Anaplasma phagocytophilum and *Ehrlichia chaffeensis*: subversive manipulators of host cells

Yasuko Rikihisa

Abstract | *Anaplasma* spp. and *Ehrlichia* spp. cause several emerging human infectious diseases. *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* are transmitted between mammals by blood-sucking ticks and replicate inside mammalian white blood cells and tick salivary-gland and midgut cells. Adaptation to a life in eukaryotic cells and transmission between hosts has been assisted by the deletion of many genes that are present in the genomes of free-living bacteria (including genes required for the biosynthesis of lipopolysaccharide and peptidoglycan), by the acquisition of a cholesterol uptake pathway and by the expansion of the repertoire of genes encoding the outer-membrane porins and type IV secretion system. Here, I review the specialized properties and other adaptations of these intracellular bacteria.

The family Anaplasmataceae (FIG. 1) is composed of pathogenic and non-pathogenic (either commensal or mutualistic) obligate intracellular bacteria that infect invertebrates; some species can also target cells of haematopoietic origin in mammals and birds (TABLE 1). The family belongs to the order Rickettsiales, along with the family Rickettsiaceae, which comprises obligate intracellular bacteria that are transmitted by blood-sucking arthropods and that cause potentially fatal diseases such as epidemic typhus. A major biological difference between members of these two families is that the Anaplasmataceae are confined within membrane-bound compartments in the host cytoplasm, whereas the Rickettsiaceae are not.

The first Anaplasmataceae species to be identified was the veterinary pathogen *Anaplasma marginale*, which was found in the red blood cells of cattle with severe anaemia in 1910 by Theiler[1]. To date, *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia canis*, *Neorickettsia sennetsu* and, potentially, *Ehrlichia ruminantium* are known to infect humans (TABLE 1). Apart from *E. chaffeensis* and *N. sennetsu*, Anaplasmataceae species were known as veterinary pathogens before they were shown to infect humans. *A. phagocytophilum*, *E. chaffeensis* and *E. ewingii* cause the important emerging zoonoses human granulocytic anaplasmosis, human monocytic ehrlichiosis and human ewingii ehrlichiosis, respectively[2]. The clinical signs of these diseases are similar and include fever, headache, myalgia, anorexia and chills, frequently accompanied by leukopenia followed by the appearance of immature cells (rebound leukocytosis), thrombocytopenia, anaemia and an increased level of serum hepatic aminotransferases[2–4]. The severity of disease varies from asymptomatic seroconversion to frequently documented severe morbidity and death[2–4]. These diseases are the most prevalent life-threatening tick-borne zoonoses in the United States[4,5] and were collectively designated nationally notifiable diseases in the United States in 1998 (REF. 6). They are less frequently reported in other parts of the world. Clinical diagnosis is made on the basis of retrospective seroconversion or PCR analysis. No vaccines are available for these diseases, and doxycycline, a broad-spectrum antibiotic, remains the drug of choice for treating patients.

The first bacterium in the family Anaplasmataceae to be identified as a cause of human ehrlichiosis was *N. sennetsu*. This bacterium has been isolated from the blood of several patients with infectious-mononucleosis-like clinical signs in Japan and Malaysia, and it has recently been detected in a patient in Laos[7]. Other *Neorickettsia* spp. are well-known veterinary pathogens in the United States. Unlike *Anaplasma* spp. and *Ehrlichia* spp., *Neorickettsia* spp. are transmitted to mammals by the ingestion of fish or aquatic insects that harbour infected trematodes. The global canine pathogen *E. canis* has been isolated from a human, and in Venezuela several patients with clinical signs compatible with human monocytic ehrlichiosis were

Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, 1925 Coffey Road, Columbus, Ohio 43210, USA.
e-mail: rikihisa.1@osu.edu
doi:10.1038/nrmicro2318
Published online 7 April 2010

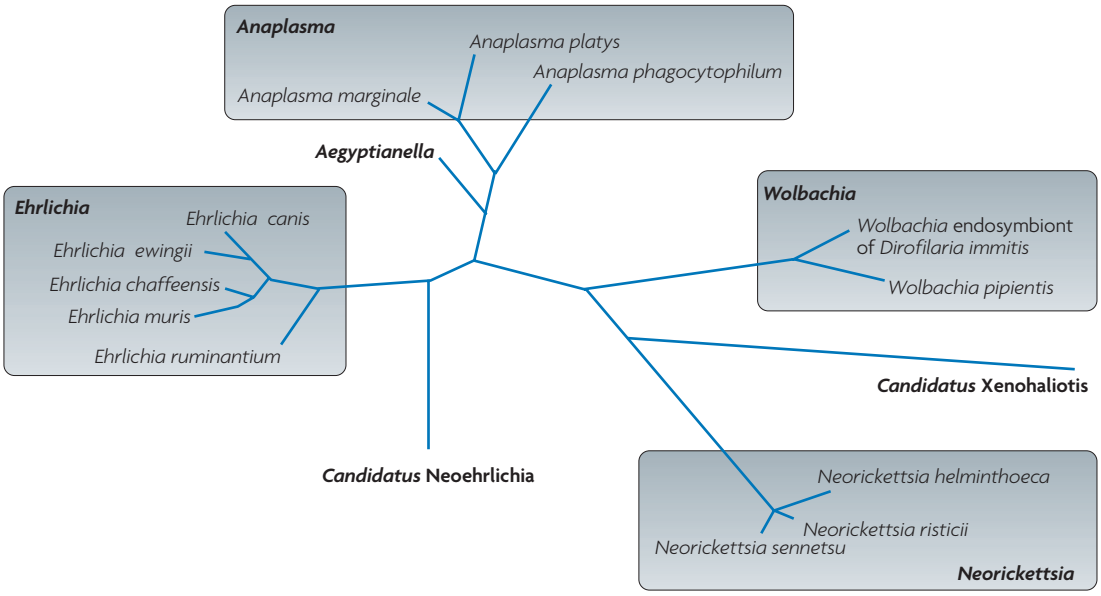

Figure 1 | **A phylogram of the family Anaplasmataceae, based on 16S ribosomal RNA gene sequences.** Genera are in bold. The phylogenetic tree was constructed from on sequence alignments by the Clustal W method using the MegAlign program from the Lasergene package.

found to be infected with *E. canis*[8,9]. The brown dog tick transmits *E. canis* between dogs, and human infestation with this species of tick is common in certain geographical regions, including Venezuela. This suggests that *E. canis* can cause human monocytic ehrlichiosis under certain circumstances. Three fatal human infections with *E. ruminantium,* a ruminant pathogen, have been reported in Africa, although they were not confirmed by cytology or bacterial isolation[10].

These species provide a useful model system to dissect the mechanisms of obligatory intracellular parasitism, inter-kingdom bidirectional signalling, vector transmission and leukocyte-targeted disease pathogenesis, thereby also contributing to our understanding of eukaryotes. Key questions that relate to *Anaplasma* spp. and *Ehrlichia* spp. include how they subvert the host immune system, how they manipulate host cells to create an effective replicative niche, how they regulate the dichotomous tasks of intracellular replication and intercellular spreading, and how they cause human disease. The focus of this Review will be on recent studies of *A. phagocytophilum* and *E. chaffeensis* that are relevant to these questions, observed primarily from the bacterial perspective. New information on the host cell response to infection and the nature of the inclusions that has come to light since this topic was last reviewed[11–15] is also included here.

Life cycle and intracellular development

In nature, the life cycles of *A. phagocytophilum*, *E. chaffeensis* and *E. ewingii* consist of mammalian and tick stages. Wild animals such as white-footed mice and white-tailed deer are primary reservoirs, and domestic animals such as dogs can occasionally serve as secondary reservoirs for human infection[16–19]. Humans are infected accidentally by the bite of infected ticks. *A. phagocytophilum* is transmitted by the black-legged tick (*Ixodes scapularis*) and the Western black-legged tick (*Ixodes pacificus*) in the United States, by the castor bean tick (*Ixodes ricinus*) in Europe and by the taiga tick (*Ixodes persulcatus*) in Asia[2]. The lone-star tick (*Amblyomma americanum*) serves as the biological vector for both *E. chaffeensis* and *E. ewingii* transmission[16,20,21] (FIG. 2). Human-to-human nosocomial transmission of *A. phagocytophilum* might have occurred in China, although this case was not confirmed by blood smears or cultures[22,23]. The fact that *E. ewingii* has not been isolated in pure culture has hindered molecular analysis of the pathogenesis of this species, and it will therefore not be covered further in this Review.

Once transmitted to mammals, *A. phagocytophilum* and *E. chaffeensis* replicate primarily in granulocytes and in monocytes or macrophages, respectively, by subverting several host innate immune responses[11]. Unlike *Rickettsia* spp. and most other Gram-negative bacteria, the genomes of *A. phagocytophilum* and *E. chaffeensis* lack the genes for biosynthesis of the lipopolysaccharide and peptidoglycan that activate host leukocytes. These bacteria must therefore incorporate cholesterol derived from host cells into their membranes to support membrane integrity[24]. The most studied host cell receptor for *A. phagocytophilum* infection is P-selectin glycoprotein ligand 1 (PSGL1); little information is available about the host cell receptors for *E. chaffeensis*. Binding of *A. phagocytophilum* to cells of the human leukaemia cell line HL-60 is dependent on the expression of both PSGL1 and an α(1,3)-fucosyltransferase[25]. However, sialic acid- and PSGL1-independent adhesion activity has been reported in a sub-line of *A. phagocytophilum* str. NCH-1[26], and *A. phagocytophilum* can infect cultured vascular endothelial cells[27], which do not usually express PSGL1. Binding of *A. phagocytophilum* to mouse neutrophils requires expression of α(1,3)-fucosyltransferases but not PSGL1 (REF. 28). The cognate *A. phagocytophilum*

Table 1 | **Well-known human pathogens in the family Anaplasmataceae**

Species	Disease	Host	Host cells	Vector	Distribution
Ehrlichia chaffeensis	Human monocytic ehrlichiosis	Humans, deer and dogs	Monocytes and macrophages	*Amblyomma americanum*	USA, South America and Asia
Ehrlichia ewingii	Human ewingii ehrlichiosis	Humans, deer and dogs	Granulocytes	*Amblyomma americanum*	USA
Anaplasma phagocytophilum	Human granulocytic anaplasmosis and tick-borne fever	Humans, horses, ruminants, rodents, dogs, cats and deer	Granulocytes and endothelial cells	*Ixodes scapularis*, *Ixodes pacificus* and *Ixodes ricinus*	USA, Europe and Asia
Neorickettsia sennetsu	Sennetsu fever and glandular fever	Humans	Monocytes and macrophages	Unknown trematode	Japan and Southeast Asia

molecules involved in PSGL1-dependent and PGSL1-independent binding and infection and the biological differences between *A. phagocytophilum* strains that infect through different routes are unknown.

Caveolae-mediated endocytosis[29] directs *A. phagocytophilum* and *E. chaffeensis* to an intracellular compartment, or inclusion, that does not acquire components of NADPH oxidase[30–33] or of late endosomes or lysosomes[34,35] (FIG. 3). *E. chaffeensis* inclusions retain characteristics of the early endosome, including the markers Rab5 and early endosome antigen 1 (EEA1) and the vacuolar (H^+)ATPase, and fuse with endosomes containing transferrin and transferrin receptors[35]. By contrast, *A. phagocytophilum* inclusions do not possess these early endosome characteristics[34,35]. However, bovine serum albumin coupled to colloidal gold particles (which traffics through the endosome pathway) has been detected in *A. phagocytophilum* inclusions[34]. Two mannose-6-phosphate receptors traffic between the *trans*-Golgi network and early and late endosomal compartments and are also present in the endocytic recycling compartment; the cation-dependent mannose-6-phosphate receptor was detected in *A. phagocytophilum* inclusions[34], whereas the cation-independent mannose-6-phosphate receptor was not[35]. During exponential growth, *A. phagocytophilum* inclusions acquire the characteristics of early autophagosomes[36] (FIG. 3). Thus, *A. phagocytophilum* and *E. chaffeensis* seem to exploit different host cell organelle biogenesis pathways to create their safe havens.

