Molecular dialogue in host-parasite interaction

18 and 19 November 2010

House of the Academies
Hertogsstraat 1, Rue Ducale
1000 • Brussels
PROGRAMME

Molecular dialogue in host-parasite interaction
November 18th - 19th 2010
Academies’ Palace, Hertogsstraat 1, Rue Ducale – 1000 Brussels

Program - Thursday November 18th

08h30  Registration desk open – Poster installation
09h00  Welcome & Opening
Chairperson: Fred Opperdoes
09u15  Etienne Pays, Belgium. Human innate resistance against African trypanosomes
10h00  Gregory A. Smith, US. Break ins and break outs: viral interactions with the cytoskeleton of mammalian cells
10h45  Break
Chairperson: Paul De Vos
11h15  Markus Engstler, Germany. How trypanosomes survive in blood
12h00  Guy R. Cornelis, Switzerland. The type III secretion injectisome: a complex nanomachine to subvert host cells
12h45  General Assembly Belgian Society Microbiology
13h00  Lunch – Poster viewing
## Parallel Sessions:

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<td>14h40</td>
<td>Jean-Jacques Letesson, Belgium. Brucellae and Their Success as Pathogens</td>
<td>Bruno Verhasselt, Belgium. Crosstalk between HIV and T cells, a molecular dialogue</td>
<td>Mike Turner, UK. Genetic variation and pathogenesis in African trypanosomiasis</td>
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<td>15h20</td>
<td>Short lecture of selected abstract</td>
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<td>14h30: Julius Lukes, Czech Republic. RNA editing in trypanosomes – ever growing complexity</td>
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<td>15h35</td>
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<td>15h00: Frédéric Bringaud, France. The essential role of acetate metabolism in procyclic trypanosomes</td>
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<td>16u00: Achim Schnaufer, UK. Dyskinetoplastic trypanosomes – how to put tsetse flies and a couple hundred mitochondrial proteins out of business</td>
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<td>16h30: Philippe Bastin, France. Amplification and specification of gene families associated to flagellar functions in <em>Trypanosoma brucei</em></td>
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17h00 Discussion and conclusion day 1
Program - Friday November 19th

08h30     Registration desk open – Poster installation
09h00     Welcome & Opening
           Chairperson: Tom Coenye
09u15     Andrea Genre, Italy. Molecular signaling and cellular responses controlling root colonization by arbuscular mycorrhizal fungi
10h00     Keith Matthews, UK. Measuring the within-host dynamics of the transmission stage of *Trypanosoma brucei* during chronic infections
10h45     Break
           Chairperson: Etienne Pays
11h15     Mark Marsh, UK. HIV assembly – can fidelity impact on pathogenesis?
12h00     Jeremy Mottram, UK. Autophagy and differentiation in trypanosomatids
12h45     Lunch – Poster viewing
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<th>Bacteriology: Chairperson: Jozef Anné</th>
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<td>14h40</td>
<td>Fabrice Vavre, France. Symbiosis in insects: the other faces of host-bacteria interactions</td>
<td>Graciela Andrei, Belgium. Papilloma virus as causative agent for cervix carcinoma</td>
<td>Dave Barry, UK. Antigenic variation in African trypanosomes</td>
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<td>14h30</td>
<td>John Mansfield, US. Regulation of the protective T cell response in trypanosomiasis</td>
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<td>15h00: Jeremy Sternberg, UK. Cytokine expression in human African trypanosomiasis</td>
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<td>Mike Lehane, UK. Tsetse serine protease inhibitors (SERPINS) protect African trypanosomes from the action of serum complement</td>
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<td>Jan Van Den Abbeele, Belgium. Tsetse fly saliva and trypanosome transmission</td>
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<td>17h00</td>
<td>Discussion and conclusion day 2 - Distribution of selected poster awards</td>
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ABSTRACTS OF INVITED LECTURES
Papilloma virus as causative agent for cervix carcinoma

Graciela Andrei

Rega Institute for Medical Research and University Hospitals Leuven, Leuven

Abstract not received
Antigenic variation in African trypanosomes

J. D. Barry, J. Hall, L. Plenderleith, B. Marchetti, M. Swiderski,
C. Tiengwe, L. Marcello, R. Mcculloch.

Wellcome Trust Centre for Molecular Parasitology, University of Glasgow, Scotland, UK.

