Molecular and Cellular Basis of the Internalization of *Bartonella henselae* by Human Endothelial Cells

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1. INTRODUCTION

1. INTRODUCTION

1.1 Biology of the Human Vasculature

This chapter highlights different aspects of the anatomy and physiology of the human vasculature and explains relevant mechanisms for establishment, maintenance and function of the human vasculature in order to provide the reader of this thesis a sound basis for understanding the subsequent discussion on the biology of endothelial cells constituting an integral part of the human vasculature.

Anatomical drawings created by Leonardo da Vinci (1452-1519) depict parts of the blood vessel system including the coronary system of the heart and the circulatory system of the lung (Da Vinci, 1509). Da Vinci was the first scientist to reveal that these "tree-like" structures form the functional connections of the human circulatory system.

In principle, the human circulatory system comprises the (cardiac) heart muscle with elastic efferent (arteries) and afferent (veins) blood vessels which branch into smaller and thinner vessels (arterioles and venules, respectively) and finally pervade visceral organs and muscles as a fine meshwork, termed capillaries or sinusoids (Schmidt and Thews, 1989). The prototype circuit leads away from the heart into the body periphery via arteries to smaller arterioles, further to capillaries or sinusoids, then to venules, to veins and back to the heart, which contracts rhythmically to enable blood perfusion. The fine network of blood vessels which pervades organs and muscles is termed the vasculature.

The vasculature functions in (i) the perfusion of oxygen, nutrients and hormones and (ii) the removal of carbon dioxide, ammonia and other metabolic waste products. Exchange of these solutes between the blood and the surrounding tissues takes place in the capillaries (e.g. in muscles) and sinusoids (e.g. in liver, spleen, bone marrow) (Schmidt and Thews, 1989).

The vasculature is formed during embryogenesis by mesoderm-inducing factors of the fibroblast growth factor (FGF) family in a process termed vasculogenesis from angioblast progenitor cells (Jain, 2003). This process is accompanied in later stages of development by maturation of blood vessels, termed arteriogenesis, and the formation of new blood vessels from pre-existing ones, in a process termed angiogenesis, by sprouting and intussusception (Carmeliet, 2005; Ferrara and Kerbel, 2005) and establishment of the lymphatic vessel system (Alitalo et al., 2005) (see Figure 1). Angiogenesis is of fundamental importance under physiological conditions, e.g. during wound healing or during the menstrual cycle of the woman, and is abberant in tumor formation and growth.

Histology of the vasculature reveals a basic building plan applying to all blood vessels: A blood vessel is a hollow tube of three different cell layers, the intima, the media and the adventitia (Risau, 1995). The intima is the innermost layer comprises the lining of the endothelial cells, termed endothelium. The endothelium is encased and stabilized by a thin basal membrane. The media comprises a lamella of elastic connective tissue, which is enveloped by a layer of vascular smooth muscle cells (SMCs). The adventitia comprises a layer of elastic connective tissue which embeds the blood vessel in the surrounding tissue. In larger vessels, the adventitia itself is is pervaded by nerves that supply the muscular layer along with nutrient capillaries.

Blood vessels dysfunction is relevant for a plethora of medical conditions, especially cardiovascular diseases such as arteriosclerosis, congestive heart failure, coronary artery disease, stroke and hypertension (WHO, 2006). In cancer, the formation of new blood vessels is a tissue response towards the elevated need for oxygen and nutrients in a growing tumour (Bergers and Benjamin, 2003). In inflammation, blood vessel permeability is increased resulting in influx of blood plasma into underlying tissues as a response to infection or irritation, giving rise to oedema and swellings (Fiedler et al., 2006; Imhof and Aurrand-Lions, 2006). Furthermore, inflammation of the blood vessel itself, termed vasculitis, is the consequence of an autoimmune response impairing vascular homeostasis. (Davies, 2005; Pendergraft et al., 2004).

1.2 Biology of Endothelial Cells

This chapter places an emphasis on one specific cell type of the vasculature, namely endothelial cells. The fundamental principles of endothelial cell biology and endothelial cell function in the context of blood vessels system are presented in order to allow the reader of this thesis to understand how bacterial pathogens manage to subvert these functions during the infection process as discussed later.

Endothelial cells (ECs) are flat and thin (squamous), of oblong to spindle-shaped appearance, lining the interior of the blood and lymphatic vessel system from the heart to capillaries. The entity of ECs lining is termed the endothelium. ECs form a slick interface between the blood vessel lumen with the circulating blood and the proper blood vessel wall.

ECs exert diverse biological functions and contribute to vascular homeostasis (Risau, 1995). These functions are highlighted in the following five paragraphs.

(1) ECs control the vascular tone by generating and transducing auto-, para-, and endocrine signals leading e.g. to contraction (vasoconstriction) or relaxation (vasodilatation) of vascular smooth muscle cells (SMCs) to regulate blood pressure. It has been found, that nitric oxide (NO) is a key signalling molecule in this process acting as a powerful vasodilator (Cockcroft, 2005). NO is produced by endothelial nitric oxide synthetase (eNOS) from L-arginine to L-citrulline (Fish and Marsden, 2006). NO activates soluble guanylate cyclases in vascular SMCs in order to lower the vascular tone.

(2) ECs are important target cells in local inflammatory processes as a response to bacterial infections by allowing adhesion, rolling and transmigration of professional phagocytes, e.g. monocytes or polymorphonucleocytes into underlying tissues (Luster et al., 2005). The pro-inflammatory response leads to the secretion of cytokines from ECs. These pro-inflammatory signals are interleukin 1 (IL-1) and tumor necrosis factor α (TNFα). Their release promotes surface expression of adhesion molecules for leukocytes, namely CD62P (P-selectin) and E-selectin (CD62E) for initial leukocyte binding to and rolling on ECs and CD54 (intercellular adhesion molecule 1, ICAM-1) and CD106 (vascular intercellular adhesion molecule-1, VCAM-1) for firm adhesion. Furthermore, the release of IL-1 and TNFα stimulates nuclear factor-κB (NF-κB)-dependent secretion of the chemokine interleukin 8 (IL-8) from ECs and engagement of CD31 (platelet endothelial cell adhesion molecule 1, PECAM-1) to stimulate transmigration of adherent leukocytes through the blood vessels into the underlying tissues and to resolve infection.

(3) Vascular endothelial growth factor (VEGF) is a potent mitogen for ECs and acts in a paracrine fashion on ECs. VEGF activates ECs to migrate and to proliferate and enhances endothelial cell survival in the process of angiogenesis, eventually leading to tube structure formation and vascular remodelling (Carmeliet, 2005; Ferrara and Kerbel, 2005). Furthermore, VEGF stimulates the aforementioned enzyme eNOS leading to increased NO production which contributes to angiogenesis.

(4) ECs control blood coagulation events such as fibrinolysis during the repair of damaged blood vessels or counteract blood vessel clotting by thrombolysis.

(5) ECs act as a selective physical diffusion barrier controlling influx and efflux of gaseous and solute substances between bloodstream and underlying tissues (e.g. in renal glomeruli, in the blood brain barrier or in the placenta).

1.3 Bacterial Interactions with Endothelial Cells

Bacterial pathogens have developed strategies to adhere to and to invade a wide range of human cell types. Establishment of a portal of entry is critical for colonization and subsequent dissemination. Entry sites for bacterial pathogens are mucosal surfaces like the gastrointestinal, the urogenital and the respiratory tract, but as well the conjunctiva, the blood-brain barrier, the maternal placental blood-barrier and the inner lining of blood vessels. These entry sites share the common property that they represent an interface between the external environment and the underlying body tissues.

Both gram-positive (e.g. group B streptococci, *Staphylococcus aureus*, *Listeria monocytogenes*) and gram-negative (*Chlamydia pneunomiae*, enterohaemorrhagic *E. coli*, *Neisseria meningitidis*, *Rickettsia* spp., *Bartonella* spp.) bacteria have been identified to display endothelial host cell tropism and to be able to infect these important target cells.