Important genomic features

The genomes of *A. phagocytophilum* str. HZ (which is 1.47 Mb in size) and *E. chaffeensis* str. Arkansas (which is 1.18 Mb in size) are approximately one quarter of the size of the *Escherichia coli* genome, and the number of ORFs in these genomes (1,369 and 1,115, respectively) is also around one quarter of the number found in *E. coli*[37]. The extent of reductive genome evolution in these species is similar to the extent seen in other sequenced members of the family Anaplasmataceae. It is also similar to the extent seen in other obligate intracellular pathogens such as *Chlamydia trachomatis* str. D/UW-3/CX (which has a genome of 1.04 Mb and contains 895 ORFs) and *Rickettsia prowazekii* str. Madrid E (which has a genome of 1.11 Mb and contains 834 ORFs), but it is greater than the extent seen in *Coxiella burnetii* str. RSA 493 (which has a genome of 2 Mb and contains 1,818 ORFs), an organism that was previously thought to be an obligate intracellular pathogen but has now been cultured successfully in a complex medium independently from eukaryotic cells[37,38].

The genes that are conspicuously absent from *A. phagocytophilum* and *E. chaffeensis* include those that are required for the biosynthesis of lipopolysaccharide and peptidoglycan[24,37]; this trend can also be seen among other sequenced members of the family Anaplasmataceae. According to the JCVI Comprehensive Microbial Resource, these obligate intracellular bacteria have a low coding capacity for genes that encode proteins belonging to the category of central intermediary metabolism. *A. phagocytophilum* and *E. chaffeensis* are unable to utilize glucose as a carbon or energy source. This is similar to *R. prowazekii* but distinct from *C. trachomatis* and *C. burnettii*. Obligate intracellular bacteria are auxotrophs for most amino acids; *A. phagocytophilum*, *E. chaffeensis* and *R. prowazekii* are auxotrophs for 14–17 amino acids[37,39], *C. trachomatis* is auxotrophic for at least this many[40] and *C. burnettii* is auxotrophic for 9 amino acids[41]. These amino acids and many metabolites must be acquired from the host. The number of *A. phagocytophilum* and *E. chaffeensis* genes encoding transport and binding proteins is 41 and 40, respectively, which is slightly more than the 34 such genes of *C. trachomatis* but less than the 66 and 122 such genes of *R. prowazekii* and *C. burnetii*, respectively.

Genes encoding some categories of proteins are conserved. All of the obligate intracellular bacteria referred to above have pathways for aerobic respiration, including pyruvate metabolism, the tricarboxylic acid cycle and the electron transport chain. However, unlike *R. prowazekii*, *C. burnetii* and *C. trachomatis*, *A. phagocytophilum* and *E. chaffeensis* do not encode cytochrome *d* ubiquinol oxidase (CydAB), a complex that has a high affinity for oxygen and that is useful for microaerophilic respiration. *A. phagocytophilum* and *E. chaffeensis* have retained genes for the biosynthesis of all of the necessary nucleotides and most vitamins and cofactors, including biotin, folate, FAD, NAD, CoA, thiamine and protohaem. This is similar to *C. burnetii* but distinct from *R. prowazekii* and *C. trachomatis*.

The outer-membrane protein 1 (OMP1)–P44 superfamily (also known as the major surface protein 2 (Msp2) superfamily or surface antigen family (Pfam accession number PF01617)) consists of proteins that are unique to the family Anaplasmataceae. The expansion of this protein family in the genomes of *Anaplasma* spp. and *Ehrlichia* spp. is noteworthy; there are > 100 members in

Caveola
A specialized lipid raft region of the plasma membrane that contains the protein caveolin and forms flask-shaped, cholesterol-rich invaginations of the membrane.

Autophagosome
An intracytoplasmic, membrane-bound vacuole containing elements of a cell's own cytoplasm and membrane proteins that are distinct from phagosomes or other intracellular vesicles. It usually fuses with a lysosome.

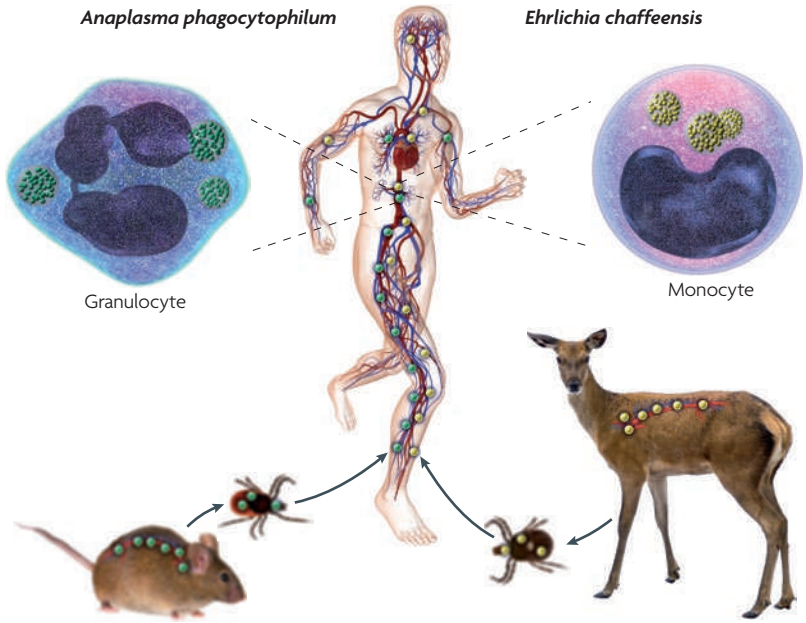

Figure 2 | **The *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* infection cycles.** Wild rodents and deer are well-known reservoirs for *Anaplasma phagocytophilum* (green) and *Ehrlichia chaffeensis* (yellow), respectively. The bacteria are usually transmitted to humans by the bite of infected ticks that have acquired the infection by taking a blood meal from infected wild animals. *A. phagocytophilum* and *E. chaffeensis* infect granulocytes and monocytes, respectively, which circulate throughout the body to cause systemic diseases. Human brain infection is rare, but *E. chaffeensis* has been observed in human cerebrospinal fluid mononuclear cells[147–149]. Image provided by T. Voigt, The Ohio State University, Columbus, USA.

A. phagocytophilum (including functional pseudogenes), 56 in *A. marginale*, 22 in *E. chaffeensis*, 16 in *E. ruminantium* (which infects endothelial cells in ruminants) and 25 in *E. canis* (which infects canine monocytes and macrophages). By contrast, only a few *omp1–p44* genes are found in the closely related genera *Neorickettsia* and *Wolbachia*[37,42–44]. Genes encoding several components of the type IV secretion system (T4SS) are duplicated in members of the Anaplasmataceae and Rickettsiaceae families[37,39,45–48].

The *A. phagocytophilum* and *E. chaffeensis* genome sequences are syntenic (but with an origin shift of ~200 kb), suggesting that genome reduction and major rearrangements occurred before these species diverged. The two species share approximately 500 genes, and 470–580 genes are unique to each species. Most of the shared genes are also shared with other bacteria, and most of the unique genes that encode hypothetical proteins are found only in the respective pathogen. As is the case for any of the obligate intracellular bacteria, genes that are essential for infecting eukaryotic host cells cannot be knocked out in *A. phagocytophilum* and *E. chaffeensis*. However, the recent development of a *Himar* transposase system to randomly insert genes[49] means that targeted genetic manipulation of these pathogens should be possible in the future.

Surface proteins

As *Ehrlichia* spp. and *Anaplasma* spp. lack lipopolysaccharide and common pili, the envelope proteins provide a critical interface between these bacteria and their hosts. Whole-genome sequencing analysis predicted 164 and 49 genes that encode envelope proteins in *A. phagocytophilum* and *E. chaffeensis*, respectively, which correspond to 12.0% and 6.6% of the total encoded proteins[37]. Most of these surface proteins evolved in this group of bacteria. The best studied surface-exposed proteins in *A. phagocytophilum* and *E. chaffeensis* are proteins belonging to the OMP1–P44 superfamily, and surface exposure of some of these proteins was confirmed by a recent proteomic analysis[50,51] (Supplementary information S1 (table)). As these proteins are highly immunogenic in infected patients, they have been the primary focus for the development of differential diagnostic antigens and vaccines[52–56].

OMP1–P44 proteins. The genome of *A. phagocytophilum* str. HZ has three copies of *omp1*, one *msp2* locus, two *msp2* homologues (which are distinct from *p44*), one copy of *msp4* and 113 copies of *p44* (which are also called *msp2*, but which are phylogenetically distinct from the *msp2* of *A. marginale*)[37]. Comparative genome hybridization showed that several of the *p44* copies are highly strain variable[37]. Most of the *p44* genes are truncated at the 5′- and/or 3′-terminal sequences and cannot be defined by a conventional ORF. Thus, *p44* genes have been defined as having a central hypervariable region of approximately 280 bp encoding a signature of four conserved amino acid regions (C, C, WP and A) and conserved flanking sequences longer than 50 bp[37,54,57–59]. These loci are considered to be functional pseudogenes (that is, silent storage copies to serve as donor *p44* genes) rather than non-functional pseudogenes that are on the way to elimination, because identical hypervariable-region nucleotide and amino acid sequences are found in multiple *p44* transcripts and P44 proteins (as deduced from the DNA sequence), respectively[50,54,57–59] (FIG. 4).

Among the 113 *p44* genes in *A. phagocytophilum* str. HZ, one is highly polymorphic and similar to the *A. marginale msp2* expression locus; this gene was designated the *p44* expression site (*p44ES*; also known as *msp2ES*) to distinguish it from the remaining *p44* loci[37,57,60,61] (FIG. 4). Multiple *p44* genes recombine at this site through a gene conversion mechanism involving the RecF pathway[57,61–63]. Without occupying a large genomic region, this mechanism allows a sizeable repertoire of P44 antigens to be rapidly exchanged but maintains transcriptional regulation from the same promoter. In fact, at a given time point during infection *p44* genes from diverse *A. phagocytophilum* loci[37] are expressed by the bacterial population in the blood of patients with human granulocytic anaplasmosis, and these genes are duplicated in *p44ES* and in the *p44* donor loci[58]. Travelling waves of sequentially expressed *p44* loci have been documented in experimentally infected horses during the initial logarithmic increase in bacterial density[64]. Recently, persistent cyclic waves of *A. phagocytophilum* populations have also been documented in sheep[65–67]. In cell culture, one *p44ES* allele seems to become dominant after several passages in human HL-60 cells or in variant cell lines, perhaps owing to its superior fitness in this environment[57,62,64,68,69]. The presence of immune serum substantially increases diversity at the *p44ES* locus[64,70].

Type IV secretion system
A multiprotein complex that mediates the translocation of macromolecules (that is, proteins, DNA or DNA–protein complexes) across the bacterial cell envelope into the extracellular medium or directly into recipient cells.

Himar transposase system
A DNA insertion system using *Himar1*, which belongs to the *mariner* family of transposable elements. *Himar1* requires no host-specific factors for transposition and has been frequently used as a prokaryotic genetic tool.