Trypanosomes are coated densely with variant surface glycoprotein (VSG), which generally is thought to prevent immune responses accessing invariant antigens. Antigenic variation is a population process in which individual trypanosomes switch from one VSG to another, evading antibodies against the first VSG and proliferating. Antigenic variation is a templated system, with each VSG encoded in an archive of hundreds or thousands of silent VSG genes and pseudogenes. As segments of pseudogenes can combinatorially yield intact, expressed mosaic VSGs, we have proposed that the scale of antigenic variation is inestimably enormous, possibly allowing reinfection of already immune mammalian hosts (Marcello and Barry 2007a,b). The VSG archive occupies chromosome subtelomeres and is extremely divergent in sequence. The trypanosome therefore is a strong example of diversification of multigene families in the subtelomere compartment of the eukaryote genome.

There are few studies of antigenic variation beyond the first week of infection. We have set out to characterize later stages, and have found that, in mice, mosaic VSGs appear with high frequency and follow patterns driven by template homology. It is likely that, in more realistic host models, mosaic VSGs will make major contribution to chronic infection.

To dissect the patterns and tempo of archive diversification, we are comparing the genomes of two isolates of the same strain, collected 17 years apart in the field. Data show how the archive hyperevolves, via several types of mutation. Present studies aim to test the relationship of sequence diversity to antigenic individuality of living trypanosomes.

Mechanisms contributing to the evolution and maintenance of the VSG archive are likely to include DNA recombination, repair and replication activities, as well as silencers. Ongoing studies aim to identify such molecules.

This work was financially supported by the Wellcome Trust.


Acetyl-CoA produced in mitochondria from carbohydrate or amino acid catabolism needs to reach the cytosol to initiate de novo synthesis of fatty acids. All eukaryotes analyzed so far use the citrate/malate shuttle to transfer acetyl group equivalents from the mitochondrial matrix to the cytosol. We have investigated how this acetyl group transfer occurs in the procyclic life cycle stage of *Trypanosoma brucei*. Deletion of the potential citrate lyase gene, a critical cytosolic enzyme of the citrate/malate shuttle, has no effect on de novo biosynthesis of fatty acids and sterols from $^{14}$C-labeled glucose, indicating that another route is used for acetyl group transfer. Since acetate is produced from acetyl-CoA in the mitochondrion of this parasite, we considered genes encoding cytosolic enzymes producing acetyl-CoA from acetate. We identified an acetyl-CoA synthetase gene encoding a cytosolic enzyme (AceCS), which is essential for cell viability of the procyclic trypanosomes. Interestingly, induction of RNAi against AceCS results in a 10- to 20-fold reduction of $^{14}$C-radiolabeled glucose or acetate incorporation in de novo synthesized fatty acids or sterols. We have demonstrated that the essential cytosolic enzyme AceCS of *T. brucei* is responsible for activation of acetate into acetyl-CoA to feed de novo biosynthesis of lipids. Therefore, we identify the procyclic form of *T. brucei* as the first eukaryotic organism where acetate instead of citrate is used to transfer acetyl groups from the mitochondria to the cytosol.
The type-III secretion injectisome:

a complex nanomachine to subvert host cells

Guy R. Cornelis

Infection Biology, Biozentrum, Universität Basel, Switzerland

The type III secretion injectisome is a nanosyringe that injects bacterial effector proteins straight into the cytosol of eukaryotic cells. It is evolutionary related to the flagellum, with which it shares structural and functional similarities. It consists of a basal body made of several rings spanning the two bacterial membranes and the peptidoglycan, five essential integral membrane proteins, which are believed to recognize export substrates and form the export channel across the inner membrane and an ATPase related to the F0 ATPase. On top of the basal body, comes a short stiff needle terminated with a tip structure. Upon contact with a target cell, a translocation pore is build around the needle tip.