To exemplify these strategies I chose to present two gram-positive bacterial pathogens and three gram-negative bacterial pathogens. The focus of these strategies centres around the question on how these pathogens are able to adhere and invade ECs to establish successful infections of the vasculature.

1.3.1 Group B Streptococci

The genus *Streptococcus* represents facultative anaerobic, gram-positive cocci growing in chains or pairs with polysaccharide capsules rich in sialic acid, muramic acid and glucosamine. Streptococci are able to colonize the oral cavity, the skin, the intestinal and respiratory tract of humans as part of the normal commensal flora. *S. agalactiae* belongs to group B streptococci (GBS) which are a leading cause of sepsis and meningitis. Meningitis is a serious infection of the central nervous system (CNS) and is frequently associated with newborn infants and immuno-compromised adults. GBS interact with cerebral endothelial cells in the process of bacterial transcytosis to breach the blood-brain barrier (BBB). Subsequent bacterial replication in the CNS provokes a massive inflammatory response leading to meningitis, septicaemia and brain injury. GBS are able to adhere to a variety of host cell surface proteins such as fibronectin (Tamura and Rubens, 1995), laminin (Spellerberg et al., 1999) and cytokeratin (Tamura and Nittayajarn, 2000). GBS invasion of human brain microvascular endothelial cells (BMEC) represents a model for transcytosis of polar BMEC monolayers *in vitro* (Nizet et al., 1997), leading to significant injury and disruption of the BBB at high bacterial concentrations correlated to β-hemolysin activity (Nizet et al., 1997). Additional cellular consequences are the release of the pro-inflammatory chemokine interleukine-8 (IL-8) and of the cytokine interleukine-6 (IL-6), and upregulation of CD54 (intercellular adhesion molecule-1, ICAM-1) that act to orchestrate neutrophil recruitment and activation to sites of bacterial infection (Doran et al., 2003). Pneumonia is often observed with early-onset meningitis in newborns. The primary portal of GBS entry into the fetus occurs over interactions with the lung epithelia by aspiration of infected amniotic fluid and dissemination into the blood-stream (Rubens et al., 1992). Subsequent invasion of pulmonary blood vessels can be mimicked by GBS infection of pulmonary artery endothelial cells (PAEC) and lung microvascular endothelial cells (LMEC) *in vitro* (Gibson et al., 1995) stimulating the realease of inflammatory eicosanoids, which are associated with bacteremia and a clear indication for sepsis that accompanies meningitis *in vivo* (Rubens et al., 1991).

1.3.2 *Staphylococcus aureus*

The gram-positive bacterium *S. aureus* is coagulase-positive and grows facultative anaerobically in grape-like clusters characterized by golden pigmentation. *S. aureus* encodes for a plethora of extracellular enzymes and exotoxins (e.g. α-toxin, leucodicin, exfoliants, entertoxins and toxic shock syndrom toxin) and is a leading cause of human disease, representing two subgroups, invasive infections and proper toxicoses. *S. aureus* has the capability to invade the vascular endothelium (Sinha and Herrmann, 2005). The invasive potential is reflected by the bacterial ability to cause serious endovascular infections, such as endocarditis and vasculitis. The major adhesins known are clumping factor A (ClfA) and fibronecting-binding protein A (FnBPA) (Massey et al., 2001). Subsequent internalization of *S. aureus* into human endothelial cells requires the host cell actin cytoskeleton (Zhang et al., 2002), is dependent on the expression of fibronectin-binding proteins (Sinha et al., 1999), and triggers recruitment of focal contact-associated proteins vinculin, tensin, zyxin and focal adhesion kinase (FAK) to the sites of bacterial attachment and invasion (Agerer et al., 2005). Dominant-negative versions of FAK block integrin-mediated internalization and FAK-deficient cells are severely impaired in their ability to internalise *S. aureus*. Pathogen binding induces tyrosine phosphorylation of several host proteins associated with bacterial attachment sites, including FAK and the Src substrate cortactin (Agerer et al., 2005) *S. aureus* internalization leads to enhanced expression of CD106 (VCAM-1) and CD54 (ICAM-1) but does not alter expression of CD62P (P-selectin), CD62E (E-selectin) and CD31 (PECAM-1). After internalization, *S. aureus* may either persist and escape host defences and antimicrobial agents or multiply and disseminate. Both vacuole-bound bacteria and cytoplasmic bacteria can be found (Peacock et al., 1999). The intracellular fate is dependent on the presence of the α-toxin, which acts as caspase-dependent and Fas-independent (Haslinger-Loffler et al., 2005) apoptotic stimulus for endothelial cells (Menzies and Kourteva, 2000).

1.3.3 *Rickettsia rickettsii*

Rickettsiae are gram-negative α-proteobacteria, exhibiting an obligate intracellular lifestyle. Bacilli are non-motile and of pleomorphic appearance. Rickettsial species are divided into two subgroups, the spotted fever group (SFG) and the typhoid group, and are transmitted to humans by arthropods, e.g. ticks. *R. rickettsii* belongs to the SFG and is the etiological agent of Rocky Mountain spotted fever (RMSF). RMSF is an acute life-threatening, febrile illness, accompanied by a typical rash on the extremities (Dumler and Walker, 2005). *R. rickettsii* spreads and replicates in the cytoplasm of endothelial cells eliciting widespread vascular inflammation (vasculitis), reduced blood perfusion (thrombosis), end-organ damage (by ischemia), which is most dangerous in lungs and brain.

Efficiency of invasion of HUVECs (Silverman, 1984; Silverman and Bond, 1984) by *R. rickettsii* is dependent on the multiplicity of infection (Silverman and Bond, 1984) and on the host cell actin cytoskeleton (Rydkina et al., 2005). Bacterial internalization into ECs leads to activation of protein kinase C (Sahni et al., 1999), which, in turn, leads to activation of NF-κB, as suggested by the activation of IκB kinase (IKBK) and phosphorylation and subsequent proteasomal degradation of the IκB inhibitory subunit (Clifton et al., 2005). This signalling cascade leads to the suppression of apoptosis by *R. rickettsii* in ECs (Joshi et al., 2004).

For the closely related species *R. conorii*, the causative agent of Mediterranean spotted fever, it has been shown, that entry into non-phagocytic cells correlates with the tyrosine phosphorylation of several host proteins, including focal adhesion kinase (FAK), depends on the Arp2/3 complex, which involves the interplay of Cdc42, phosphatidylinositol 3-kinase (PI3K), c-Src and cortactin leading to localized actin rearrangements (Martinez and Cossart, 2004) (see Figure 1).

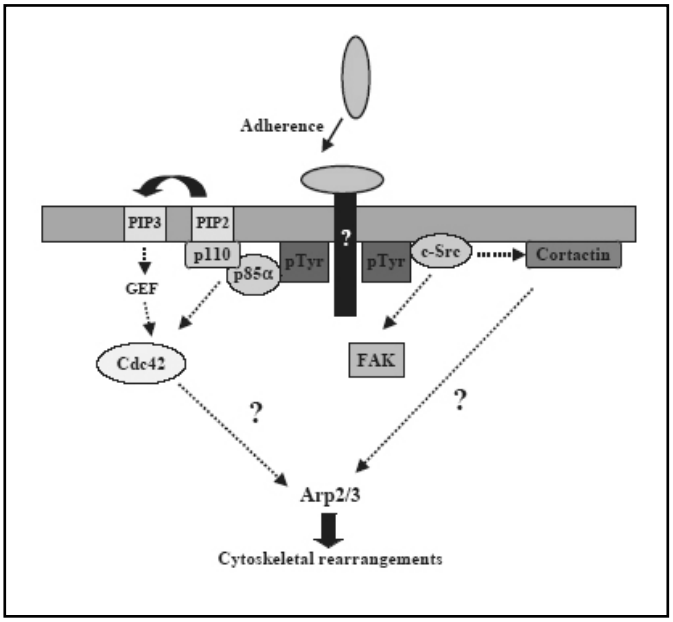


Figure 1

Early signalling events involved in the host cell entry of *Rickettsia conorii*
(Martinez J.J. and Cossart P., Journal of Cell Science, 2005)

1.3.4 *Neisseria meningitidis*

Neisseria meningitidis (or simply the meningococcus) is a gram-negative bacterium, of coccoid shape, which is protected by a polysaccharide capsule, and which belongs to the order of β-proteobacteria. *N. meningitidis* specifically infects humans, there is no animal reservoir known. Serogroups A, B, C and W135 are the most important clinical subtypes of *N. meningitidis*.