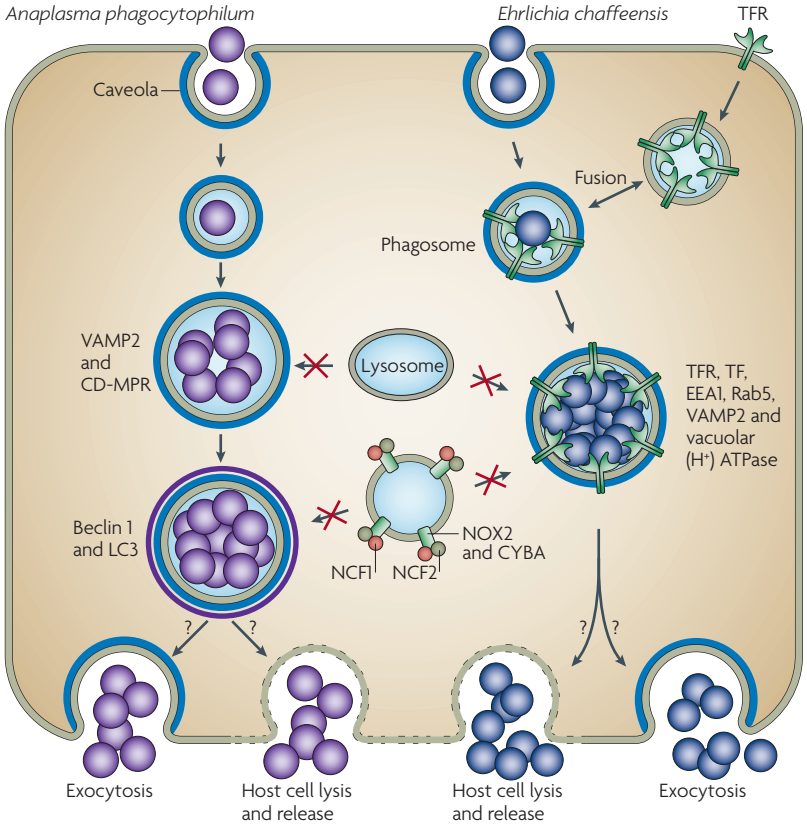

Figure 3 | **The intracellular niches of *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* in human cells.** Caveolae-mediated endocytosis directs *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* into intracellular compartments (morulae) that do not fuse with lysosomes or assemble NADPH oxidase (the components of which are NADPH oxidase 2 (NOX2; also known as gp91^{phox} homologue 2), cytochrome *b*245 light chain (CYBA, also known as p22^{phox}), neutrophil cytosol factor 1 (NCF1; also known as p47^{phox}) and NCF2 (also known as p67^{phox})), thus providing protection from non-oxidative and oxidative damage. *E. chaffeensis* inclusions are early endosomes, being positive for early-endosome antigen 1 (EEA1), Rab5, transferrin (TF), transferrin receptor (TFR), vacuolar (H$^+$)ATPase and vesicle-associated membrane protein 2 (VAMP2). Some *A. phagocytophilum* morulae are positive for VAMP2 and cation-dependent mannose-6-phosphate receptor (CD-MPR; also known as M6PR) and acquire early-autophagosome-like features (they are positive for beclin 1 and LC3). It is thought that these bacteria exit through host cell lysis or exocytosis, but this has yet to be proved experimentally (as indicated by the question marks).

An *A. phagocytophilum* population therefore naturally encodes a mixture of *p44ES* alleles, and a cloned *A. phagocytophilum* expressing a single *p44* gene was used to show that antigenic variation of *p44* occurs in infected horses and severe combined immunodeficient (SCID) mice[62]. Donor *p44* genes that are frequently expressed were found to be concentrated near the *p44ES*[62]. Diverse *p44* genes were also expressed in various developmental stages of *I. scapularis* ticks[71]. Furthermore, the populations of expressed *p44* genes were different in the blood and spleen of *A. phagocytophilum*-infected mice and also in the salivary glands and midgut of *I. scapularis* removed from these mice following *A. phagocytophilum* transmission[72]. Diverse *p44* sequences were also detected in the *p44ES* loci of *A. phagocytophilum* strains from various animals and geographical locations[73]. Taken together, these data indicate that *p44* recombination is an inherent property of *A. phagocytophilum*.

The expression of *p44* mRNA by *A. phagocytophilum* is significantly upregulated in the spleens of mice compared with expression in salivary glands of *I. scapularis*, and mRNA levels are also higher in HL-60 cells than in ISE6 tick cells[74]. ApxR, a hypothetical protein from *A. phagocytophilum* that was originally isolated by DNA affinity purification and then identified by proteomics[75], can bind to the promoter regions of *p44ES* and *apxR* and transactivate *p44ES*[74]. In addition, *apxR* is upregulated approximately 1,000-fold in HL-60 cells compared with levels in ISE6 tick cells[74]. These results indicate that *p44ES* and *apxR* are upregulated in the mammalian host environment and suggest that ApxR not only positively autoregulates itself but also transcriptionally regulates *p44ES*[74]. A recent study reported that 43% of *A. phagocytophilum* genes that are upregulated more than twofold in HL-60 cells compared with levels in ISE6 tick cells are predicted to encode membrane-associated proteins and 54% encode hypothetical proteins. Similarly, 46% of the genes that are upregulated more than twofold in ISE6 tick cells compared with levels in HL-60 cells are predicted to encode membrane-associated proteins and 93% encode hypothetical proteins[76]. This finding reinforces the notion that *A. phagocytophilum* is a 'microbial chameleon' that can change its surface coating to adapt to the host environment.

OMP1–P28 proteins. Members of the OMP1–P28 protein family in *E. chaffeensis* are encoded by a polymorphic multigene family composed of 22 paralogues that are clustered in a 29 kb gene locus, and this locus is downstream of the transcriptional regulator gene *tr1*, as is *p44ES* in *A. phagocytophilum*[77] (FIG. 4). The gene organization and genomic locus of the *E. chaffeensis omp1–p28* gene cluster are conserved among *Ehrlichia* species, including *E. canis*, *E. ruminantium* and *E. ewingii*[77–79]. The *omp1–p28* locus is among the most strain-variable genomic regions found to date[80–83]. Although there is no evidence for recombination, multiple OMP1–P28 proteins are expressed by *E. chaffeensis* in infected animals and in cell culture[51,77,82,84–86]. Given that most of the *omp1–p28* paralogues are transcribed in DH82 cells[77], it is possible that each organism expresses more than one of these proteins. Alternatively, each organism might express a single OMP1–P28 protein, but in a given bacterial population multiple OMP1–P28 family proteins could be expressed. Of note, OMP1B (also known as OMP14–P28) is the only OMP1–P28 paralogue for which a transcript is detected in three developmental stages of *A. americanum* ticks (which are a proven vector of *E. chaffeensis* transmission[11]) before or after *E. chaffeensis* transmission to naive dogs[84]. OMP1B was also the only OMP1–P28 paralogue detected by proteomics in *E. chaffeensis* cultured in the ISE6 tick cell line[86]. OMP1B is an orthologue of *E. canis* P30-10, the expression of which is upregulated in cell culture at 25 °C compared with expression at 37 °C[87]. Therefore, the *E. chaffeensis* OMP1–P28 multigene family may be differentially expressed in mammalian and tick hosts[84,86].

Functions of P44 and OMP1–P28 proteins. Antibodies specific to P44 inhibit *A. phagocytophilum* infection in mice and HL-60 cell culture[64,88–90], indicating that

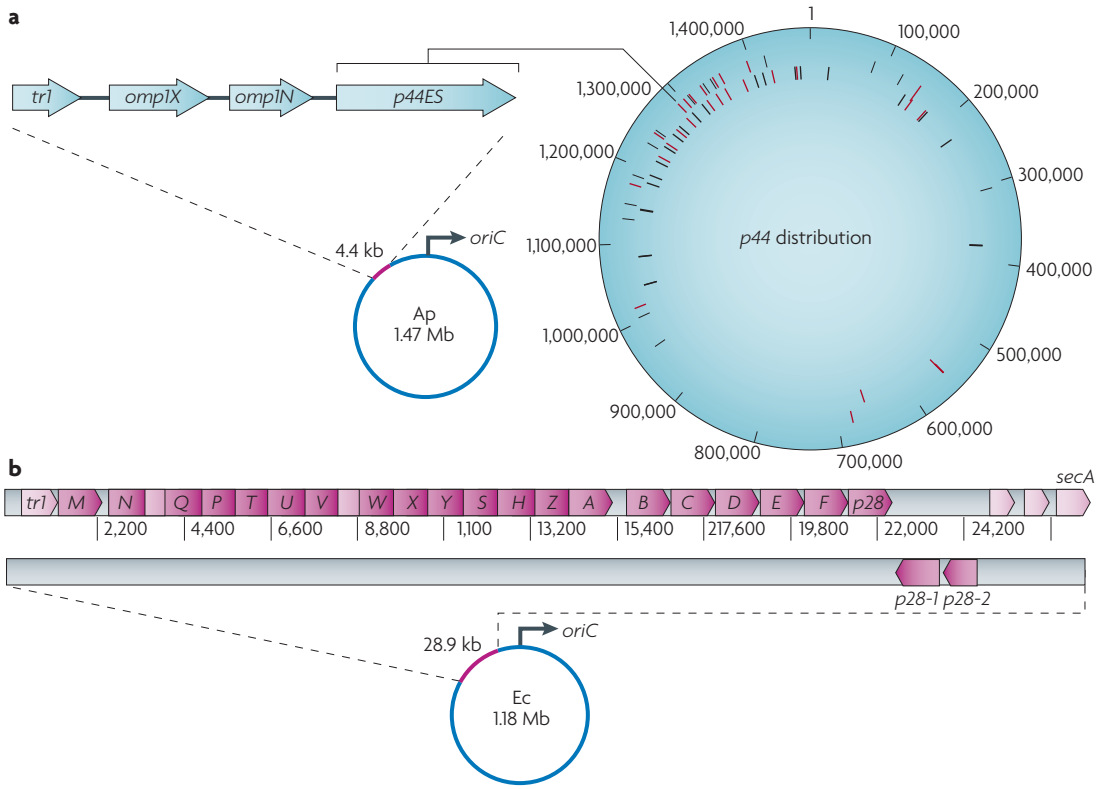

Figure 4 | **The *p44* and *omp1–p28* loci. a** | The *Anaplasma phagocytophilum* (Ap) *p44* expression locus (*p44ES*) is downstream of transcriptional regulator 1 (*tr1*), outer-membrane protein 1X (*omp1X*) and *omp1N*, near the predicted replication origin (*oriC*). More than 100 *p44* genes are concentrated around *p44ES*, as shown in the genome representation, and serve as donors for recombination at *p44ES*. From outside to inside in the distribution diagram, the two circles represent *p44* genes on the plus and minus strands. The full-length *p44* genes (that each have an ORF longer than 1,000 bp and contain conserved start and stop codons and a central hypervariable region of approximately 280 bp) are indicated in red. The silent *p44* genes (that each have an ORF less than 1,000 bp long, contain either conserved or alternative start or stop codons and cannot be expressed as full-length *p44* genes at the current genomic location) are shown in black. **b** | The *Ehrlichia chaffeensis* (Ec) *omp1–p28* locus contains a cluster of 22 *omp1–p28* genes (shown in magenta; letters represent the relevant *omp1* gene, such that *M* is *omp1M* and so on) flanked by *tr1* and *secA* near the predicted *oriC*.

antigenic variation of P44 proteins may help *A. phagocytophilum* to escape host immune surveillance. Likewise, immunization with recombinant P28 protects mice from *E. chaffeensis* challenge[91]. Polyclonal antibodies specific to *E. chaffeensis* or monoclonal antibodies specific to P28 (also known as OMP1G) mediate protection of SCID mice from fatal infection with *E. chaffeensis*[92,93]. So what is the physiological function, if any, of P44 and OMP1–P28 proteins in bacteria? Isolated native P44, P28 and OMP1F have transmembrane β-strands and exhibit porin activity[94,95]. Porin activity allows passive diffusion across the outer membrane of L-glutamine, the monosaccharides arabinose and glucose, the disaccharide sucrose and even the tetrasaccharide stachyose. Notably, P28 and OMP1F of *E. chaffeensis* have different solute diffusion rates[94], suggesting that differential expression of this gene family could affect the effectiveness of nutrient acquisition by the bacteria.

Other surface proteins. In addition to P44, proteomics analysis in *A. phagocytophilum* has shown bacterial surface expression of two hypothetical proteins (named in this study as Asp55 and Asp62) and OMP85, OMP1A and components of the T4SS apparatus[50] (see Supplementary information S1 (table)). Asp55 and Asp62 are predicted to contain 22 transmembrane β-strands forming a β-barrel and, thus, might be involved in membrane transport. Sera specific to Asp55 and Asp62 partially inhibit *A. phagocytophilum* infection of HL-60 cells *in vitro*[50]. The genes encoding Asp55 and Asp62 are co-transcribed and are conserved among members of the family Anaplasmataceae[51]. OMP85 is a conserved outer-membrane protein in Gram-negative bacteria[96] and a central component of the apparatus for outer-membrane protein assembly[97].