The assembly of the basal body was investigated by grafting fluorescent proteins onto four structural components. The recombinant injectisomes were functional and appeared as fluorescent spots at the cell periphery. Epistasis experiments with the hybrid alleles in an array of injectisome mutants revealed an outside-in assembly order: the assembly starts with outer membrane ring and proceeds via a connector YscD to the plasma membrane ring. Completion of the two membrane rings allows the subsequent assembly of cytosolic components.

After completion of the basal body, the export machine pursues the assembly by sequentially exporting the needle and needle-tip subunits. Needle elongation is controlled by protein YscP. There is a linear correlation between the length of YscP and the length of the needle, suggesting that YscP acts as a molecular ruler. To address the question whether one molecule of YscP suffices to control the length of one needle, strains expressing simultaneously a short and a long version of YscP were engineered. The experimentally obtained needle length distribution was compared to the distributions predicted by stochastic modeling of the various possible scenarios. The experimental data are compatible with the single ruler model.
How trypanosomes survive in blood.

Markus Engstler

Biozentrum of the University of Würzburg, Germany

"Blood is an extraordinary fluid: it is highly viscous, contains a dense packaging of cells and is constantly flowing at velocities varying over three orders of magnitude. Consequently, only few pathogens brave the harsh physical conditions within the vertebrate bloodstream and can prosper despite being constantly attacked by host antibodies. African trypanosomes are strictly extracellular blood parasites, which escape immune destruction through a system of antigenic variation, plasma membrane recycling and incessant, directional motility. How the flagellates actually move in blood remains to be elucidated. We provide evidence that the mechanics of trypanosome locomotion is perfectly adapted to life within a crowded environment."
Molecular signaling and cellular responses controlling root colonization by arbuscular mycorrhizal fungi

Andrea Genre and Paola Bonfante

University of Torino, Dept. of Plant Biology, Torino, Italy.

Arbuscular mycorrhizas (AM) are symbiotic associations between 90% of land plants and obligate fungal symbionts belonging to Glomeromycota (Schussler et al., 2001). AM fungi improve plant nutrient uptake and resistance against pathogens by colonizing the root through intra/intercellular hyphal development and the formation of arbuscules, the highly branched structures that mediate nutrient exchange (Parniske, 2008). The presence of a symbiotic interface compartment around the intracellular fungal structures is a landmark of AM establishment and allows fungal development inside the cell lumen whilst maintaining host cell integrity (Bonfante, 2001).

This presentation focuses on the events associated with the perception of the AM fungus and its accommodation inside the lumen of the host plant cell. Our findings, based on an in vivo confocal microscopy approach, demonstrate that root cells perceive AM fungal signals and trigger calcium-mediated signaling in their nucleoplasm, both before and upon direct contact with the fungus (Chabaud et al., 2010). Such calcium signals position within the so-called SYM pathway, the signaling pathway that controls AM establishment. Nuclear calcium spiking is a pre-requisite to the cellular reorganization that initiates the process of interface construction. This starts after the adhesion of the fungal hyphopodium to the root and leads to the assembly of the so-called prepenetration apparatus (PPA) inside one or a few contacted epidermal cells (Genre et al., 2005). The PPA is a columnar aggregation of cytoplasm containing all the elements of the secretory pathway. The exploitation of a range of fluorescent protein markers indicates that host plasma membrane proliferation takes place within the PPA. This leads to the assembly of the perifungal membrane and symbiotic interface in advance of hyphal tip growth.

References


Amplification and specification of gene families associated to flagellar functions in *Trypanosoma brucei*

*Daria Julkowska, Ines Subota, Johanna Buisson, Thierry Blisnick, Brice Rotureau and Philippe Bastin*

*Trypanosome Cell Biology, Institut Pasteur & CNRS, Paris, France.*

*Trypanosoma brucei* is a flagellated protozoan parasite responsible for sleeping sickness in Africa. During its complex life cycle the parasite has to deal with different environmental changes in both its mammal (human, animal) and its invertebrate (tsetse fly) hosts. It is now well established that flagella (cilia) play sensory roles in different organisms. Proteins involved in signalling often localize in the membrane or matrix compartments. Regarding the course of its cycle, we proposed that the trypanosome flagellum could be involved in sensory and signalling functions. The aim of our study was the purification of intact flagella from *T. brucei* in order to analyze matrix and membrane fractions and identify proteins of potential sensing function.