Meningococcal meningitis and sepsis frequently affect infants and adolescents, even at epidemic scales. Avirulent meningococci persistently and asymptomatically colonize the nasophrarynx. In contrast, virulent meningococci penetrate mucosal surfaces of the nasopharynx, spread haematogenously, eventually crossing the BBB causing systemic meningitis and sepsis. *N. meningitidis* adherence to and invasion of HUVECs (Virji et al., 1994) and HBMECs (Unkmeir et al., 2002) has been demonstrated.

Adherence of meningococci to epithelial and endothelial cells is mediated by type IV pili (Nassif et al., 1994) and CD46 (membrane cofactor protein, MCP) is considered to constitute the host cell receptor (Kallstrom et al., 1997; Kirchner and Meyer, 2005). In addition to type IV pilus-mediated adhesion, meningococcal attachment to ECs is mediated as well by the bacterial outer membrane protein Opc that binds fibronectin, thereby anchoring the bacterium to the integrin α5 β1-receptor on the endothelial cell surface (Sokolova et al., 2004). Bacterial adhesion results in the formation of cellular protrusions at the site of bacterial attachment. These microvilli-like protrusions are highly enriched for ezrin and moesin, two members of the ERM (ezrin/radixin/moesin) family of actin-binding proteins, whereas the focal adhesion proteins, vinculin and paxillin, are absent (Eugene et al., 2002). Formation of membrane ruffles and subsequent internalisation requires cortical actin polymerization and depends on the activation of the small GTPases Rho and Cdc42, but not of Rac1 (Eugene et al., 2002).

Proper internalisation of *N. meningitidis* into ECs involves the activation of the ErbB2 tyrosine kinase receptor and the c-Src kinase, leading to tyrosine phosphorylation of cortactin (Hoffmann et al., 2001). *N. meningitidis* mutants expressing a deglycosylated lipooligosaccharide (LOS) are poorly invasive. These mutants show structurally altered actin polymerization. Moreover, although they efficiently recruit and activate the kinases ErbB2 and c-Src, these mutants are defective in the recruitment and phosphorylation of cortactin (Lambotin et al., 2005) (see Figure 2).

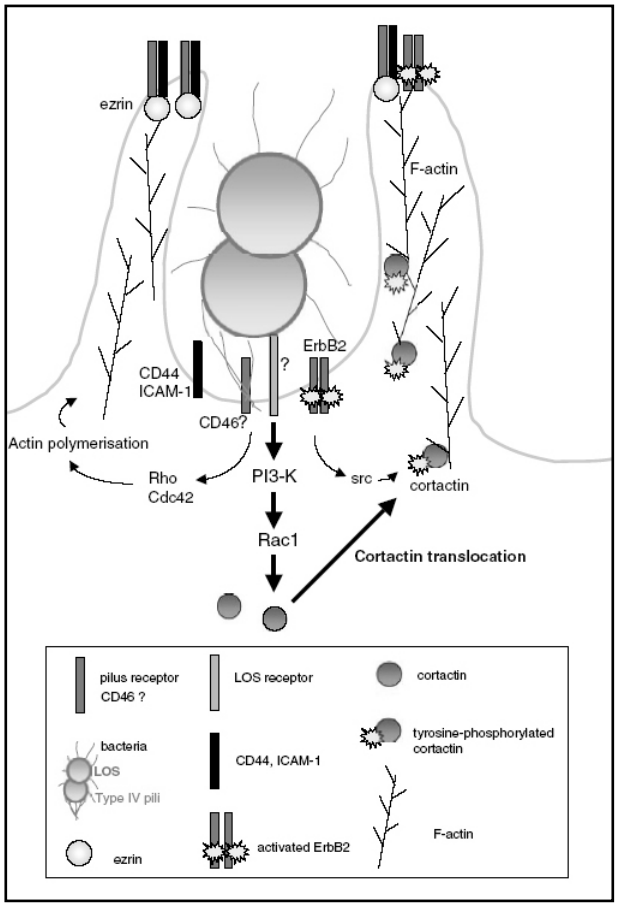


Figure 2

Neisseria meningitidis invasion of endothelial cells

(Lambotin et al., Journal of Cell Science, 2005)

Phosphorylated cortactin controls cortical actin polymerization, which leads to membrane protrusion formation. In addition, cortactin recruitment is dependent on the activation of a PI3K/Rac1-GTPase signalling pathway, which is required for actin polymerization and internalization of *N. meningitides* in ECs, and is not activated by the mutant strains (Lambotin et al., 2005).

Furthermore, it has been shown that binding of meningococci to HBMEC phosphorylates and activates c-Jun N-terminal kinases 1 and 2 (JNK1 and JNK2) and p38 mitogen-activated protein kinase (p38 MAPK) as well as their direct substrates c-Jun and MAP kinase activated protein kinase-2 (MAPKAPK-2), respectively (Sokolova et al., 2004). Non-invasive meningococcal strains lacking the *opc* locus still activate p38 MAPK, but fail to activate JNK. Inhibition of JNK1 and JNK2 significantly reduces internalization of *N. meningitidis* by HBMEC without affecting its adherence. Blocking the endothelial integrin $\alpha 5\beta 1$ also decreases *N. meningitidis*-induced JNK activation in HBMEC. These findings indicate the crucial role of JNK signalling pathway in *N. meningitidis* invasion in HBMEC.

In contrast, the p38 MAPK pathway is important for the control of IL-6 and IL-8 release by HBMEC in eliciting a pro-inflammatory response (Sokolova et al., 2004). Genistein, a protein tyrosine kinase inhibitor, decreases both invasion of *N. meningitidis* into HBMEC and IL-6 and IL-8 release, indicating that protein tyrosine kinases, which link signals from integrins to intracellular signalling pathways, are essential for both bacterial internalization and cytokine secretion by HBMEC (Sokolova et al., 2004).

1.3.5 *Bartonella henselae*

Bartonella henselae is a gram-negative, α-proteobacterial zoonotic pathogen and exhibits a strong host cell tropism for ECs. *B. henselae* is able to adhere to and invade ECs and serves as a model organism for vascular colonization. Clinical manifestations, modes of infection, and virulence factors of *B. henselae* involved in pathogenesis are highlighted in the following three subchapters. These introductory chapters provide the knowledge on the basic mechanisms of *B. henselae* host cell interactions relevant for the experimental approaches presented in the results section.

1.3.5.1 Pathogenesis of *B. henselae*

B. henselae is a pleomorphous, rod-shaped bacterium that is fastidious in growth. It causes longstanding intraerythrocytic bacteraemia in its natural reservoir, the cat. Transmission from cat to cat occurs mainly by the cat flea. Transmission from cat to the incidental human host occurs by cat flea or a cat scratch or bite. The infection in cats is asymptomatic or associated with light fever. Importantly, *B. henselae* is responsible for most cases of human bartonellosis (Dehio, 2004) and the outcome of disease in humans is dependent on the immune status of the infected individual (see Figure 3).