In addition to the OMP1–P28 family proteins, there are other surface-exposed proteins in *E. chaffeensis*; including the hypothetical protein Esp73 (which is an Asp55 orthologue), OMP85, tandem-repeat protein 47 (TRP47; also known as gp47), proteases and components of the T4SS apparatus[51]. TRP47, TRP32 and TRP120 (also known as gp120) were previously reported to be exposed on the surface of *E. chaffeensis*[98–100]. None of these three proteins has a predicted signal peptide or transmembrane segment, and they probably adhere to the bacterial surface. TRP120 was suggested to play a part in adhesion or invasion of mammalian cells on the basis of experiments using TRP120-transformed *E. coli*, although its expression on the surface of *E. coli* was not shown[98]. In addition,

these proteins are highly immunogenic in infected animals and patients[99,101,102]. Although genes encoding the proteins involved in glycosylation have not been found in *A. phagocytophilum* and *E. chaffeensis*[37], the tandem-repeat proteins were reported to be glycosylated[99,103]. Recently, however, the same laboratory reported that some of these proteins are not glycosylated[104].

In *E. chaffeensis*, 15 predicted lipoproteins are expressed in cell culture, and orthologues of these lipoproteins are encoded in the *A. phagocytophilum* genome[105]. Some are predicted to be exposed on the bacterial surface and are thought to be responsible for the delayed hypersensitivity reaction in infected mammals[105]. Dynamic expression of diverse surface proteins, both as a bacterial population and during the life cycle of an individual cell, is key for bacterial survival and persistence and, in addition to the variation of surface proteins according to bacterial strain, poses a challenge to effective vaccine design.

Intracellular replication and maturation

Anaplasma spp. and *Ehrlichia* spp. form dense intracellular microcolonies, which are called morulae, as they look like mulberries when blood smears, tissue samples or cultured cells are Romanowsky stained and observed under the light microscope (the term 'morulae' is derived from the Latin word for mulberry: 'morus') (reviewed in REF 106). Under an electron microscope, *A. phagocytophilum* and *E. chaffeensis* can be seen to be enveloped with inner and outer membranes. They are polymorphic bacteria (ranging from 0.2 to 2.0 μm in diameter) that can sometimes be categorized into dense-cored cells and reticulate cells in cultured human cells as well as in infected blood and tick midgut cells[27,106–112]. In cell culture, a biphasic developmental cycle has been reported (FIG. 5): initially, small dense-cored cells bind to host cells, are internalized and develop into large reticulate cells; these reticulate cells then mature into dense-cored cells or compact into clumps[110–112]. The infectivity of populations that are rich in dense-cored cells is notably greater than that of populations that are rich in reticulate cells[111,112]. Dense-cored cells and reticulate cells might not always be distinct, because various intermediate or aberrant forms exist[110,112]. In addition, caution should be taken, as the ultrastructure of this group of bacteria is influenced not only by the physiological conditions in the host cell, but also by fixation and other sample preparation methods used in electron microscopy[113].

When an *A. phagocytophilum* or *E. chaffeensis* population enriched in dense-cored cells was used to inoculate a human leukaemia cell line, quantitative PCR showed that after a lag phase of growth lasting for approximately 24 h, an exponential growth phase occurred from 24 h to 72 h followed by a short stationary phase from 72 h to 96 h[114,115]. Under different culture conditions, transmission electron microscopy showed a considerable amount of replication by 24 h, and shortly thereafter the reticulate cells recondensed to dense-cored cells to re-initiate the infection cycle[111,112]. During the first hour, only one to a few bacteria per host cell can be seen under the light microscope (at this stage, the bacteria are generally not distinct and immunofluorescence labelling using bacteria-specific antibodies is required to confirm their presence)[25,114–116]. During exponential growth, both the number and the size of the morulae increase in every infected cell. During stationary phase, morulae become loose and swollen and the bacteria disperse and begin to be liberated from the host cells, eventually resulting in bursting of the infected cell[114–116].

Proteins belonging to the *A. phagocytophilum* and *E. chaffeensis* T4SS apparatus and two-component systems as well as several *E. chaffeensis* surface proteins (P28, OMP1F, TRP47 and TRP120) are differentially expressed during intracellular development[94,98,99,112]. These proteins can therefore be used as surface markers to distinguish dense-cored cell and reticulate cell populations by immunofluorescence microscopy. Within less than 1 h of incubation, only small-form *A. phagocytophilum* (consisting of mostly dense-cored cells) in which the VirB9 component of the T4SS cannot be detected are associated with human peripheral blood neutrophils, whereas in HL-60 cells a VirB9-expressing form (consisting of mostly reticulate cells) is also found[116]. Most dense-cored cells remain attached to the HL-60 cell surface, but reticulate cells are internalized and co-localize with lysosome-associated membrane glycoprotein 1 (LAMP1), a lysosomal marker[116], suggesting that some reticulate cells are rapidly internalized and degraded, depending on the cell type. Taken together, the different stages of the *A. phagocytophilum* and *E. chaffeensis* developmental cycle influence the nature of binding to host cells and the early avoidance of the late-endosome–lysosome pathway.

Although the *A. phagocytophilum* and *E. chaffeensis* developmental cycles resemble those of *Chlamydia* spp. and *Coxiella* spp., eukaryotic histone H1 homologues, which are required for chromosomal condensation during the elementary body stages of the *Chlamydia* spp. and *Coxiella* spp. cycles[117,118], are not found in members of the family Anaplasmataceae. Many questions remain regarding the intracellular replication and maturation of *Erlichia* spp. and *Anaplasma* spp., including how these processes are regulated and coordinated. Although conversion to dense-cored cells resembles stress or quorum responses, genes encoding the nutritional stress response proteins RelA and SpoT or proteins required for the biosynthesis of a quorum-sensing pheromone have not been found in the genomes of *Anaplasma* spp. or *Ehrlichia* spp. Furthermore, *Anaplasma* spp. and *Ehrlichia* spp. encode only two RNA polymerase σ-factor homologues: a constitutive σ^{70} (also known as RpoD) and a single alternative σ-factor, σ^{32} (also known as RpoH). The paucity of alternative σ-factors suggests that the intracellular development of these bacteria requires regulation of constitutive σ^{70}-type promoters by transcription factors. In fact, DNA-binding proteins that can transactivate downstream genes have been identified: ApxR in *A. phagocytophilum*[75] and EcxR in *E. chaffeensis*[114]. In addition, genome sequence analysis predicts several DNA-interacting proteins: a putative transcriptional regulator encoded by *tr1* that has a winged helix–turn–helix motif, a basic histone-like HU protein, a putative transcription factor of the barrier to autointegration factor (BAF) family, a transcriptional regulator of the MerR (mercuric resistance operon regulator) family, integration host factor α-subunit (IHFα) and IHFβ, and

Alternative σ-factor
A σ-factor that is produced under specific conditions, allowing the RNA polymerase to transcribe a different set of genes than the housekeeping σ factor, σ^{70}, allows.

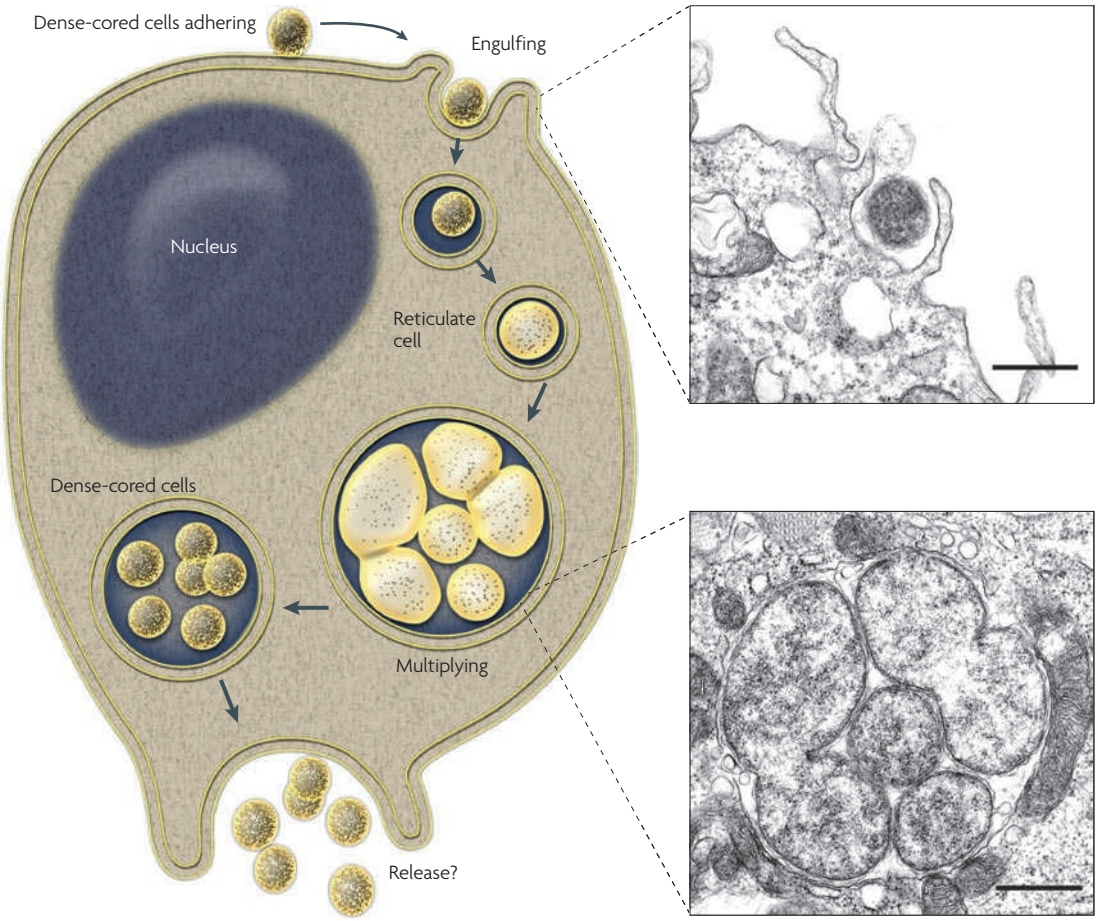

Figure 5 | **Proposed intracellular development cycle of *Ehrlichia chaffeensis*.** The dense-cored cells enter host cells and develop into reticulate cells, which replicate and mature into dense-cored cells. Later, a mixture of dense-cored cells and reticulate cells (not shown) is liberated by exocytosis or rupture of host cells. The electron micrographs show *Ehrlichia chaffeensis* bound to the surface of host cells (top panel) and replicating in a morula (bottom panel). The scale bar represents 0.5 μm. Electron micrographs are modified, with permission, from REF. 112 © (2007) Blackwell Science.

DnaA. Interestingly, ApxR is upregulated in HL-60 cells, whereas Tr1 is upregulated in ISE6 tick cells[74,76].

Two-component systems, which comprise a histidine kinase sensor and a response regulator, are signal-sensing and signal transduction mechanisms that are widely distributed among bacteria, but their functions are known in only a limited number of species. The *A. phagocytophilum* and *E. chaffeensis* genomes each encode three histidine kinases[119]: CckA, nitrogen regulation protein Y (NtrY) and PleC, each of which has specific histidine-dependent autokinase activity and is sensitive to the histidine kinase inhibitor closantel. Kinase activity is essential for both *A. phagocytophilum* and *E. chaffeensis* infection, as infection is inhibited by closantel treatment[115,119,120]. The *A. phagocytophilum* and *E. chaffeensis* genomes also encode three response regulators containing a conserved receiver domain with an aspartate phosphorylation site[119,120]: cell cycle transcriptional regulator A (CtrA), NtrX and PleD. CtrA and NtrX both have a carboxy-terminal DNA-binding domain, and PleD has a C-terminal GGDEF domain. The specific aspartate-dependent phosphotransfer occurs from PleC to PleD, from NtrY to NtrX and from CckA to CtrA[115,120]. The GGDEF domain is associated with a diguanylate cyclase that produces bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP), a bacterial second messenger[121]. Recombinant PleD from *A. phagocytophilum* can generate c-di-GMP[115]. A hydrophobic c-di-GMP analogue, 2′-*O*-di(tert-butyldimethysilyl)-c-di-GMP, inhibits the interaction of c-di-GMP with c-di-GMP-binding proteins and inhibits *A. phagocytophilum* infection[115]. These data suggest that both CtrA and PleD regulate the intracellular growth and maturation of these bacteria.

