabolism, enzymatic/catalytic activity, structural molecules, transporter activity, DNA/RNA metabolism and stress but most of the molecules affected are of unknown function (Fig. 3). To complement the results of SSH analysis, the proteome of IDE8 cells was compared between uninfected and *A. marginale*-infected cells by differential in-gel electrophoresis and matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS)/MS analysis (de la Fuente et al., 2007c). Finally, RNAi was used to study the function of these molecules during *A. marginale* infection and multiplication in *D. variabilis* and cultured IDE8 tick cells (de la Fuente et al., 2007c). In these experiments, four genes (encoding for putative glutathione S-transferase (GST), salivary selenoprotein M, vATPase and ubiquitin) coincided with significantly lower *A. marginale* infection levels after RNAi in *D. variabilis* guts and/or salivary glands. Six genes (encoding for putative selenoprotein W2a, hematopoietic stem/progenitor cells protein-like, proteasome 26S subunit, ferritin, GST and subolesin) affected *A. marginale* infection levels in IDE8 tick cells after RNAi (de la Fuente et al., 2007c). These genes are good candidates to develop vaccines for the control of *A. marginale* transmission through reduction of tick vector capacity.

The genes differentially expressed after *A. phagocytophilum* infection were characterized in *I. scapularis* nymphs and ISE6 cells. Differentially expressed genes included some genes such as GST and ferritin shown previously to affect *A. marginale* infection and/or multiplication in *D. variabilis* and/or IDE8 tick cells (de la Fuente et al., 2007c). Interestingly, GST and ferritin have been reported to be regulated by tick feeding or infection with other pathogens (Macaluso et al., 2003; Mulenga et al., 2004; Rudenko et al., 2005; Dreher-Lesnick et al., 2006; Ribeiro et al., 2006). These results also showed that *A. phagocytophilum*

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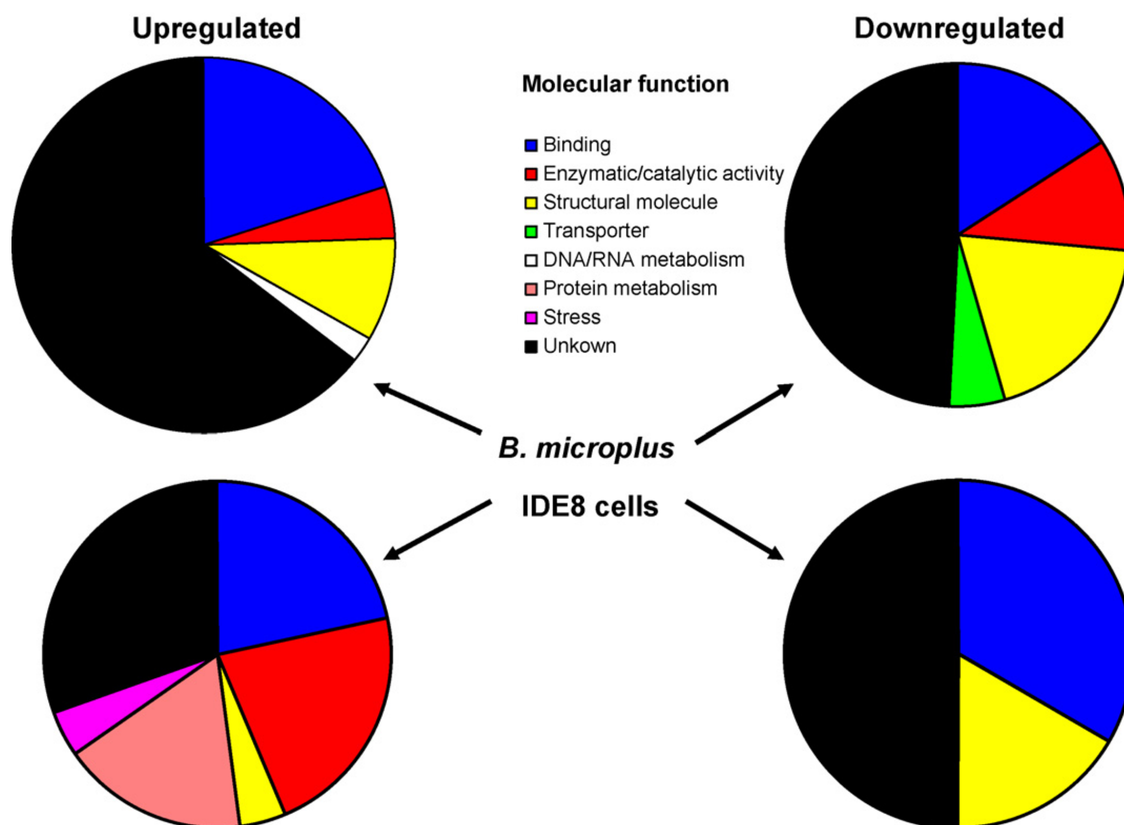