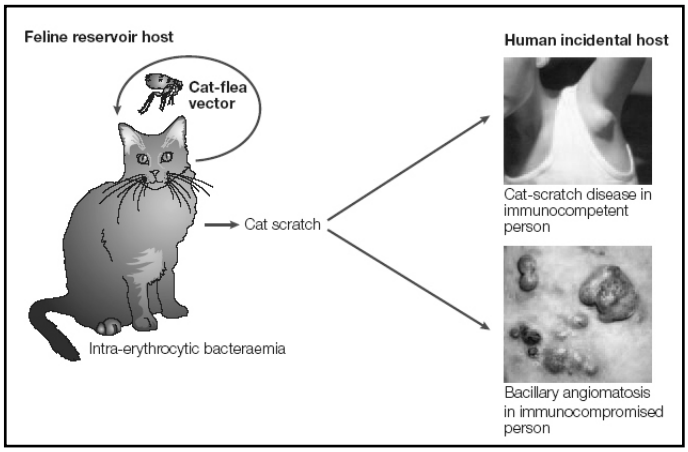


Figure 3
Pathogenesis of *B. henselae*
(Dehio C., Nature Reviews in Microbiology, 2005)

Upon infection with *B. henselae*, immunocompetent individuals establish a clinical condition usually referred to as cat-scratch disease (CSD). CSD is most commonly characterized by a regional inflammation and swelling of lymph nodes, termed lymphadenopathy, which is a self-limiting disease with no adverse consequences. Bacillary angiomatosis (BA) is the most common clinical manifestation of *B. henselae* infection in immunocompromised individuals, especially in AIDS patients. Cutaneous lesions in BA are bacterially-induced, with bacterial aggregates found in intimate contact with proliferating ECs. BA lesions are also infiltrated with macrophages/monocytes and polymorphonuclear neutrophils (PMN). BA lesions provoked by *B. henselae* are indistinguishable from BA lesions elicited by the related species *Bartonella quintana*. However, other than *B. quintana*, *B. henselae* can also cause a similar vasoproliferative disorder in inner organs, bacillary peliosis (BP), which is characterized by vascular proliferation in liver and spleen. BA and BP lesions always contain proliferating ECs and both conditions are characterized by the formation of benign, untransformed, vascular tumours. These tumours are caused by a mitogenic bacterial factor leading to enhanced cell migration and proliferation of ECs (Dehio, 2005). Furthermore, *B. henselae* is a major cause of endocarditis, which represents an inflammation of the endocardium, the inner layer of the heart, usually affecting cardiac valves. Homeless and chronically alcoholic individuals are particularly susceptible to this disease, which can be established as well by the aforementioned species *B. quintana* and several other zoonotic *Bartonella* species (Dehio, 2004). *B. henselae* exhibits a facultative intracellular life-style and is able to infect ECs in the feline reservoir and the incidental human host. Primary human umbilical vein endothelial cells (HUVEC) have been used as an appropriate *in vitro* system to study the interactions of *B. henselae* with the human vascular endothelium, i.e. (i) endothelial cell proliferation (ii) activation of the transcription factor NF-κB and stimulation of a pro-inflammatory response, which both are considered to contribute to *Bartonella*-triggered vasoproliferation, (iii) inhibition of endothelial cell apoptosis, and (iv) cellular invasion by rearrangement of host cell actin cytoskeleton (Schmid et al., 2004).

1.3.5.2 Type IV Secretion Systems in *B. henselae*

Activation of the transcription factor NF-κB and stimulation of a pro-inflammatory response, inhibition of endothelial cell apoptosis, and cellular invasion by rearrangement of the actin cytoskeleton elicited by *B. henselae* can be associated genetically to one virulence determinant of *B. henselae*, the VirB/VirD4 type IV secretion system (T4SS) (Padmalayam et al., 2000; Schmid et al., 2004; Schmiederer and Anderson, 2000; Schulein and Dehio, 2002). The VirB/VirD4 T4SS of *B. henselae* is chromosomally encoded and is closely related to the bacterial conjugation system AvhB/TraG of the *Agrobacterium tumefaciens* cryptic plasmid pAT18 based on the conservation of amino acid identities (see Figure 4).

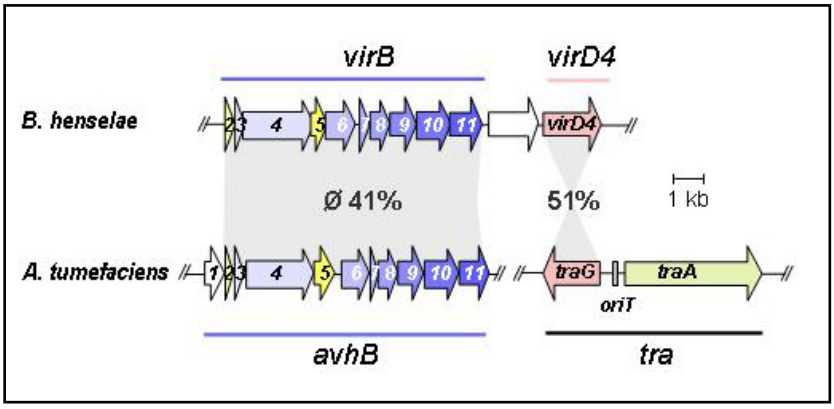


Figure 4

Genetic organisation of the *virB/virD4* loci of *Bartonella henselae* and *avhB/tra* loci of *Agrobacterium tumefaciens*. (adapted from Schröder G. and Dehio C., Trends in Microbiology, 2005)

T4SS are versatile macromolecular secretion machineries found in many gram-negative bacteria, and are evolutionarily derived from conjugation systems involved in horizontal gene transfer. T4SS are either plasmid-borne or chromosomally encoded, genetically organised into operons, usually constituting a proper pathogenicity island.

Upon induction, the T4SS is expressed and assembles into a membrane-spanning multiprotein complex (see Figure 5) competent for the secretion of either singular effector proteins, multiprotein complexes or even nucleoprotein complexes (Cascales and Christie, 2003).

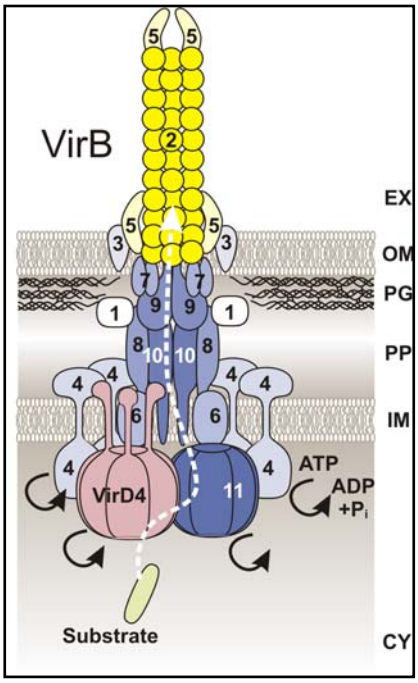


Figure 5

Macromolecular Assembly of the VirB/VirD4 T4SS of *Bartonella henselae*.
(adapted from Schröder G. and Dehio C., Trends in Microbiology, 2005)

The role in pathogenesis of the VirB/VirD4 T4SS has been studied in an appropriate animal model *in vivo* (Schulein and Dehio, 2002). Experimental infection of rats by *Bartonella tribocorum* – a close relative of *B. henselae* - revealed the initial colonization of a yet unidentified niche outside of circulating blood, presumably represented by the vascular endothelium. This primary niche periodically seeds bacteria into the bloodstream, resulting in the invasion and persistent intracellular colonisation of erythrocytes (Schulein et al., 2001).

Introduction of non-polar in-frame deletions in structural components of the VirB/VirD4 T4SS (e.g. *ΔvirB4* or *ΔvirD4*) completely abrogates the capability to cause bacteraemia, whereas complementation with the full-length genes *in trans* restored infectivity, clearly indicating that the VirB/VirD4 T4SS is essential for pathogenesis (Schulein and Dehio, 2002). *B. henselae* harbours a second functional T4SS, termed Trw (Seubert et al., 2003), which is highly similar to the Trw conjugation machinery of the broad-host-range antibiotic resistance plasmid R388. The Trw T4SS is upregulated intracellularly during the interaction of *B. henselae* with HUVECs. However, the contribution to pathogenesis of *Bartonella* on ECs remains to be demonstrated (Seubert et al., 2003).