Subverting and hijacking host cellular processes

The type IV secretion system. T4SSs are ATP-dependent bacterial secretion systems that can deliver proteins or DNA from bacteria into eukaryotic cells[122]. In *A. phagocytophilum* and *E. chaffeensis*, the T4SS comprises eight and four *virB2* genes, respectively, and one *virB3*, two *virB4*, four *virB6*, two *virB8*, two *virB9*, one *virB10*, one *virB11* and one *virD4* in each species (REF. 123). Notably, the four non-identical, large VirB6 homologue proteins in *E. chaffeensis*, which are between 800 and 2,800 amino acids long, are co-expressed in human leukocyte THP1 cells and ISE6 tick cells and form a protein complex with VirB9 (REF. 124). During intracellular development, EcxR

coordinately regulates the five *virB* and *virD* loci that are dispersed throughout the *E. chaffeensis* genome[114]. In addition, differential transcription of the eight tandem *virB2* homologues of *A. phagocytophilum* has been reported in infected ISE6 tick cells and HL-60 cells[76].

The presence of multiple copies of T4SS components may facilitate environment- or host cell-dependent delivery of effector molecules. Using the Cre recombinase reporter assay from *Agrobacterium tumefaciens*, the *A. phagocytophilum* str. HZ ankyrin-repeat-domain-containing protein AnkA was shown to be secreted in a T4SS-dependent manner[125]. After delivery into the host cell cytoplasm, AnkA binds to Abl interactor 1 (ABI1), a host adaptor protein that interacts with the tyrosine kinase ABL1, thus mediating AnkA phosphorylation. AnkA and ABL1 are crucial for *A. phagocytophilum* infection, as infection is inhibited by delivery of AnkA-specific antibody to the host cytoplasm, by ABL1 knockdown with targeted small interfering RNA or by treatment with Gleevec, a specific pharmacological inhibitor of ABL1 (REF. 125). *A. phagocytophilum* str. NCH-1 infection induces tyrosine phosphorylation of AnkA, and the phosphorylated protein then interacts with the host cell tyrosine phosphatase, SHP1[126]. It has been proposed that AnkA of *A. phagocytophilum* str. Webster regulates host cell transcription by directly binding to DNA and nuclear proteins after translocation to the nucleus[127,128]. In addition, host chromatin modifications are linked to the transcription of host defence genes during *A. phagocytophilum* infection[129]. Similarly, it has been reported that *E. chaffeensis* AnkA translocates into the host cell nucleus and binds to Alu elements, although an association between *E. chaffeensis* AnkA and the T4SS has not been shown[130]. Other bacterial ankyrin-repeat-domain-containing proteins are also secreted by the T4SS and serve as effectors for bacterial infection of their hosts[131]. In addition, *E. chaffeensis* TRP47 interacts with multiple target proteins in the host cytoplasm[132]; whether TRP47 is a T4SS substrate is unknown. Thus, expression of the T4SS is regulated in *A. phagocytophilum* and *E. chaffeensis*, and the secreted substrates influence the activity of host cells, which, in turn, facilitate intracellular bacterial survival, growth and, perhaps, virulence.

Inhibition of host cell apoptosis. *A. phagocytophilum* inhibits spontaneous apoptosis of human neutrophils, allowing the bacterium sufficient time (> 24 h post-infection) to develop morulae[133,134]. This phenomenon has been confirmed by several *in vitro* studies on human neutrophils as well as by an *ex vivo* study on ovine neutrophils infected *in vivo* with a sheep isolate[134–138]. *A. phagocytophilum* prevents human neutrophils from reducing the expression of *BFL1* (also known as *BCL-2A1*), a member of the anti-apoptotic B cell lymphoma 2 (BCL-2) family, and it also prevents the loss of mitochondrial membrane potential and inhibits the activation of caspase 3[134,139]. Microarray data derived from human neutrophils and NB4 cells corroborate these findings, showing that *A. phagocytophilum* infection upregulates the expression of members of the BCL-2 family[136,138,140]. *A. phagocytophilum* also blocks anti-CD95-induced programmed cell death in human neutrophils[136,139] and blocks clustering of CD95 at the cell surface during spontaneous neutrophil apoptosis[139]. Furthermore, in neutrophils the cleavage of pro-caspase 8, the activation of caspase 8 and the cleavage of BH3-interacting domain death agonist (BID), which link the intrinsic and extrinsic pathways of apoptosis, are inhibited by *A. phagocytophilum* infection[139]. Likewise, *A. phagocytophilum* infection inhibits translocation of the pro-apoptotic protein BAX to mitochondria as well as inhibiting the activation of caspase 9 (the initiator caspase in the intrinsic pathway) and the degradation of a potent caspase inhibitor, X-linked inhibitor of apoptosis protein (XIAP), during spontaneous neutrophil apoptosis[139]. It has been reported that *A. phagocytophilum* inhibits apoptosis in human neutrophils through activation of p38 mitogen-activated protein (MAP) kinases[137]. *E. chaffeensis* upregulates the expression of nuclear factor-κB and other apoptosis inhibitors and differentially regulates cell cyclins and cyclin-dependent kinase expression in THP1 cells[141]. A recently identified *A. phagocytophilum* VirD4-interacting protein, *Anaplasma* translocation substrate 1 (Ats1)[142], seems to have a role in preventing host cell apoptosis at mitochondria, because native and ectopically expressed Ats1 is translocated across the bacterial and inclusion membrane, localized to the host cell mitochondrial matrix and inhibits induced apoptosis[142].

Subversion of autophagy. Autophagy helps to clear intracellular infections and process non-self and self antigens in the host cytoplasm as part of the innate and adaptive immune responses[143–145]. Several hallmarks of early autophagosomes are present in *A. phagocytophilum* morulae, including a double lipid bilayer and co-localization with GFP-tagged LC3 and beclin 1, the human homologues of the *Saccharomyces cerevisiae* autophagy-related proteins Atg8 and Atg6, respectively[36]. Stimulation of autophagy by rapamycin favours *A. phagocytophilum* infection. Inhibition of the autophagosomal pathway by 3-methyladenine does not prevent *A. phagocytophilum* internalization but reversibly arrests its growth[36]. Thus, *A. phagocytophilum* subverts autophagy to facilitate infection.

Hijacking cholesterol. In contrast to eukaryotes, most bacteria do not synthesize, acquire or require cholesterol. However, like mycoplasma, *A. phagocytophilum* and *E. chaffeensis* require cholesterol for survival and growth, but they lack any genes for cholesterol biosynthesis or modification[24]. *A. phagocytophilum* acquires host cellular cholesterol from the low-density lipoprotein (LDL)-mediated uptake pathway but not from the *de novo* biosynthesis pathway[146]. Sterol-regulatory-element-binding proteins, which are key transcription factors for cholesterol-mediated feedback regulation, maintaining intracellular cholesterol homeostasis, cannot respond to the increased intracellular cholesterol in *A. phagocytophilum*-infected cells[146]. Rather, *A. phagocytophilum* upregulates LDL receptor expression in HL-60 cells by increasing the levels of LDL receptor mRNA through a post-transcriptional mechanism[146]. *A. phagocytophilum*

Alu elements
Short interspersed DNA elements that are abundant in the human genome.

Apoptosis
Programmed cell death. In contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis usually confers advantages during an organism's life cycle.

Autophagy
A catabolic process involving the degradation of a cell's own components through the lysosomal machinery. It is a tightly regulated process that plays a normal part in cell growth, development and homeostasis, helping to maintain a balance between the synthesis, degradation and subsequent recycling of cellular products.

therefore hijacks cholesterol by manipulating the cellular LDL cholesterol uptake system to facilitate bacterial replication.

Future prospects

Since the first complete genome sequence data for *A. phagocytophilum* and *E. chaffeensis* became available 4 years ago, we have made good progress in understanding the surface proteins of these bacteria and their signalling and regulatory potential. Many 'hypothetical' proteins have been shown to be expressed, and several have been shown to have, or are predicted to have, important functions. Our eventual understanding of how *A. phagocytophilum* and *E. chaffeensis* cause disease will entail integration of this knowledge with an understanding of the host cell response to infection. It is likely that further surprising functions will be discovered for 'hypothetical' proteins in these bacteria. On the one hand, the unique surface proteins that have evolved in this group of pathogens continue to provide insights into the niche adaptation of these bacteria and their interplay with the host immune system. On the other hand, *A. phagocytophilum* and *E. chaffeensis* proteins that are found in other bacteria, including certain two-component systems and the T4SS, offer the opportunity to study the roles of these proteins in the pathogenesis of eukaryotic host cells. Although space limitations precluded their discussion in this Review, ongoing studies of immunity and host defence against these pathogens, of tick gene knockdowns and of the ecology and evolution of this group of bacteria should also fill important gaps in our knowledge.

As the choice of antibiotics available to treat infection with this group of pathogens is limited and the effectiveness of treatment is reduced when the initiation of therapy is delayed, non-antimicrobial compounds that inhibit the replication of these bacteria may have therapeutic and prophylactic applications. Recombinant bacterial antigens and synthetic peptides offer a novel set of compounds that could be used to aid rapid diagnosis and to develop a vaccine for high-risk populations.