The trypanosome flagellum is attached along the length of the cell body via the flagellum attachment zone (FAZ). It was shown that the flagellum adhesion glycoprotein 1 (FLA1) plays a critical role in flagellum attachment. Since FLA1<sup>RNAi</sup> mutant detaches flagellum from the cell body, we decided to use this cell line to mechanically shear flagella and purify them in their intact form. The yield and purity were verified by light, scanning and transmission electron microscopy. Skeletal, membrane and matrix components were separated and protein identification was performed using mass spectrometry. Among different matrix and membrane proteins identified, about twenty could be involved in the life cycle. Identified genes encode proteins involved in metabolism, exchange with the environment, calcium binding or differentiation. Using immunofluorescence and western blotting assays on purified flagella fractions, we confirmed flagellar localization for several interesting candidates. Strikingly, detailed genomic analysis revealed that these proteins derive from duplicated genes that differ by their N- or C-terminal extensions. Peptides found during proteomic analysis and western blotting investigation demonstrated that the flagellar isoform is coming from a single gene. Moreover, in all cases examined so far, this gene was exclusively present in the *T. brucei* subgroup and not in other trypanosome subspecies raising issue of a specific amplification and gene specialization for flagellum function in this group of parasites. It is known that *T. brucei*, compared to other subspecies, undergoes the most complex cycle in its fly vector. This journey requires specific adaptation of the parasite to changing environment. Functional *in vitro* and *in vivo* investigation is now in progress to evaluate further the significance of this specific gene amplification in trypanosomes.
African trypanosomes alternate their life cycles between a vertebrate host and the tsetse. When an infected bloodmeal is ingested by the fly, bloodstream form (BSF) trypanosomes of *Trypanosoma brucei brucei* quickly transform into procyclics (PCF) within the midgut. While BSF survive in the mammal using antigenic variation and are resistant to serum complement (SC), PCF are very susceptible to the action of SC. This observation is puzzling considering that tsetse flies feed every two days and therefore, procyclic trypanosomes should be in close contact with a fresh source of SC after every bloodmeal. Thus, African trypanosomes must have developed ways to avoid the lethal effect of SC in order to establish a midgut infection. Here we show that procyclic form *T. b. brucei* exploit a series of tsetse fly Serine Protease Inhibitors (SERPINS) to escape vertebrate SC and that this process may explain the complex migratory pattern shown by procyclics in the tsetse midgut.

*In vitro* assays showed that both cultured and gut extracted procyclcics display a similar pattern of mortality when exposed to SC, which is reverted in the presence of cobra venom factor (CVF), an SC inhibitor. Interestingly, susceptibility to killing by SC occurs rapidly (within 1 h) upon transformation from BSF into PCF. RNAi knockdown experiments of several *Glossina m. morsitans* serpin-like genes resulted in a decrease in the percentage of infected midguts. This effect was by-passed when knockdown flies were fed with bloodmeals spiked with CVF. Moreover, recombinant tsetse serpins protect procyclic trypanosomes from SC both *in vitro* and when added to infected bloodmeals. Taken together, these experiments suggest that midgut serpins are important in modulating the infection of trypanosomes in tsetse flies by mitigating effects of SC.
Brucellae: their success as pathogens

J.J. Letesson

URBM, University of Namur, Namur, Belgium

*Brucella* is a facultative intracellular bacteria responsible for brucellosis, a worldwide zoonosis that remains of public health concern in endemic countries. Even if we lack informations about the infectious cycle within its natural mammalian host, it is commonly assumed that *Brucella* spends most of its time inside phagocytic cells. Its impressive ability to subvert the intracellular trafficking pathway to promote its survival and replication in those cells and its ability to modulate the innate immune response are the keystones of its chronicity. The virulence of *Brucella* depends on Quorum Sensing regulatory system, based, as in many others gram-negative bacteria, on the production of *N*-acyl homoserine lactone (AHL) as a signal. The AHLs regulate the expression of both the T4SS (crucial for the vacuolar trafficking) and of the flagellum (crucial for modulating the innate immune system). During its entire intracellular life, *Brucella* is individually enclosed in a membrane-bound compartment. In this context, it seems likely that QS regulation is not a matter of social behavior, AHLs could rather monitor the confinement state, a situation in which a single bacteria can be the quorum.
RNA editing in trypanosomes – ever growing complexity