1.3.5.3 Other Virulence Determinants of *B. henselae*

Further virulence factors of *B. henselae* important for the interaction with ECs are outer membrane proteins, namely (i) HbpA, (ii), Omp43, and (iii) BadA. HbpA (Pap31) has been originally identified as a hemin-binding protein of *B. henselae*, but appears to mediate as well adhesion to fibronection and to heparin on HUVECs (Dabo et al., 2006), while the prototypical β-barrel Omp43 might be an adhesin for HUVECs (Burgess and Anderson, 1998; Burgess et al., 2000).

The non-fimbrial adhesin BadA mediates the binding of *B. henselae* to extracellular matrix proteins and adhesion to ECs and is an immunodominant protein detectable in *B. henselae*-infected patients and rodents (Riess et al., 2004). BadA expression is important for the activation of hypoxia-inducible factor 1 (HIF-1) (Kempf et al., 2005) and the secretion of vascular endothelial growth factor (VEGF), which acts as proangiogenic mediators in EC proliferation induced by *B. henselae* (Kempf et al., 2005).

1.4 Bacterial Subversion of the Host Cell Actin Cytoskeleton Function

Modulation of components of the actin cytoskeleton machinery is frequently observed in the interaction of pathogenic bacteria with host cells. This involves (i) the stimulation of actin polymerization/depolymerization, (ii) the modulation of the activity of actin-tethering/bundling/branching proteins and finally (iii) the modulation of the activity of proteins which itself are in control of actin-dependent processes in response to cellular cues under physiological situations. Control of the dynamics and the assembly of the actin cytoskeleton converge on a specific protein family, the Rho-family of small GTPases (Hall, 1998), that regulate actin-dependent processes and function as molecular switches. Thus, bacterial subversion of the host cell actin cytoskeleton machinery frequently triggers the Rho GTPase switch leading to rearrangement, polymerization or disruption of the host cell actin cytoskeleton in order to gain access into non-phagocytic cells or to prevent uptake into phagocytic cells (Gruenheid and Finlay, 2003).

The following chapters are dedicated to five topics. The first chapter introduces the GTPase switch as a target for bacterial effector proteins. The second chapter introduces the invasion strategy of the *Salmonella typhimurium* to get access into non-phagocytic cells, whereas the third chapter introduces the strategy of *Yersinia enterocolitica* to prevent its own uptake into phagocytic cells. The fourth chapter highlights how *Listeria monocytogenes* not only promotes its own uptake into host cells, but as well how it employs the actin cytoskeleton machinery to spread intra- and intercellulary. The fifth and sixth chapter deal with *Helicobacter pylori* and *B. henselae* and how they gain access to their target cells.

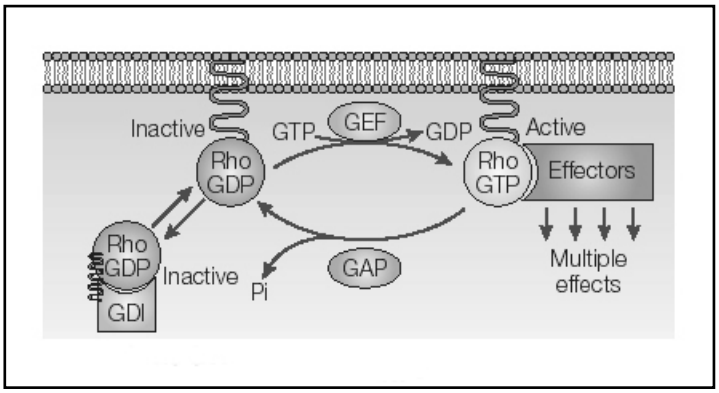
1.4.1 The GTPase Switch

Rho-family small GTPases belong to the Ras superfamily of small GTPases and share a high degree of conservation in their amino acid sequence from yeast to man. Twenty genes encoding different family members have been identified in the human genome (Hall, 1998). Out of these, the most important members are Rho, Rac and Cdc42 (Hall, 2005; Hall and Nobes, 2000). Rho, Rac and Cdc42 each control a signal transduction pathway linking membrane receptors to the assembly and disassembly of the actin cytoskeleton. Rho elicits the formation of stress fibers and focal adhesion contacts, Rac controls the formation of membrane ruffles and lamellipodia, and Cdc42 engages the formation of filopodial cell extensions (Nobes and Hall, 1995). The Rho-family small GTPases comprise versatile molecular players and regulate many cellular events of fundamental importance such as cell growth, cell morphogenesis, cytokinesis, cell movement, and lipid trafficking (Nobes and Hall, 1999).

Rho-family small GTPases act as molecular switches (Jaffe and Hall, 2005). They exist in an inactive GDP-bound and an active GTP-bound form that is able to signal to downstream effector proteins (see Figure 6). Cycling between these two states is regulated by the action of GTPase exchange factors (GEFs) which stimulate exchange of GDP for GTP, whereas GTPase activating proteins (GAPs) stimulate the GTP hydrolysis to GDP. Over 80 GEFs have been identified in mammals, which fall, based on their domain structure into two families. The larger family of Rho GEFs contains a characteristic DH (Dbl homology) domain adjacent to a PH (pleckstrin homology) domain (Rossman et al., 2005), whereas the smaller family of Rho GEFs shares homology with a protein called DOCK180 (dedicator of cytokinesis 180) (Meller et al., 2005).

On the other hand, Rho GAPs are more variable and do not share significant sequence homologies between each other (Bernards and Settleman, 2004; Moon and Zheng, 2003). Finally, GTPase dissociation inhibitors (GDIs) inhibit not only the exchange of GDP for GTP but as well recruitment of C-terminally prenylated Rho-family small GTPases to the plasma membrane, where they excert their biological function (Olofsson, 1999).

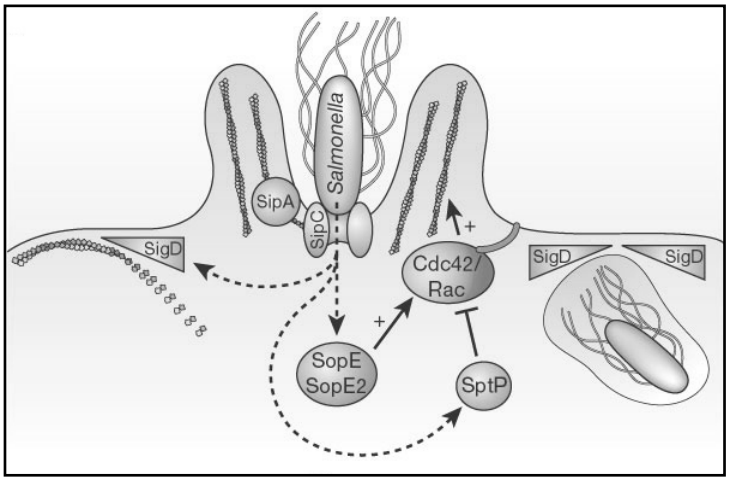
Figure 6
The Rho-family GTPase switch (Aktories P. and Barbieri J.T., Nature Reviews in Microbiology, 2005)



1.4.2 *Salmonella enterica* serovar *Typhimurium*

Salmonella is a ubiquitous gram-negative intestinal pathogen and the causative agent of several food-borne diarrheal diseases. *Salmonella* gains access to the intracellular environment by localized actin polymerization at the cell cortex, leading to extensive membrane ruffling and subsequent uptake (Altmeyer et al., 1993; Pace et al., 1993). This mode of forced cell entry is usually referred to as "trigger"-mechanism (Francis et al., 1993; Jones et al., 1993). Host cell invasion requires the concerted action of several bacterial effector proteins (see Figure 7). Translocation of these proteins from the bacterium into the host cell cytoplasm is accomplished by a specific macromolecular machine, termed injectisome. Injectisomes are found in many gram-negative bacterial pathogens and are collectively referred to as type III secretion system (T3SS). The T3SS of *Salmonella* required for invasion is termed SPI-1 (*Salmonella* Pathogenicity Island 1) (Kaniga et al., 1995; Mills et al., 1995).

Figure 7
Salmonella entry into host cells is mediated by the SPI-1 T3SS and its effectors (Gruenheid S. and Finlay B.B., Nature, 2003)



The following paragraphs introduce the most important effector proteins translocated by the SPI-1 T3SS of *Salmonella*.