1. Theiler, A. *Anaplasma marginale* (gen. and spec. nov.) The marginal points in the blood of cattle suffering from specific disease. *Transvaal S. Afr. Rep.Vet. Bacteriol. Dept. Agr. 1908–1909,* 7–64 (1910).
2. Thomas, R. J., Dumler, J. S. & Carlyon, J. A. Current management of human granulocytic anaplasmosis, human monocytic ehrlichiosis and *Ehrlichia ewingii* ehrlichiosis. *Expert Rev. Anti Infect. Ther.* **7**, 709–722 (2009).
3. Paddock, C. D. & Childs, J. E. *Ehrlichia chaffeensis*: a prototypical emerging pathogen. *Clin. Microbiol. Rev.* **16**, 37–64 (2003).
4. Bakken, J. S. & Dumler, S. Human granulocytic anaplasmosis. *Infect. Dis. Clin. North Am.* **22**, 433–448 (2008).
5. Olano, J. P. & Walker, D. H. Human ehrlichioses. *Med. Clin. North Am.* **86**, 375–392 (2002).
6. Gardner, S. L., Holman, R. C., Krebs, J. W., Berkelman, R. & Childs, J. E. National surveillance for the human ehrlichioses in the United States, 1997–2001, and proposed methods for evaluation of data quality. *Ann. N.Y. Acad. Sci.* **990**, 80–89 (2003).
7. Newton, P. N. *et al.* Sennetsu neorickettsiosis: a probable fish-borne cause of fever rediscovered in Laos. *Am. J. Trop. Med. Hyg.* **81**, 190–194 (2009).
8. Perez, M., Bodor, M., Zhang, C., Xiong, Q. & Rikihisa, Y. Human infection with *Ehrlichia canis* accompanied by clinical signs in Venezuela. *Ann. N.Y. Acad. Sci.* **1078**, 110–117 (2006).
9. Perez, M., Rikihisa, Y. & Wen, B. *Ehrlichia canis*-like agent isolated from a man in Venezuela: antigenic and genetic characterization. *J. Clin. Microbiol.* **34**, 2133–2139 (1996).
10. Allsopp, M. T., Louw, M. & Meyer, E. C. *Ehrlichia ruminantium*: an emerging human pathogen? *Ann. N.Y. Acad. Sci.* **1063**, 358–360 (2005).
11. Rikihisa, Y. *Ehrlichia* subversion of host innate responses. *Curr. Opin. Microbiol.* **9**, 95–101 (2006).
12. Rikihisa, Y. Molecular events involved in cellular invasion by *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum*. *Vet. Parasitol.* **167**, 155–166 (2010).
13. Winslow, G. M. & Bitsaktsis, C. Immunity to the ehrlichiae: new tools and recent developments. *Curr. Opin. Infect. Dis.* **18**, 217–221 (2005).
14. Carlyon, J. A. & Fikrig, E. Mechanisms of evasion of neutrophil killing by *Anaplasma phagocytophilum*. *Curr. Opin. Hematol.* **13**, 28–33 (2006).
15. Rikihisa, Y. in *Intracellular Niches of Microbes. A Pathogen's Guide Through the Host Cell* (eds Schaible, U. & Haas, A.) 301–314 (Wiley-VCH, Weinheim, 2009).
16. Ewing, S. A. *et al.* Experimental transmission of *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) among white-tailed deer by *Amblyomma americanum* (Acari: Ixodidae). *J. Med. Entomol.* **32**, 368–374 (1995).
17. Telford, S. R. 3rd *et al.* Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. *Proc. Natl Acad. Sci. USA* **93**, 6209–6214 (1996).
18. Gieg, J., Rikihisa, Y. & Wellman, M. Diagnosis of *Ehrlichia ewingii* infection by PCR in a puppy from Ohio. *Vet. Clin. Pathol.* **38**, 406–410 (2009).
19. Buller, R. S. *et al. Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. *N. Engl. J. Med.* **341**, 148–155 (1999).
20. Steiert, J. G. & Gilfoy, F. Infection rates of *Amblyomma americanum* and *Dermacentor variabilis* by *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in southwest Missouri. *Vector Borne Zoonotic Dis.* **2**, 53–60 (2002).
21. Anziani, O. S., Ewing, S. A. & Barker, R. W. Experimental transmission of a granulocytic form of the tribe Ehrlichieae by *Dermacentor variabilis* and *Amblyomma americanum* to dogs. *Am. J. Vet. Res.* **51**, 929–931 (1990).
22. Zhang, L. *et al.* Nosocomial transmission of human granulocytic anaplasmosis in China. *JAMA* **300**, 2263–2270 (2008).
23. Krause, P. J. & Wormser, G. P. Nosocomial transmission of human granulocytic anaplasmosis? *JAMA* **300**, 2308–2309 (2008).
24. Lin, M. & Rikihisa, Y. *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* lack genes for lipid A biosynthesis and incorporate cholesterol for their survival. *Infect. Immun.* **71**, 5324–5331 (2003).
The work described in this article shows that *E. chaffeensis* and *A. phagocytophilum* lack lipid A and peptidoglycan and that incorporation of host cholesterol is essential for their survival.
25. Herron, M. J. *et al.* Intracellular parasitism by the human granulocytic ehrlichiosis bacterium through the P-selectin ligand, PSGL-1. *Science* **288**, 1653–1656 (2000).
The authors show that PSGL1-specific antibody blocks *A. phagocytophilum* binding and infection of host cells and that dual transfection of non-permissive cells with PSGL1 and sialyl Lewis^x confers the ability to bind *A. phagocytophilum* and become infected.
26. Reneer, D. V. *et al.* Characterization of a sialic acid- and P-selectin glycoprotein ligand-1-independent adhesin activity in the granulocytotropic bacterium *Anaplasma phagocytophilum*. *Cell. Microbiol.* **8**, 1972–1984 (2006).
27. Munderloh, U. G. *et al.* Infection of endothelial cells with *Anaplasma marginale* and *A. phagocytophilum*. *Vet. Microbiol.* **101**, 53–64 (2004).
28. Carlyon, J. A. *et al.* Murine neutrophils require α1,3-fucosylation but not PSGL-1 for productive infection with *Anaplasma phagocytophilum*. *Blood* **102**, 3387–3395 (2003).
29. Lin, M. & Rikihisa, Y. Obligatory intracellular parasitism by *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* involves caveolae and glycosylphosphatidylinositol-anchored proteins. *Cell. Microbiol.* **5**, 809–820 (2003).
30. Mott, J., Rikihisa, Y. & Tsunawaki, S. Effects of *Anaplasma phagocytophila* on NADPH oxidase components in human neutrophils and HL-60 cells. *Infect. Immun.* **70**, 1359–1366 (2002).
31. Carlyon, J. A., Abdel-Latif, D., Pypaert, M., Lacy, P. & Fikrig, E. *Anaplasma phagocytophilum* utilizes multiple host evasion mechanisms to thwart NADPH oxidase-mediated killing during neutrophil infection. *Infect. Immun.* **72**, 4772–4783 (2004).
32. IJdo, J. & Mueller, A. C. Neutrophil NADPH oxidase is reduced at the *Anaplasma phagocytophilum* phagosome. *Infect. Immun.* **72**, 5392–5401 (2004).
33. Lin, M. & Rikihisa, Y. Degradation of p22^{phox} and inhibition of superoxide generation by *Ehrlichia chaffeensis* in human monocytes. *Cell. Microbiol.* **9**, 861–874 (2007).
34. Webster, P., IJdo, J. W., Chicoine, L. M. & Fikrig, E. The agent of Human Granulocytic Ehrlichiosis resides in an endosomal compartment. *J. Clin. Invest.* **101**, 1932–1941 (1998).
35. Mott, J., Barnewall, R. E. & Rikihisa, Y. Human granulocytic ehrlichiosis agent and *Ehrlichia chaffeensis* reside in different cytoplasmic compartments in HL-60 cells. *Infect. Immun.* **67**, 1368–1378 (1999).
36. Niu, H., Yamaguchi, M. & Rikihisa, Y. Subversion of cellular autophagy by *Anaplasma phagocytophilum*. *Cell. Microbiol.* **10**, 593–605 (2008).
This study shows that *A. phagocytophilum* subverts autophagy to establish itself in an early-autophagosome-like compartment that is segregated from lysosomes and that the induction of autophagy is required for bacterial growth.
37. Dunning Hotopp, J. C. *et al.* Comparative genomics of emerging human ehrlichiosis agents. *PLoS Genet.* **2**, e21 (2006).
A detailed analysis of the genomes of three human pathogens, *A. phagocytophilum, E. chaffeensis* and *N. sennetsu*.
38. Omsland, A. *et al.* Host cell-free growth of the Q fever bacterium *Coxiella burnetii*. *Proc. Natl Acad. Sci. USA* **106**, 4430–4434 (2009).
39. Andersson, S. G. *et al.* The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* **396**, 133–140 (1998).
40. Stephens, R. S. *et al.* Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science* **282**, 754–759 (1998).
41. Seshadri, R. *et al.* Complete genome sequence of the Q-fever pathogen *Coxiella burnetii*. *Proc. Natl Acad. Sci. USA* **100**, 5455–5460 (2003).
42. Lin, M., Zhang, C., Gibson, K. & Rikihisa, Y. Analysis of complete genome sequence of *Neorickettsia risticii*: causative agent of Potomac horse fever. *Nucleic Acid Res.* **37**, 6076–6079 (2009).
43. Foster, J. *et al.* The *Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode. *PLoS Biol.* **3**, e121 (2005).