Fig. 3. Differential gene expression in *A. marginale*-infected tick cells. Differential gene expression was analyzed by suppression subtractive hybridization in IDE8 cultured tick cells and *B. microplus* male salivary glands. Gene ontologies of genes up- and downregulated in infected ticks and cultured tick cells were determined using the VectorBase GO (http://www.vectorbase.org/) and plotted as percent of each molecular function category. The data was collected from de la Fuente et al. (2007c) and unpublished results.

modulates gene expression in infected *I. scapularis* nymphs and cultured ISE6 cells but in a different manner when compared with *A. marginale* (de la Fuente et al., 2007c and unpublished results) (Fig. 4). In most cases, genes upregulated by *A. marginale* were downregulated or expression levels did not change after *A. phagocytophilum* infection and vice versa (Fig. 4 and unpublished results). These results suggest that *A. marginale* and *A. phagocytophilum* produce different differential gene expression profiles in infected ticks. These differences in *Anaplasma*–tick interactions may reflect differences in pathogen developmental cycle.

Cultured tick cells have provided a valuable tool for the study of tick–pathogen interactions and gene function in ticks (Blouin et al., 2002; Bell-Sakyi et al., 2007; de la Fuente et al., 2007d). Most of these studies have been done using *I. scapularis* cell lines, IDE8 and ISE6, developed by Munderloh et al. (1999). However, recent developments are opening the possibility to work with other tick cell lines, particularly those obtained from the important vector of *A. marginale*, *B. microplus* (Bell-Sakyi et al., 2007; Esteves et al., 2008 and unpublished results). Despite the impact of tick cell lines on tick research, differences in the results obtained in cultured tick cells and ticks suggest that the results of gene expression studies in vitro should be corroborated in vivo in different tick tissues. For example, differences were observed in the genes differentially expressed in IDE8 cultured cells and *B. microplus* salivary glands (Fig. 3). Functional genomics experiments demonstrated that some tick genes had different expression patterns in tick guts and salivary glands and affected the *A. marginale* life cycle at different sites in the tick, thus providing additional evidence of the distinct role that guts and salivary glands play on *Anaplasma* infection and transmission by ticks (Ueti et al., 2007; de la Fuente et al., 2007c). Differences were also observed in differential gene expressions between ticks and cultured tick cells infected with *A. marginale* and *A. phagocytophilum* (de la Fuente et al., 2007c and unpublished results).

4.3. Subolesin and tick–Anaplasma interactions

The tick subolesin is a good example of the role of tick proteins in the infection and transmission of *A. marginale* and *A. phagocytophilum*. Subolesin was discovered as a tick protective antigen in *I. scapularis* and has been shown to be conserved in different tick species (Almazán et al., 2003; de la Fuente et al., 2006b). Subolesin was shown by both RNAi gene knockdown and immunization trials using the recombinant protein to protect hosts against tick infestations, reduce tick survival and reproduction, and cause degeneration of gut, salivary gland, reproductive tissues and embryos (Almazán et al., 2003, 2005a,b; de la Fuente et al., 2006b,c; Nijhof et al., 2007; Kocan et al., 2007). The targeting of subolesin by RNAi or vaccination also decreased tick vector capacity for *A. marginale* and *A.*

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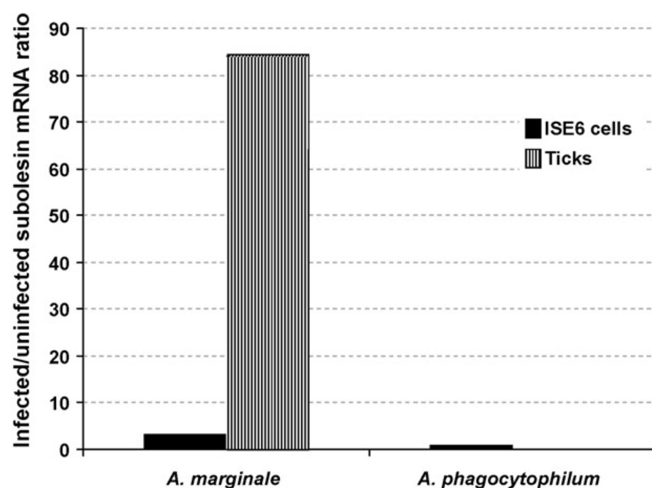

Fig. 4. *A. marginale* and *A. phagocytophilum* modify tick gene expression in different ways. Subolesin mRNA levels were determined by real-time PCR (de la Fuente et al., 2008c) in *A. marginale* and *A. phagocytophilum* infected ISE6 cultured tick cells, *I. scapularis* nymphs infected with *A. phagocytophilum* and *D. variabilis* salivary glands infected with *A. marginale* and compared to uninfected controls. All values are statistically significant ($P < 0.05$ by Student's *t*-test; $N = 3$).