The effector proteins SopE/SopE2 stimulate cytoskeletal reorganization during *Salmonella* invasion in a Rac1 and Cdc42 dependent manner, interacts with Rac1 and Cdc42 *in vivo* and stimulates nucleotide exchange of Rac1 and *Cdc42 in vitro* (Hardt et al., 1998; Stender et al., 2000). This action is reversed by SptP which in turn functions as a GAP for Rac1 and Cdc42 (Fu and Galan, 1999). Both, SopE and SptP are delivered early during infection in equal amounts into the host cell cytoplasm. However, SopE is rapidly cleared through proteasomal degradation, while SptP is degraded with much slower kinetics. This regulatory mechanism allows activation and inactivation of Rac1/Cdc42 during invasion in a precisely timed manner (Kubori and Galan, 2003).

The effector protein SigD/SopB is an inositol 3-phosphatase increasing cellular levels of phosphatidylinositol 4,5-bisphosphate leading to indirect activation of Cdc42 (Hernandez et al., 2004; Terebiznik et al., 2002). Activation of Cdc42 and Rac1 lead to recruitment and activation of WASP and Scar/WAVE family proteins together with the Arp2/3 complex involved in initiating actin polymerization (Shi et al., 2005; Unsworth et al., 2004).

The effector protein SipA nucleates and bundles actin filaments (Zhou et al., 1999a; Zhou et al., 1999b) and SipC stabilizes bundles of actin filaments (Hayward and Koronakis, 1999) which are formed during membrane ruffling and bacterial engulfment.

Loss-of-function mutants in one or more of these effector loci result in a significant impairment of invasion. Several other gram-negative bacteria such as *Shigella flexneri* use comparable strategies by injecting effector proteins via their T3SS to promote invasion and to force uptake into non-phagocytic cells.

Intracellular *Salmonella* reside in a membrane-bound compartment permissive for replication, termed *Salmonella*-containing vacuole (SCV) (Cuellar-Mata et al., 2002; Steele-Mortimer et al., 1999). Biogenesis, maturation and maintenance of the SCV is dependent on the *Salmonella* pathogenicity island 2 (SPI-2) T3SS in epithelial cells (Steele-Mortimer et al., 2002). After bacterial uptake, *Salmonella* induces the formation of a fine meshwork of F-actin decorating the SCV established in epithelial cells, fibroblasts and macrophages which is SPI-2 dependent. Treatment of infected cells with actin-depolymerizing drugs inhibits *Salmonella* replication and results in the loss of the SCV membrane and the release of bacteria into the cytoplasm indicating that actin recruitment to the SCV is essential. Intracellular *Salmonella* produce another T3SS-translocated effector protein, termed SpvB, which triggers actin depolymerization in host cells (Lesnick et al., 2001). SpvB is encoded by the *spv* locus, a regulon consisting of the spvABCD structural genes controlled by spvR (Coynault et al., 1992). SpvB is an actin-ADP-ribosylating toxin that modifies several isoforms and mediates disruption of actin around the SCV and at other host cell sites and induces the loss of cytoskeletal integrity.

1.4.3 *Yersinia enterocolitica*

Yersinia enterocolitica is a gram-negative pathogen and the etiological agent of food-borne illnesses, which are usually self-limiting. *Y. enterocolitica* is able to penetrate the intestinal mucosa, to cross the epithelial cell lining and to multiply in the lymphoid tissues of the gastrointestinal tract, such as Peyer's patches (Cornelis, 2002a; Cornelis, 2002b). *Yersinia* has developed two strategies during host cell interaction to modulate the host cell actin cytoskeleton, namely invasion of non-phagocytotic epithelial cells and inhibition of phagocytosis into macrophages and PMNs.

The *Yersinia* outer membrane protein invasin is an adhesin and is chromosomally encoded by the *inv* locus (Isberg et al., 1987). Invasin binds to the heterodimeric transmembrane receptor β1-integrin (Isberg and Leong, 1990). Upon binding, β1-integrins oligomerize into clusters to initiate Cdc42-controlled downstream signalling promoting bacterial entry into specialized intestinal cells, termed M cells, at the phagocytic cup (McGee et al., 2001; Wiedemann et al., 2001). Invasin/β1-integrin interaction is highly similar to the interaction of Intimin/Tir in enteropathogenic *E. coli* which directs actin pedestal formation on host epithelial cells at sites of bacterial attachment (Kenny et al., 1997).

Inhibition of phagocytosis into macrophages and PMNs by *Yersinia* is another example of subversion of the host cell actin cytoskeleton. For that purpose *Yersinia* engages a subset of Ysc T3SS-translocated effector proteins (Michiels and Cornelis, 1991; Michiels et al., 1990), termed YopE, YopT, YopO/YpkA and YopH that are injected intracellularly into phagocytes and paralyze them by inactivation of components of the actin cytoskeleton (see Figure 12). The first three effectors target Rho-family small GTPases and YopH acts as a powerful tyrosine phosphatase.

(1) The effector protein YopE is translocated into host cells by the Ysc T3SS injectisome (Sory et al., 1995; Sory and Cornelis, 1994). YopE is a single-domain protein and functions as a GTPase-activating protein (GAP) (Black and Bliska, 2000; Von Pawel-Rammingen et al., 2000) for the small GTPases RhoA, Rac1 and Cdc42 by direct binding and promoting efficient GTP hydrolysis (Andor et al., 2001). YopE-induced disintegration of the actin cytoskeleton causes cell rounding and detachment of infected cells, suggesting cytotoxic effects (Von Pawel-Rammingen et al., 2000).

(2) YopT is a cysteine protease that inactivates RhoA, Rac1 and Cdc42 (Aepfelbacher et al., 2003; Iriarte and Cornelis, 1998; Zumbihl et al., 1999). YopT cleaves small GTPases at a specific C-terminal cysteine which lies in a conserved amino acid motif, termed CAAX (Shao et al., 2002; Shao et al., 2003). This tetrapeptid motif is the site of isoprenylation of Rho-family GTPases which is essential for membrane association. Thus YopT causes membrane release of Rho-family GTPases Rho, Rac and Cdc42 (Sorg et al., 2001) and blocks interaction with GDIs (Zumbihl et al., 1999). The cellular consequence of YopT action is the disruption of actin filaments in professional phagocytes in order to prevent uptake of *Yersinia*, a process termed antiphagocytosis (Grosdent et al., 2002).

(3) The YopO/YpkA is a serine/theronine kinase that becomes autophosphorylated upon contact with F-actin, RhoA and Rac1 (Barz et al., 2000). YopO binds to GDP- and GTP-bound forms of RhoA and Rac1 with similar affinity, but this interaction does not affect GDP/GTP exchange by the GTPase and is independent from the YopO kinase activity. The target of the N-terminal kinase domain remains to be elucidated. During infections, YopO disrupts the actin cytoskeleton of cultured cells and leads to the complete loss of actin stress fibers (Nejedlik et al., 2004).

(4) YopH, the most powerful phosphotyrosine phosphatases (PTPase) known (Green et al., 1995; Persson et al., 1995), is targeted to focal adhesions and to other protein complexes where it dephosphorylates proteins such as the tyrosine kinase FAK, the adaptor protein $p130^{Cas}$ (Black and Bliska, 1997; Persson et al., 1997), the scaffolding protein SKAP-HOM (Black et al., 2000) and Fyn kinase binding protein Fyb (Hamid et al., 1999) which are engaged in the assembly of focal adhesions.

Yersinia lacking one of these four Yops exhibit stronger phagocytosis by PMNs and macrophages, indicating that there is no redundancy between these Yops but rather synergy in establishing the antiphagocytic phenotype (Grosdent et al., 2002).