44. Wu, M. *et al.* Phylogenomics of the reproductive parasite *Wolbachia pipientis* wMel: a streamlined genome overrun by mobile genetic elements. *PLoS Biol.* **2**, e69 (2004).
45. Brayton, K. A. *et al.* Complete genome sequencing of *Anaplasma marginale* reveals that the surface is skewed to two superfamilies of outer membrane proteins. *Proc. Natl Acad. Sci. USA* **102**, 844–849 (2005).
46. Collins, N. E. *et al.* The genome of the heartwater agent *Ehrlichia ruminantium* contains multiple tandem repeats of actively variable copy number. *Proc. Natl Acad. Sci. USA* **102**, 838–843 (2005).
47. Mavromatis, K. *et al.* The genome of the obligately intracellular bacterium *Ehrlichia canis* reveals themes of complex membrane structure and immune evasion strategies. *J. Bacteriol.* **188**, 4015–4023 (2006).
48. Ogata, H. *et al.* Mechanisms of evolution in *Rickettsia conorii* and R. *prowazekii*. *Science* **293**, 2093–2098 (2001).
49. Felsheim, R. F. *et al.* Transformation of *Anaplasma phagocytophilum*. *BMC Biotechnol.* **6**, 42 (2006).
This article describes the first transformation of *A. phagocytophilum* with the *Himar* transposase system.
50. Ge, Y. & Rikihisa, Y. Identification of novel surface proteins of *Anaplasma phagocytophilum* by affinity purification and proteomics. *J. Bacteriol.* **189**, 7819–7828 (2007).
51. Ge, Y. & Rikihisa, Y. Surface-exposed proteins of *Ehrlichia chaffeensis*. *Infect. Immun.* **75**, 3833–3841 (2007).
52. Unver, A. *et al.* Western blot analysis of sera reactive to human monocytic ehrlichiosis and human granulocytic ehrlichiosis agents. *J. Clin. Microbiol.* **39**, 3982–3986 (2001).
53. Unver, A. *et al.* Western and dot blotting analyses of *Ehrlichia chaffeensis* indirect fluorescent-antibody assay-positive and -negative human sera by using native and recombinant *E. chaffeensis* and *E. canis* antigens. *J. Clin. Microbiol.* **37**, 3888–3895 (1999).
54. Zhi, N. *et al.* Cloning and expression of the 44-kilodalton major outer membrane protein gene of the human granulocytic ehrlichiosis agent and application of the recombinant protein to serodiagnosis. *J. Clin. Microbiol.* **36**, 1666–1673 (1998).
55. Zhi, N., Rikihisa, Y., Kim, H. Y., Wormser, G. P. & Horowitz, H. W. Comparison of major antigenic proteins of six strains of the human granulocytic ehrlichiosis agent by Western immunoblot analysis. *J. Clin. Microbiol.* **35**, 2606–2611 (1997).
56. IJdo, J. W., Wu, C., Magnarelli, L. A. & Fikrig, E. Serodiagnosis of human granulocytic ehrlichiosis by a recombinant HGE-44-based enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **37**, 3540–3544 (1999).
57. Lin, Q., Rikihisa, Y., Ohashi, N. & Zhi, N. Mechanisms of variable *p44* expression by *Anaplasma phagocytophilum*. *Infect. Immun.* **71**, 5650–5661 (2003).
58. Lin, Q. *et al.* Analysis of sequences and loci of p44 homologs expressed by *Anaplasma phagocytophila* in acutely infected patients. *J. Clin. Microbiol.* **40**, 2981–2988 (2002).
59. Zhi, N., Ohashi, N. & Rikihisa, Y. Multiple *p44* genes encoding major outer membrane proteins are expressed in the human granulocytic ehrlichiosis agent. *J. Biol. Chem.* **274**, 17828–17836 (1999).
This is the first study to show that there are many expressible *p44* genes with the signature central hypervariable region in the *A. phagocytophilum* genome.
60. Barbet, A. F., Lundgren, A., Yi, J., Rurangirwa, F. R. & Palmer, G. H. Antigenic variation of *Anaplasma marginale* by expression of MSP2 mosaics. *Infect. Immun.* **68**, 6133–6138 (2000).
The first paper about the *msp2* expression locus, proposing the segmental gene conversion mechanism of expression.
61. Barbet, A. F. *et al.* Expression of multiple outer membrane protein sequence variants from a single genomic locus of *Anaplasma phagocytophilum*. *Infect. Immun.* **71**, 1706–1718 (2003).
62. Lin, Q. & Rikihisa, Y. Establishment of cloned *Anaplasma phagocytophilum* and analysis of *p44* gene conversion within an infected horse and infected SCID mice. *Infect. Immun.* **73**, 5106–5114 (2005).
63. Lin, Q., Zhang, C. & Rikihisa, Y. Analysis of involvement of the RecF pathway in *p44* recombination in *Anaplasma phagocytophilum* and in *Escherichia coli* by using a plasmid carrying the *p44* expression and *p44* donor loci. *Infect. Immun.* **74**, 2052–2062 (2006).
A description of RecF-dependent *p44* recombination, showing a half-crossover structure in *A. phagocytophilum* and using a double-origin plasmid carrying *p44ES* and a donor *p44* locus in RecF-active and RecF-inactive mutant strains of *E. coli*.
64. Wang, X. *et al.* Rapid sequential changeover of expressed *p44* genes during the acute phase of *Anaplasma phagocytophilum* infection in horses. *Infect. Immun.* **72**, 6852–6859 (2004).
65. Granquist, E. G. *et al.* Variant-specific and diminishing immune responses towards the highly variable MSP2(P44) outer membrane protein of *Anaplasma phagocytophilum* during persistent infection in lambs. *Vet. Immunol. Immunopathol.* **133**, 117–124 (2009).
66. Granquist, E. G., Stuen, S., Lundgren, A. M., Braten, M. & Barbet, A. F. Outer membrane protein sequence variation in lambs experimentally infected with *Anaplasma phagocytophilum*. *Infect. Immun.* **76**, 120–126 (2008).
67. Ladbury, G. A. *et al.* Dynamic transmission of numerous *Anaplasma phagocytophilum* genotypes among lambs in an infected sheep flock in an area of anaplasmosis endemicity. *J. Clin. Microbiol.* **46**, 1686–1691 (2008).
68. Sarkar, M. *et al. Anaplasma phagocytophilum* MSP2(P44)-18 predominates and is modified into multiple isoforms in human myeloid cells. *Infect. Immun.* **76**, 2090–2098 (2008).
69. Troese, M. J. *et al.* Differential expression and glycosylation of *Anaplasma phagocytophilum* major surface protein 2 paralogs during cultivation in sialyl Lewis x-deficient host cells. *Infect. Immun.* **77**, 1746–1756 (2009).
70. Scorpio, D. G., Caspersen, K., Ogata, H., Park, J. & Dumler, J. S. Restricted changes in major surface protein-2 (*msp2*) transcription after prolonged *in vitro* passage of *Anaplasma phagocytophilum*. *BMC Microbiol.* **4**, 1 (2004).
71. Zhi, N. *et al.* Transcript heterogeneity of the *p44* multigene family in a human granulocytic ehrlichiosis agent transmitted by ticks. *Infect. Immun.* **70**, 1175–1184 (2002).
72. Felek, S., Telford, S. 3rd, Falco, R. C. & Rikihisa, Y. Sequence analysis of *p44* homologs expressed by *Anaplasma phagocytophilum* in infected ticks feeding on naive hosts and in mice infected by tick attachment. *Infect. Immun.* **72**, 659–666 (2004).
73. Barbet, A. F. *et al.* Structure of the expression site reveals global diversity in MSP2 (P44) variants in *Anaplasma phagocytophilum*. *Infect. Immun.* **74**, 6429–6437 (2006).
74. Wang, X., Cheng, Z., Zhang, C., Kikuchi, T. & Rikihisa, Y. *Anaplasma phagocytophilum p44* mRNA expression is differentially regulated in mammalian and tick host cells: involvement of the DNA binding protein ApxR. *J. Bacteriol.* **189**, 8651–8659 (2007).
75. Wang, X., Kikuchi, T. & Rikihisa, Y. Proteomic identification of a novel *Anaplasma phagocytophilum* DNA binding protein that regulates a putative transcription factor. *J. Bacteriol.* **189**, 4880–4886 (2007).
This study identifies a novel transcription factor in *A. phagocytophilum* by proteomics, followed by DNA footprint analysis and a *lacZ* reporter assay.
76. Nelson, C. M. *et al.* Whole genome transcription profiling of *Anaplasma phagocytophilum* in human and tick host cells by tiling array analysis. *BMC Genomics* **9**, 364 (2008).
The authors describe for the first time the whole-genome transcriptome of *A. phagocytophilum* in human cell and tick cell culture.
77. Ohashi, N., Rikihisa, Y. & Unver, A. Analysis of transcriptionally active gene clusters of major outer membrane protein multigene family in *Ehrlichia canis* and *E. chaffeensis*. *Infect. Immun.* **69**, 2083–2091 (2001).
78. Zhang, C., Xiong, Q., Kikuchi, T. & Rikihisa, Y. Identification of 19 polymorphic major outer membrane protein genes and their immunogenic peptides in *Ehrlichia ewingii* for use in a serodiagnostic assay. *Clin. Vaccine Immunol.* **15**, 402–411 (2008).
79. van Heerden, H., Collins, N. E., Brayton, K. A., Rademeyer, C. & Allsopp, B. A. Characterization of a major outer membrane protein multigene family in *Ehrlichia ruminantium*. *Gene* **330**, 159–168 (2004).
80. Miura, K. & Rikihisa, Y. Virulence potential of *Ehrlichia chaffeensis* strains of distinct genome sequences. *Infect. Immun.* **75**, 3604–3613 (2007).
81. Cheng, C., Paddock, C. D. & Reddy Ganta, R. Molecular heterogeneity of *Ehrlichia chaffeensis* isolates determined by sequence analysis of the 28-kilodalton outer membrane protein genes and other regions of the genome. *Infect. Immun.* **71**, 187–195 (2003).
82. Long, S. W. *et al.* Antigenic variation of *Ehrlichia chaffeensis* resulting from differential expression of the 28-kilodalton protein gene family. *Infect. Immun.* **70**, 1824–1831 (2002).
83. Yu, X. J., McBride, J. W. & Walker, D. H. Genetic diversity of the 28-kilodalton outer membrane protein gene in human isolates of *Ehrlichia chaffeensis*. *J. Clin. Microbiol.* **37**, 1137–1143 (1999).
84. Unver, A., Rikihisa, Y., Stich, R. W., Ohashi, N. & Felek, S. The *omp-1* major outer membrane multigene family of *Ehrlichia chaffeensis* is differentially expressed in canine and tick hosts. *Infect. Immun.* **70**, 4701–4704 (2002).
85. Zhang, J. Z., Guo, H., Winslow, G. M. & Yu, X. J. Expression of members of the 28-kilodalton major outer membrane protein family of *Ehrlichia chaffeensis* during persistent infection. *Infect. Immun.* **72**, 4336–4343 (2004).
86. Singu, V., Liu, H., Cheng, C. & Ganta, R. R. *Ehrlichia chaffeensis* expresses macrophage- and tick cell-specific 28-kilodalton outer membrane proteins. *Infect. Immun.* **73**, 79–87 (2005).
87. Unver, A. *et al.* Transcriptional analysis of *p30* major outer membrane multigene family of *Ehrlichia canis* in dogs, ticks, and cell culture at different temperatures. *Infect. Immun.* **69**, 6172–6178 (2001).
88. Kim, H. Y. & Rikihisa, Y. Characterization of monoclonal antibodies to the 44-kilodalton major outer membrane protein of the human granulocytic ehrlichiosis agent. *J. Clin. Microbiol.* **36**, 3278–3284 (1998).
89. Wang, X., Kikuchi, T. & Rikihisa, Y. Two monoclonal antibodies with defined epitopes of P44 major surface proteins neutralize *Anaplasma phagocytophilum* by distinct mechanisms. *Infect. Immun.* **74**, 1873–1882 (2006).
90. Park, J., Choi, K. S. & Dumler, J. S. Major surface protein 2 of *Anaplasma phagocytophilum* facilitates adherence to granulocytes. *Infect. Immun.* **71**, 4018–4025 (2003).
91. Ohashi, N., Zhi, N., Zhang, Y. & Rikihisa, Y. Immunodominant major outer membrane proteins of *Ehrlichia chaffeensis* are encoded by a polymorphic multigene family. *Infect. Immun.* **66**, 132–139 (1998).
The first description of the major outer-membrane OMP1–P28 family proteins of *E. chaffeensis*, which are encoded by a multigene family.
92. Li, J. S., Chu, F., Reilly, A. & Winslow, G. M. Antibodies highly effective in SCID mice during infection by the intracellular bacterium *Ehrlichia chaffeensis* are of picomolar affinity and exhibit preferential epitope and isotype utilization. *J. Immunol.* **169**, 1419–1425 (2002).
93. Winslow, G. M. *et al.* Antibody-mediated elimination of the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis* during active infection. *Infect. Immun.* **68**, 2187–2195 (2000).
94. Kumagai, Y., Huang, H. & Rikihisa, Y. Expression and porin activity of P28 and OMP-1F during intracellular *Ehrlichia chaffeensis* development. *J. Bacteriol.* **190**, 3597–3605 (2008).
95. Huang, H., Wang, X., Kikuchi, T., Kumagai, Y. & Rikihisa, Y. Porin activity of *Anaplasma phagocytophilum* outer membrane fraction and purified P44. *J. Bacteriol.* **189**, 1998–2006 (2007).
The first study to show porin activity of the major outer-membrane protein of a rickettsial pathogen.
96. Gentle, I. E., Burri, L. & Lithgow, T. Molecular architecture and function of the Omp85 family of proteins. *Mol. Microbiol.* **58**, 1216–1225 (2005).
97. Voulhoux, R., Bos, M. P., Geurtsen, J., Mols, M. & Tommassen, J. Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science* **299**, 262–265 (2003).
98. Popov, V. L., Yu, X. & Walker, D. H. The 120 kDa outer membrane protein of *Ehrlichia chaffeensis*: preferential expression on dense-core cells and gene expression in *Escherichia coli* associated with attachment and entry. *Microb. Pathog.* **28**, 71–80 (2000).
99. Doyle, C. K., Nethery, K. A., Popov, V. L. & McBride, J. W. Differentially expressed and secreted major immunoreactive protein orthologs of *Ehrlichia canis* and *E. chaffeensis* elicit early antibody responses to epitopes on glycosylated tandem repeats. *Infect. Immun.* **74**, 711–720 (2006).
100. Luo, T., Zhang, X., Wakeel, A., Popov, V. L. & McBride, J. W. A variable-length PCR target protein of *Ehrlichia chaffeensis* contains major species-specific antibody epitopes in acidic serine-rich tandem repeats. *Infect. Immun.* **76**, 1572–1580 (2008).