Julius Lukeš

Biology Centre, Institute of Parasitology, Czech Academy of Sciences and Faculty of Sciences, University of South Bohemia, České Budějovice (Budweis), Czech Republic

The mitochondrion of trypanosomes, leishmanias and related flagellates is a remarkable organelle that contains huge amount of circular DNA molecules intercatenated into a densely packed network. The mitochondrial RNA processing is also highly unusual, as the majority of mRNAs are extensively changed by RNA editing, which is the extensive insertions and less frequent deletions of uridines at multiple sites. Only after being edited, the mRNAs become translatable, making the editing machinery essential. Hundreds of different small guide RNA molecules are required for RNA editing and, as was shown only recently, a highly coordinated action of several large protein complexes is also needed for this process. Thus, several hundreds of different RNA molecules and close to a hundred of proteins are needed to perform a process, the only function of which seems to be correcting mistakes in DNA on the level of RNA. Our current understanding of this sophisticated process, unique for the parasite, will be discussed.
Regulation of the protective T cell response in trypanosomiasis

John Mansfield

Department of Bacteriology, University of Wisconsin-Madison, Madison, USA.

Helper T lymphocyte responses to the African trypanosome variant surface glycoprotein (VSG) play a central role in host resistance in trypanosomiasis by secreting interferon-gamma (IFN-γ). This cytokine activates macrophages to produce trypanocidal factors such as reactive nitrogen and oxygen species as well as TNFα that destroy the parasites in extravascular tissues. This presentation examines the early polarization of VSG-specific Th cells induced by the GPI residues of shed VSG, and examines mechanisms associated with GPI exposure that subsequently regulate the VSG-specific T cell response. Further, additional infection-associated events that impact on T cell activation, specificity and memory will be examined, including the ability of trypanosomes to alter their biological behavior and escape from IFN-γ dependent resistance mechanisms.
HIV assembly – can fidelity impact on pathogenesis?

Mark Marsh

Cell Biology Unit, University College London, UK

The cellular and molecular mechanisms that underlie human immunodeficiency virus (HIV) replication are becoming increasingly well understood, but major gaps in our knowledge remain. Macrophages are a major target for HIV infection in vivo and have been implicated in pathogenesis and as drug resistant reservoirs for virus. A significant feature of these cells, at least when cultured ex vivo, is their ability to form intracellular, sequestered plasma membrane domains that appear to be the principle sites of HIV assembly and a site for virus particle sequestration. These sites appear to provide a secluded environment where virion formation may be invisible to humoral immune responses. In addition, sequestration of infectious virions can allow virus release to be temporally and spatially regulated to facilitate efficient cell-to-cell transfer of virus through so-called virological synapses. The virus particles formed in these assembly compartments may be also biochemically different to virions generated at the surface of infected T cells, in that it is more infectious and potentially more difficult to neutralise with antibodies directed against the envelope protein (Env). Similar compartments have been described in HIV infected dendritic cells, where it has been observed that intracellularly sequestered internalised or newly synthesised viruses can be delivered to uninfected T cells through virological synapses.
Autophagy and differentiation in trypanosomatids

Jeremy C. Mottram¹ and Graham H. Coombs²

¹Wellcome Trust Centre for Molecular Parasitology, Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, G12 8TA, UK. ²Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow G4 0NR, UK