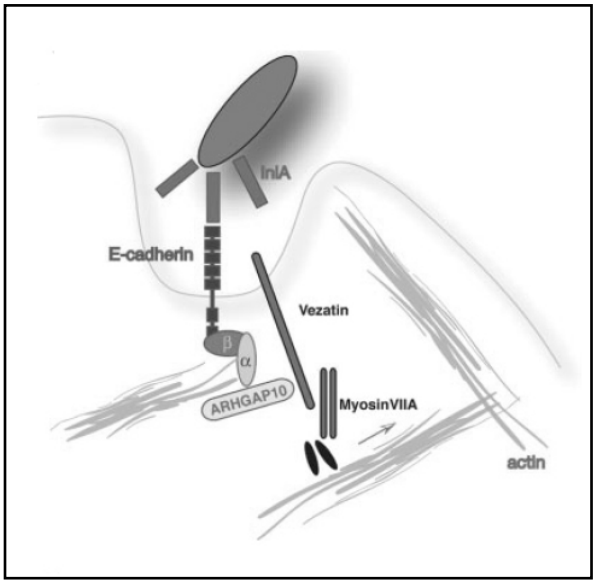
1.4.4 *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive, opportunistic rod with peritrichous flagella and the etiological agent of listeriolosis, a clinical condition which may lead to severe gastroenteritis in immuno competent individuals and to sepsis or meningoencephalitis in infants, elderly or immuno compromised individuals.

Subversion of the host cell actin cytoskeleton by *L. monocytogenes* serves two purposes, namely uptake into non-phagocytic cells and intracellular movement and cell-to-cell spread (Cossart et al., 2003; Pizarro-Cerda and Cossart, 2006b). Invasion of polarized epithelial cells by *L. monocytogenes* involves distinct changes in the host cell actin cytoskeleton by two alternative pathways, which are dependent on two bacterial surface proteins, internalin A (InlA) and internalin B (InlB) respectively (Cossart et al., 2003).

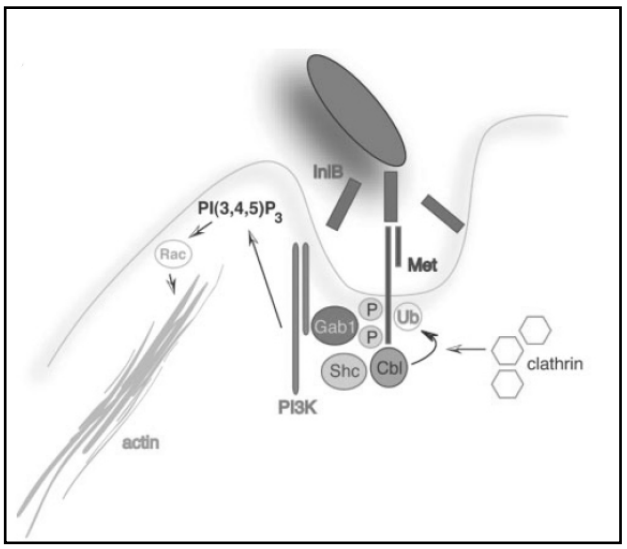
Key steps of the InlA-invasion pathway (Gaillard et al., 1991; Mengaud et al., 1996) are (1) the interaction of InlA with its host cell surface receptor E-cadherin, (2) recruitment of α-and β-catenins, which modulate and stabilize anchoring of the cortical actin cytoskeleton, (3) recruitment of the Rho GAP ARHGAP10, which, in turn, recruits vezatin and the unconventional myosin VIIA (Sousa et al., 2005) (see Figure 8).

Figure 8
InternalinA-dependent host cell entry of *Listeria monocytogenes*
(Pizarro-Cerdo J. and Cossart P., Journal of Pathology, 2006)



Key steps of the InlB-invasion pathway (Dramsi et al., 1995) are (1) the interaction of InlB with its host cell surface receptor, the hepatocyte growth factor receptor, Met (Shen et al., 2000), leading to receptor dimerization and autophosphorylation, (2) recruitment of several kinases such as Shc, Gab1 and Cbl (Ireton et al., 1999), (3) recruitment of type IA PI3Kto the plasma membrane (Ireton et al., 1996), (4) generation of phosphatidylinositol 3,4,5-trisphosphate, (5) subsequent activation of Rac1 leading to (6) the activation of WASP-related proteins, Abi1 and Ena/VASP (Bierne et al., 2005) involved in Arp2/3 complex-mediated actin polymerization (Bierne et al., 2001) (see Figure 9).

Figure 9
InternalinB-dependent host cell entry of *Listeria monocytogenes*
(Pizarro-Cerdo J. and Cossart P., Journal of Pathology, 2006)



Thus, *L. monocytogenes* uptake redirects actin cytoskeletal functions of the host cell to promote entry at sites of bacterial attachment by exploiting the cellular machinery of adherens junctions (InlA-dependent invasion) (Sousa et al., 2005) and receptor ubiquitination and endocytosis (InlB-dependent invasion) (Veiga and Cossart, 2005).

After escape from the membrane-bound vacuole by the action of listerolysin O (Mengaud et al., 1987), *L. monocytogenes* subverts the actin cytoskeletal machinery from within the infected cells to promote its own spread by actin-based motility. ActA (Kocks et al., 1992) is a non-covalently attached cell wall protein, which functions in (1) assembly of a scaffold for actin polymerization by recruitment of the adaptor protein Ena/VASP to one cell pole, (Laurent et al., 1999) (2) subsequent recruitment of the actin monomer-binding protein profilin (Grenklo et al., 2003) and the Arp2/3 complex (Welch et al., 1997), (3) initiation of actin polymerization and assembly into parallel actin filaments (David et al., 1998), and finally the (4) propulsion through the cytoplasm driven by a characteristic "actin comet tail" (see Figure 10).

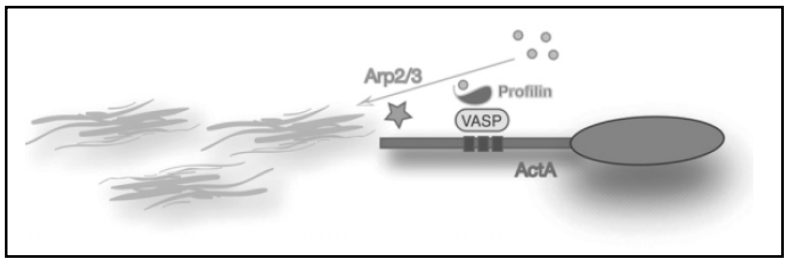


Figure 10
Actin-based motility of *Listeria monocytogenes* (Pizarro-Cerdo J. and Cossart P., Journal of Pathology, 2006)

Thus, *L. monocytogenes* harnesses the machinery that controls cellular actin nucleation under normal physiological conditions such as cell migration or pseudopod extension for the purpose of actin-based motility. Intracellular propulsion by "actin comet tails" can be found in other bacteria such as *Shigella* and *Rickettsia* (Gouin et al., 1999). These intracellular pathogens encode proteins that are functionally similar to ActA, namely IcsA/VirG (Goldberg and Theriot, 1995) and RickA (Gouin et al., 2004), respectively.

1.4.5 *Helicobacter pylori*

Helicobacter pylori is a gram-negative microaerophilic rod with lophotrichous flagella. *H. pylori* is the etiological agent of chronic gastritis, gastric ulcers and adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. *H. pylori* invades cultured gastric epithelial cells (AGS cells) (Segal et al., 1996) by a zipper-like mechanism (Kwok et al., 2002), which comprises receptor-mediated internalization by β1-integrins (Segal et al., 1996; Su et al., 1999). Internalisation coincides with *cag* T4SS-dependent translocation of the effector protein CagA into the host cell cytoplasm where it is tyrosine-phosphorylated (Odenbreit et al., 2000; Stein et al., 2000) by the Src family kinases, c-Src and Lyn (Stein et al., 2002).