101. McBride, J. W., Yu, X. J. & Walker, D. H. A conserved, transcriptionally active *p28* multigene locus of *Ehrlichia canis*. *Gene* **254**, 245–252 (2000).
102. Yu, X. J., McBride, J. W., Diaz, C. M. & Walker, D. H. Molecular cloning and characterization of the 120-kilodalton protein gene of *Ehrlichia canis* and application of the recombinant 120-kilodalton protein for serodiagnosis of canine ehrlichiosis. *J. Clin. Microbiol.* **38**, 369–374 (2000).
103. McBride, J. W., Yu, X. J. & Walker, D. H. Glycosylation of homologous immunodominant proteins of *Ehrlichia chaffeensis* and *Ehrlichia canis*. *Infect. Immun.* **68**, 13–18 (2000).
104. Luo, T., Zhang, X. & McBride, J. W. Major species-specific antibody epitopes of the *Ehrlichia chaffeensis* p120 and, *E. canis* p140 orthologs in surface-exposed tandem repeat regions. *Clin. Vaccine Immunol.* **16**, 982–990 (2009).
105. Huang, H. *et al.* Proteomic analysis of and immune responses to *Ehrlichia chaffeensis* lipoproteins. *Infect. Immun.* **76**, 3405–3414 (2008).
106. Rikihisa, Y. The tribe *Ehrlichieae* and ehrlichial diseases. *Clin. Microbiol. Rev.* **4**, 286–308 (1991).
107. Reichard, M. V. *et al.* Inoculation of white-tailed deer (*Odocoileus virginianus*) with Ap-V1 or NY-18 strains of *Anaplasma phagocytophilum* and microscopic demonstration of Ap-V1 In *Ixodes scapularis* adults that acquired infection from deer as nymphs. *Vector Borne Zoonotic Dis.* **9**, 565–568 (2009).
108. Woldehiwet, Z. & Scott, G. R. Stages in the development of *Cytoecetes phagocytophila*, the causative agent of tick-borne fever. *J. Comp. Pathol.* **92**, 469–474 (1982).
109. Popov, V. L., Chen, S. M., Feng, H. M. & Walker, D. H. Ultrastructural variation of cultured *Ehrlichia chaffeensis*. *J. Med. Microbiol.* **43**, 411–421 (1995).
110. Munderloh, U. G. *et al.* Invasion and intracellular development of the human granulocytic ehrlichiosis agent in tick cell culture. *J. Clin. Microbiol.* **37**, 2518–2524 (1999).
111. Troese, M. J. & Carlyon, J. A. *Anaplasma phagocytophilum* dense-cored organisms mediate cellular adherence through recognition of human P-selectin glycoprotein ligand 1. *Infect. Immun.* **77**, 4018–4027 (2009).
112. Zhang, J. Z., Popov, V. L., Gao, S., Walker, D. H. & Yu, X. J. The developmental cycle of *Ehrlichia chaffeensis* in vertebrate cells. *Cell. Microbiol.* **9**, 610–618 (2007).
An analysis of the *E. chaffeensis* intracellular developmental cycle and the associated differential expression of cell surface antigens.
113. Ito, S. & Rikihisa, Y. in*Rickettsiae and Rickettsial Diseases* (eds Burgdorfer, W. & Anacker, R. L.) 213–227 (Academic Press, New York, 1981).
114. Cheng, Z., Wang, X. & Rikihisa, Y. Regulation of type IV secretion apparatus genes during *Ehrlichia chaffeensis* intracellular development by a previously unidentified protein. *J. Bacteriol.* **190**, 2096–2105 (2008).
115. Lai, T. H., Kumagai, Y., Hyodo, M., Hayakawa, Y. & Rikihisa, Y. The *Anaplasma phagocytophilum* PleC histidine kinase and PleD diguanylate cyclase two-component system and role of cyclic di-GMP in host cell infection. *J. Bacteriol.* **191**, 693–700 (2009).
This article describes the PleCD two-component system and the importance of c-di-GMP for *A. phagocytophilum* infection.
116. Niu, H., Rikihisa, Y., Yamaguchi, M. & Ohashi, N. Differential expression of VirB9 and VirB6 during the life cycle of *Anaplasma phagocytophilum* in human leucocytes is associated with differential binding and avoidance of lysosome pathway. *Cell. Microbiol.* **8**, 523–534 (2006).
117. Heinzen, R. A. & Hackstadt, T. A developmental stage-specific histone H1 homolog of *Coxiella burnetii*. *J. Bacteriol.* **178**, 5049–5052 (1996).
118. Hackstadt, T., Baehr, W. & Ying, Y. *Chlamydia trachomatis* developmentally regulated protein is homologous to eukaryotic histone H1. *Proc. Natl Acad. Sci. USA* **88**, 3937–3941 (1991).
119. Cheng, Z., Kumagai, Y., Lin, M., Zhang, C. & Rikihisa, Y. Intra-leukocyte expression of two-component systems in *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* and effects of the histidine kinase inhibitor closantel. *Cell. Microbiol.* **8**, 1241–1252 (2006).
Analysis of the three pairs of two-component systems, their potential downstream effectors and their requirement for *E. chaffeensis* infection.
120. Kumagai, Y., Cheng, Z., Lin, M. & Rikihisa, Y. Biochemical activities of three pairs of *Ehrlichia chaffeensis* two-component regulatory system proteins involved in inhibition of lysosomal fusion. *Infect. Immun.* **74**, 5014–5022 (2006).
121. Romling, U. Cyclic di-GMP (c-di-GMP) goes into host cells—c-di-GMP signaling in the obligate intracellular pathogen *Anaplasma phagocytophilum*. *J. Bacteriol.* **191**, 683–686 (2009).
122. Alvarez-Marinez, C. E. & Christie, P. J. C. Biological diversities of prokaryotic type IV secretion systems. *Microbiol. Mol. Biol. Rev.* **73**, 775–808 (2009).
123. Rikihisa, Y. & Lin, M. *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* type IV secretion and Ank proteins. *Curr. Opin. Microbiol.* **13**, 59–66 (2010).
124. Bao, W. *et al.* Four VirB6 paralogs and VirB9 are expressed and interact in *Ehrlichia chaffeensis*-containing vacuoles. *J. Bacteriol.* **191**, 278–286 (2009).
125. Lin, M., den Dulk-Ras, A., Hooykaas, P. J. & Rikihisa, Y. *Anaplasma phagocytophilum* AnkA secreted by type IV secretion system is tyrosine phosphorylated by Abl-1 to facilitate infection. *Cell. Microbiol.* **9**, 2644–2657 (2007).
This study shows that *A. phagocytophilum* AnkA can be delivered into host cells by a T4SS and binds to ABI1 to activate the tyrosine kinase ABL1and that ABL1 is required for *A. phagocytophilum* infection.
126. IJdo, J., Carlson, A. C. & Kennedy, E. L. *Anaplasma phagocytophilum* AnkA is tyrosine-phosphorylated at EPIYA motifs and recruits SHP-1 during early infection. *Cell. Microbiol.* **9**, 1284–1296 (2007).
127. Park, J., Kim, K. J., Choi, K. S., Grab, D. J. & Dumler, J. S. *Anaplasma phagocytophilum* AnkA binds to granulocyte DNA and nuclear proteins. *Cell. Microbiol.* **6**, 743–751 (2004).
128. Garcia-Garcia, J. C., Rennoll-Bankert, K. E., Pelly, S., Milstone, A. M. & Dumler, J. S. Silencing of host cell *CYBB* gene expression by the nuclear effector AnkA of the intracellular pathogen *Anaplasma phagocytophilum*. *Infect. Immun.* **77**, 2385–2391 (2009).
129. Garcia-Garcia, J. C., Barat, N. C., Trembley, S. J. & Dumler, J. S. Epigenetic silencing of host cell defense genes enhances intracellular survival of the rickettsial pathogen *Anaplasma phagocytophilum*. *PLoS Path.* **5**, e1000488 (2009).
The authors show that host chromatin modifications by histone deacetylase 1 are linked to the downregulation of host defence genes during *A. phagocytophilum* infection.
130. Zhu, B. *et al.* Nuclear translocated *Ehrlichia chaffeensis* ankyrin protein interacts with a specific adenine-rich motif of host promoter and intronic Alu elements. *Infect. Immun.* **77**, 4243–4255 (2009).
131. Pan, X., Luhrmann, A., Satoh, A., Laskowski-Arce, M. A. & Roy., C. R. Ankyrin repeat proteins comprise a diverse family of bacterial type IV effectors. *Science* **320**, 1651–1654 (2008).
132. Wakeel, A., Kuriakose, J. A. & McBride, J. W. An *Ehrlichia chaffeensis* tandem repeat protein interacts with multiple host targets involved in cell signaling, transcriptional regulation, and vesicle trafficking. *Infect. Immun.* **77**, 1734–1745 (2009).
133. Yoshiie, K., Kim, H. Y., Mott, J. & Rikihisa, Y. Intracellular infection by the human granulocytic ehrlichiosis agent inhibits human neutrophil apoptosis. *Infect. Immun.* **68**, 1125–1133 (2000).
The first demonstration that *A. phagocytophilum* blocks the spontaneous apoptosis of human neutrophils.
134. Ge, Y., Yoshiie, K., Kuribayashi, F., Lin, M. & Rikihisa, Y. *Anaplasma phagocytophilum* inhibits human neutrophil apoptosis via upregulation of *bfl-1*, maintenance of mitochondrial membrane potential and prevention of caspase 3 activation. *Cell. Microbiol.* **7**, 29–38 (2005).
135. Scaife, H., Woldehiwet, Z., Hart, C. A. & Edwards, S. W. *Anaplasma phagocytophilum* reduces neutrophil apoptosis *in vivo*. *Infect. Immun.* **71**, 1995–2001 (2003).
136. Borjesson, D. L. *et al.* Insights into pathogen immune evasion mechanisms: *Anaplasma phagocytophilum* fails to induce an apoptosis differentiation program in human neutrophils. *J. Immunol.* **174**, 6364–6372 (2005).
This study shows that *A. phagocytophilum* fails to induce apoptotic differentiation of human neutrophils in cell culture by global microarray analysis.
137. Choi, K. S., Park, J. T. & Dumler, J. S. *Anaplasma phagocytophilum* delay of neutrophil apoptosis through the p38 mitogen-activated protein kinase signal pathway. *Infect. Immun.* **73**, 8209–8218 (2005).
138. Lee, H. C. & Goodman, J. L. *Anaplasma phagocytophilum* causes global induction of antiapoptosis in human neutrophils. *Genomics* **88**, 496–503 (2006).
139. Ge, Y. & Rikihisa, Y. *Anaplasma phagocytophilum* delays spontaneous human neutrophil apoptosis by modulation of multiple apoptotic pathways. *Cell. Microbiol.* **8**, 1406–1416 (2006).
140. Pedra, J. H., Sukumaran, B., Carlyon, J. A., Berliner, N. & Fikrig, E. Modulation of NB4 promyelocytic leukemic cell machinery by *Anaplasma phagocytophilum*. *Genomics* **86**, 365–377 (2005).
141. Zhang, J. Z., Sinha, M., Luxon, B. A. & Yu, X. J. Survival strategy of obligately intracellular *Ehrlichia chaffeensis*: novel modulation of immune response and host cell cycles. *Infect. Immun.* **72**, 498–507 (2004).
142. Niu, H., Kozjak-Pavlovic, V., Rudel, T. & Rikihisa, Y. *Anaplasma phagocytophilum* Asts-1 is Imported into host cell mitochondria. *PLoS Path.* **6**, e1000774 (2010).
This work finds that *A. phagocytophilum* Ats1 is translocated into the mitochondrial matrix of host cells and inhibits apoptosis.
143. Amano, A., Nakagawa, I. & Yoshimori, T. Autophagy in innate immunity against intracellular bacteria. *J. Biochem.* **140**, 161–166 (2006).
144. Ling, Y. M. *et al.* Vacuolar and plasma membrane stripping and autophagic elimination of *Toxoplasma gondii* in primed effector macrophages. *J. Exp.Med.* **203**, 2063–2071 (2006).
145. Schmid, D., Dengjel, J., Schoor, O., Stevanovic, S. & Munz, C. Autophagy in innate and adaptive immunity against intracellular pathogens. *J. Mol. Med.* **84**, 194–202 (2006).
146. Xiong, Q., Lin, M. & Rikihisa, Y. Cholesterol-dependent *Anaplasma phagocytophilum* exploits the low-density lipoprotein uptake pathway. *PLoS Path.* **5**, e1000329 (2009).
This article shows that *A. phagocytophilum* acquires cholesterol from the host low-density-lipoprotein uptake pathway by upregulating the low-density-lipoprotein receptor and increasing host cell cholesterol homeostasis.
147. Berry, D. S. *et al.* Ehrlichial meningitis with cerebrospinal fluid morulae. *Pediatr. Infect. Dis. J.* **18**, 552–555 (1999).
148. Dunn, B. E. *et al.* Identification of *Ehrlichia chaffeensis* morulae in cerebrospinal fluid mononuclear cells. *J. Clin. Microbiol.* **30**, 2207–2210 (1992).
149. Ratnasamy, N., Everett, E. D., Roland, W. E., McDonald, G. & Caldwell, C. W. Central nervous system manifestations of human ehrlichiosis. *Clin. Infect. Dis.* **23**, 314–319 (1996).

Acknowledgements

I thank all former and current members of my laboratory for their scientific contributions, T. Vojt for preparing the figures, V. L. Popov for the electron micrographs and K. Hayes-Ozello for editorial assistance. I also acknowledge grants from the US National Institutes of Health.

Competing interests statement

The author declares no competing financial interests.

DATABASES

Entrez Genome Project: http://www.ncbi.nlm.nih.gov/genomeprj
Amblyomma americanum | *Anaplasma marginale* | *Anaplasma phagocytophilum* | *Chlamydia trachomatis* str. D/UW-3/CX | *Coxiella burnetii* str. RSA 493 | *Ehrlichia canis* | *Ehrlichia chaffeensis* | *Ehrlichia ruminantium* | *Escherichia coli* | *Ixodes scapularis* | *Neorickettsia sennetsu* | *Rickettsia prowazekii* str. Madrid E

Pfam: http://pfam.sanger.ac.uk
PF01617

UniProtKB: http://www.uniprot.org
σ^{32} | σ^{70} | ABL1 | ABL1 | AnkA | ApxR | Asp62 | Asp55 | Ats1 | DnaA | Esp73 | HU | IHFα | IHFβ | OMP1A | OMP1B | OMP1F | OMP85 | P30-10 | PSGL1 | RecF | TRP47

FURTHER INFORMATION

Yasuko Rikihisa's homepage: http://riki-lb1.vet.ohio-state.edu/ehrlichia

JCVI Comprehensive Microbial Resource: http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi

SUPPLEMENTARY INFORMATION

See online article: S1 (table)

ALL LINKS ARE ACTIVE IN THE ONLINE PDF