Autophagy is a catabolic process involving the degradation and recycling of a cell’s own constituents, through the action of lysosomal enzymes. Analysis of the *Leishmania major* and *Trypanosoma brucei* genomes reveals that these trypanosomatids contain many of the *ATG* genes demonstrated to be involved in autophagy in yeast and mammalian cells. These include the small ubiquitin-like modifiers *ATG8* and *ATG12* and we have used GFP-ATG8 and GFP-ATG12 fusion proteins as molecular markers for monitoring the pathway in *L. major* and *T. brucei*. In *Leishmania*, autophagosomes are most abundant during differentiation of the cell from multiplicative promastigotes to metacyclic promastigotes and from metacyclic promastigotes to amastigotes, suggesting an important role for autophagy in these remodelling processes. *Leishmania* apparently contains four types of ATG8, which are selectively cleaved by the two ATG4 cysteine peptidases of the parasite. Parasites lacking one ATG4 have a growth defect and fail to undergo metacyclogenesis. The data suggest that autophagy is required for efficient metacyclogenesis and transformation to amastigotes. Autophagy is induced when *Leishmania* is placed in starvation conditions, presumably as a survival mechanism. However, if this is unsuccessful *Leishmania* undergoes autophagic cell death. The importance of peptidases, lysosome function and autophagy in life-cycle progression in *Leishmania* and *T. brucei* will be discussed.
Human innate resistance against African trypanosomes.

Etienne Pays

Laboratoire de Parasitologie Moléculaire, ULB, Gosselies

Presumably due to their evolutionary origin in Africa, humans and some primates have acquired specific innate immunity against African trypanosomes. Two Trypanosoma brucei subspecies termed rhodesiense and gambiense have managed to resist this immunity, and this is why these trypanosomes cause human sleeping sickness. Through the identification of the protein that confers resistance to human serum in T. b. rhodesiense, we have discovered that apoL1 is the human serum protein responsible for killing trypanosomes. This protein is taken up in the parasite by different pathways, one of which involves a surface receptor for internalization of the haptoglobin-hemoglobin complex. We recently discovered that some apoL1 mutations allow this protein to bypass the resistance mechanism of T. b. rhodesiense. Interestingly, human individuals with recent African origin were found to bear such mutations while developing end-stage renal disease. Therefore, it appears that apoL1 mutants have been selected to afford protection of humans to T. b. rhodesiense, but the price to pay for this selection is chronic kidney disease.
Dyskinetoplastic trypanosomes – how to put tsetse flies and a couple hundred mitochondrial proteins out of business

Achim Schnaufer

University of Edinburgh, Scotland, UK

The defining feature of the order Kinetoplastida, to which the trypanosomatids belong, is the organization of mitochondrial DNA into a structure called kinetoplast. Nonetheless, there are cases where kinetoplast DNA (kDNA) has been lost, giving rise to dyskinetoplastic (dk) bloodstream forms. Examples are the naturally occurring (sub)species Trypanosoma brucei evansi and T. b. equiperdum. Transmission of the dk forms between mammals occurs mechanically: venereally in case of T. b. equiperdum and mostly via biting flies in case of T. b. evansi. This permits their wide geographical distribution, whereas T. b. brucei, dependent on cyclical development in the tsetse vector, is restricted to sub-Saharan Africa. Since replication and expression of kDNA are normally essential processes in T. brucei, the dk forms must have developed mechanisms to compensate for loss of the essential mitochondrial gene product(s).

To test the hypothesis that mutations in the nuclearly encoded gamma subunit of the mitochondrial ATP synthase complex are involved in these compensatory mechanisms, we have generated transgenic T. b. brucei bloodstream forms expressing various mutated versions of this gene. Evidence will be presented that a single amino acid change in this subunit is necessary and sufficient to permit survival of bloodstream form T. b. brucei in the absence of kDNA. Genetic and physiological implications of this finding will be discussed, as will be open questions regarding the evolutionary history of dk forms.
Several herpesviruses are neuroinvasive pathogens of humans and domesticated animals. A hallmark of these infections is the processive transport of viral particles in axons of the nervous system. The mechanism by which these viruses recruit cellular motor complexes to move long distances in axons and ultimately cause peripheral and central nervous system disease is unknown. Data will be presented to demonstrate that a large viral structural protein, VP1/2, is initially expressed in an inactive form, but when bound to the herpesvirus capsid recruits cellular motor complexes and is sufficient to mediate microtubule-dependent transport in cells. VP1/2-mediated recruitment of the dynein motor complex in particular is required for efficient retrograde axon transport and neuroinvasion in vivo.
Cytokine expression in human African trypanosomiasis.