Invasion of gastric epithelial cells has two outcomes that involve modulation of the host cell actin cytoskeleton, namely (1) stimulation of cellular motility (Churin et al., 2003) and (2) host cell elongation (Higashi et al., 2004; Moese et al., 2004), which collectively result in the induction of the hummingbird phenotype (Segal et al., 1999). CagA interacts with the receptor tyrosine kinase c-Met/HGF in a phospholipase C γ-dependent manner enhancing the motogenic response of AGS cells upon *H. pylori* infection (Churin et al., 2003). Upon activation, PLC γ cleaves its membrane-bound substrate phosphatidylinositolbisphosphate (PIP_2). PIP_2 release stimulates actin-modifiying proteins such as gelsolin, profiling and cofilin, which interact with the cortical actin cytoskeleton to sustain cell motility (Chen et al., 1996). CagA promotes host cell elongation by interaction with a series of host cell proteins to form long actin-rich cell protrusions (Brandt et al., 2005; Higashi et al., 2002; Suzuki et al., 2005). Three pathways are known to participate in this process.

(1) CagA is phosphorylated by the tyrosine kinase c-Src, which, in turn, is inactivated by CagA by a negative feedback loop. c-Src inactivation leads to a dramatic decrease in tyrosine phosphorylation of the c-Src substrates ezrin (Selbach et al., 2004) and cortactin (Selbach et al., 2003). Dephosphorylated cortactin has enhanced actin cross-linking and nucleation activity, thereby modulating actin dynamics and thus contributing to host cell elongation.

(2) CagA stimulates the activation of the small GTPases Rac1 and Cdc42 leading to the subsequent activation of the downstream effector kinase PAK1 (p21-activated kinase) that promotes cytoskeletal changes culminating in host cell elongation (Churin et al., 2001).

(3) Finally, phosphorylation-dependent interaction of CagA with the tyrosine kinase Crk results in various cellular changes, one of which is WAVE-dependent and WASP-independent induction of actin nucleation as observed in actin-rich cell protrusions (Suzuki et al., 2005).

1.4.6 *Bartonella spp.*

B. bacilliformis, *B. quintana* and *B. henselae* are known to be able to induce vasoproliferative lesions in humans as a result of intimate interaction with ECs. All of these species are competent for adhesion to and invasion of HUVECs by an actin-dependent process. Bacterial internalization is reminiscent of conventional phagocytosis. Within few hours, single bacteria adhering to the host cell membrane are engulfed by membrane protrusions in a phagocytic cup and are internalized subsequently. This results in the establishment of perinuclearly located *Bartonella*-containing vacuoles (BCVs) whose nature is poorly understood (Dehio et al., 1997).

Cytoskeletal remodelling during internalisation of single bacilli has been studied at the example of *B. bacilliformis* invading ECs. Invasion of ECs by *B. bacilliformis* is dependent on the Rho-family small GTPases RhoA, Rac1 and Cdc42. Pretreament of HUVECs with C3 exoenzyme, which inactivates small GTPases, blocked bacterial internalization (Verma et al., 2000). RhoA is activated during *B. bacilliformis* invasion of ECs leading to (1) the formation of actin stress fibres orientated in parallel to the long axis of the cells and (2) the establishment of an increased number of focal adhesion contacts, which coincides with reduced cell motility (Verma et al., 2001). Furthermore, *B. bacilliformis* invasion of ECs leads to the formation of membrane ruffles and lamellipodia. Rac1 and Cdc42 are activated and recruited to the plasma membrane to sites of bacterial entry. Inhibiton of Rac1 and Cdc42 function by toxin treatment significantly reduces invasion frequencies of *B. bacilliformis* (Verma and Ihler, 2002).

Uptake of *B. henselae* into ECs induces re-organization of the host cell plasma membrane and re-arrangement of the actin cytoskeleton, resulting in the internalization of single bacilli as aforementioned and in addition uptake of bacterial aggregates, by a unique invasive structure, termed invasome (Dehio et al., 1997).

The process of invasome formation is accompanied by massive rearrangements of the underlying F-actin cytoskeleton and can be inhibited by the use of the drug cytochalasin D (Wakatsuki et al., 2001). Invasome formation of *B. henselae* into primary human umbilical vein endothelial cells (HUVECs) and into the hybridoma cell line Eahy.926 cells (fusion of HUVECs and A549 lung carcinoma cells) has been shown. Typically, an invasome comprises a ring-like basal part of twisted F-actin stress fibres anchored by focal adhesion plaques. The bacterial aggregate resides on top of this structure engulfed by membrane protrusions, which are enriched for cortical F-actin, intercellular adhesion molecule-1, and phosphotyrosines (Dehio et al., 1997) (see Figure 11).

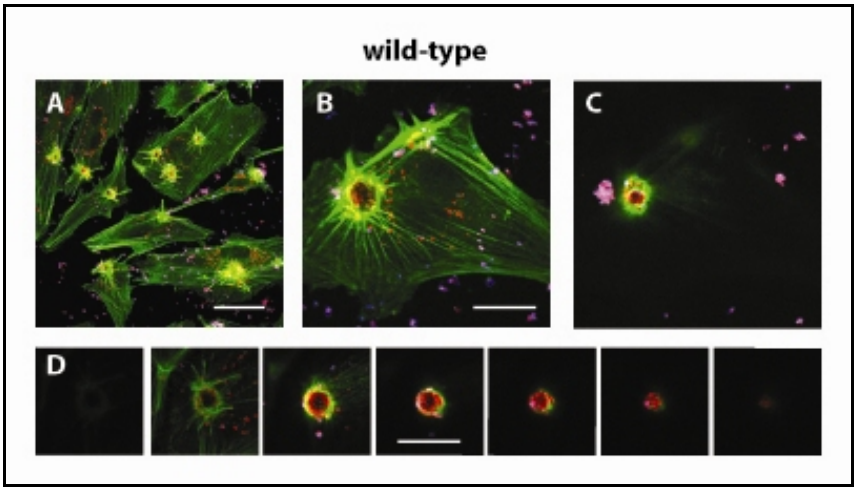


Figure 11

Invasome Formation as seen by confocal laser scanning microscopy. Endothelial cells were infected with *B. henselae* wild-type for 48 hours (MOI=100). (A) bottom view, 40x magnification; (B) bottom view, 126x magnification; (C) top view, 126x magnification; (D) serial cross sections from bottom to top. Scale (20μm).

Invasome formation requires 24 hours for completion and represents a three-step process, which is characterized by initial adherence and accumulation of dozens of bacteria on the cell surface leading to (i) aggregation, followed by (ii) engulfment and (iii) internalization of the bacterial aggregate by membrane protrusions. The relevance of invasome-mediated uptake *in vivo* remains to be demonstrated.

4. SUMMARY

4. SUMMARY

The facultative intracellular bacterium *Bartonella henselae* enters human endothelial cells either passively by conventional phagocytosis or actively by a pathogen-triggered process known as invasome-mediated internalization. The latter involves the formation of a cell-surface-associated bacterial aggregate, which is subsequently engulfed by host cell membranes eventually resulting in its complete internalization.

Recent work indicated that invasome formation of *B. henselae* depends on its VirB/VirD4 T4SS.

This work describes that the VirB/VirD4 T4SS of *B. henselae* injects a cocktail of seven effector proteins into endothelial host cells to subvert cellular functions and that one of these translocated effector proteins, BepG, mediates the sustained cytoskeletal changes leading to invasome formation. Moreover, this work indicates the existence of two non-redundant pathways to promote invasome formation, one of which is governed by the action of BepG and another one, involving further Bep proteins, which is BepG-independent.

On the host cell side, Rho-family small GTPases Cdc42 and Rac1, but not RhoA are shown to be required for invasome-mediated internalization. Furthermore, it is shown that invasome formation leads both to the rearrangement of pre-existing F-actin fibers and to localized actin polymerization enriched for Arp2/3, which occurs in a Scar1/WAVE-dependent manner. Finally, this work provides evidence that after complete internalization the invasome membranes do not fuse with Lamp-1 positive lysosomes, indicating that invasome-mediated invasion represents a novel mechanism allowing the uptake of bacteria without entering the endocytic-lysosomal pathway.

Importantly, this is the first report, which attributes the orchestrated action of more than one effector protein of *B. henselae* to a known VirB/VirD4 T4SS-dependent phenotype, namely invasome formation, which represents a multifacetted example for the complexity of host cell subversion by a bacterial pathogen.