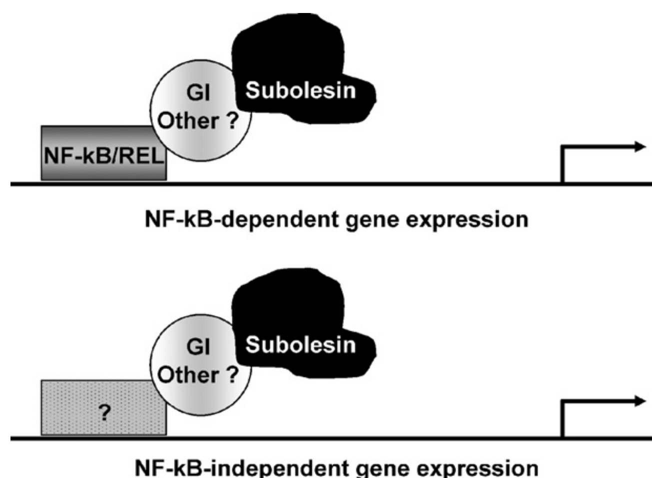

Fig. 5. Model for subolesin function in ticks. Subolesin may function in the regulation of NF-κB-dependent and independent gene expression through interaction with other regulatory factors such as GI, GII and other as yet unidentified proteins (de la Fuente et al., 2008d; Galindo et al., 2009).

phagocytophilum (de la Fuente et al., 2006d). In addition, subolesin was shown to be differentially expressed in *Anaplasma*-infected ticks and cultures tick cells (de la Fuente et al., 2007c, 2008c) (Fig. 4). Subolesin was differentially expressed in *A. marginale*-infected ticks in a tissue-specific manner in which mRNA levels increased in response to *A. marginale* infection in tick salivary glands but not in the guts (de la Fuente et al., 2007c, 2008c). Subolesin was also differentially expressed in *I. scapularis* nymphs and ISE6 cultured tick cells infected with *A. phagocytophilum* (de la Fuente et al., 2008c) (Fig. 4). Subolesin knockdown by RNAi reduced *Anaplasma* infection/multiplication only in cells in which infection increased subolesin expression, i.e. in *A. marginale*-infected *D. variabilis* salivary glands and IDE8 cultured tick cells (de la Fuente et al., 2008c).

Recently, subolesin was shown to function in the control of gene expression in ticks through the interaction with other regulatory proteins (de la Fuente et al., 2008d; Galindo et al., 2009) (Fig. 5). The results showed that tick subolesin is an ortholog of insect and vertebrate akirins and suggested that these proteins function in the regulation of NF-κB-dependent and independent expression of signal transduction and innate immune response genes (Galindo et al., 2009). These studies demonstrated a role of subolesin in the control of multiple cellular pathways by exerting a regulatory function on global gene expression in ticks and provided further support for the use of subolesin in vaccines for the control of tick infestations and the transmission of tick-borne pathogens (de la Fuente and Kocan, 2006; de la Fuente et al., 2008e).

5. Conclusions

Anaplasma and tick-derived molecules participate in the regulation of tick–pathogen interactions. For *Anaplasma* spp., MSPs and other proteins are involved in interactions with both vertebrate and invertebrate hosts. In ticks, several cellular pathways are affected by *Anaplasma* infection but differential gene expression varies between *A. marginale* and *A. phagocytophilum*. Tick-transmission studies and phylogenetic analyses of *Anaplasma* MSPs provide evidence of tick–pathogen co-evolution. The data from functional genomic studies support the hypothesis that *A. marginale* traffics through ticks by means of a molecular mechanism, and the pathogen's subsequent transmission is mediated by tick cell gene expression. As demonstrated for subolesin, these studies will likely be important for providing the basis for new strategies for development of effective dual-action vaccines for the control of both tick infestations and the transmission of *Anaplasma* spp. These vaccines are likely to contain a combination of pathogen and tick-derived antigens.

Conflict of interest statement

None declared.

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