Jeremy M Sternberg and V. Pius Alibu

Institute of Biological and Environmental Sciences,
University of Aberdeen, Aberdeen, UK

Experimental model studies reveal a complex interplay between the host-response, pathology, and parasite-control in African trypanosomiasis. Early control of acute infection depends on the expansion of classically activated macrophages while longer term survival and control of pathology requires a tipping of the balance towards counter-inflammatory regulation, this being mediated by alternatively activated macrophages and regulatory T-cells. We aim to determine if this is also the case in human African trypanosomiasis and whether changes in the host-response to genetically distinct parasites may account for the variation of virulence observed in the field. We analyse total and cell-specific cytokine expression profiles in the bloodstream and CSF to characterise the immune response in clinical infection with T.b.rhodesiense. The results provide new insights into mechanisms of virulence variation in natural infections and also offer opportunities to develop novel diagnostic tools.
The variation in pathology during trypanosome infections can be due to both host or pathogen. Whilst host genetic variation has been well studied, parasite genetic variation has not. We therefore analysed the pathology induced by infection of mice with two strains of *Trypanosoma brucei* and found that pathogenesis is partly strain-specific. Infections with one strain resulted in greater anaemia and erythropoietin production; infections of the other displayed greater splenomegaly and reticulocytosis. Expression microarray analysis of host genes showed that 40% of the most up- or down-regulated genes were specific to infections with one or other trypanosome strain. LXR/RXR signaling, IL010 signalling and alternative macrophage activation pathways were identified as being the most significantly differentially activated host processes. We then used forward genetics to identify parasite loci that determine this variation. A major quantitative trait locus (QTL) was identified on *T. brucei* chromosome 3 (LOD = 7.2) for splenomegaly and hepatomegaly. A second locus was identified that contributed to splenomegaly, hepatomegaly and reticulocytosis. These data show that the degree of pathogenesis is determined by the activation (or not) of specific host response pathways which is in turn driven by defined genetic loci in the parasites.
Tsetse fly saliva and trypanosome transmission

Jan Van Den Abbeele
Instituut voor Tropische Geneeskunde, Antwerpen

Trypanosoma brucei parasites, including the two human-pathogenic species causing African sleeping sickness, rely for their transmission on an obligatory, complex developmental cycle within the blood feeding tsetse fly (Glossina sp.). The fly salivary gland plays a key role as it is the biotope where the parasites adhere, multiply and undergo the final re-programming into the metacyclic stage. Only the latter trypanosome stage can initiate a new infection in the mammalian host which occurs through the co-injection of parasites with the tsetse saliva during the bite of the fly. Our basic knowledge on the molecular composition of the salivary gland micro-environment, on the biology of the specific trypanosome stages in this tissue and on the trypanosome/tsetse molecular interactions is still very limited and can be considered as a real blind spot in the understanding of the parasite life cycle. We will present an update of our current knowledge on the tsetse fly saliva proteins and how the trypanosome parasites modify this protein environment thereby favouring their spread within the mammalian host population.
All Eukaryotes interact with bacteria. While pathogenic interactions have attracted most of the research efforts for obvious reasons, most interactions are however commensal or even mutualistic. Understanding the functioning of non-pathogenic interactions may shed light on the evolution and mechanisms of host-bacteria associations, including interactions with the immune system. Insects are particularly interesting in this context since they are often associated with vertically transmitted (from mother to offspring) intracellular endosymbionts. The objectives of this talk will be to: (i) describe the diversity of interactions insects have with symbionts; (ii) review the current knowledge on the mechanisms underlying these interactions; and (iii) illustrate how the functioning and evolution of organisms can be better understood when individuals are viewed as community of interacting partners.
Viruses can only replicate in living cells. To achieve this, an intense crosstalk between viral and host proteins has evolved. Since the challenges are in common between viruses, analogue mechanisms were developed by different viral species (convergent evolution). From intensely studied viruses like HIV, considerable knowledge has been obtained in recent years. This virus, that infects CD4 positive cells like T cells subsets, encodes for both catalytic and non-catalytic proteins. However both types are known to interact with host proteins to function in the cell.

As an example, the HIV Nef protein functions as an adapter protein that associates with cellular host proteins. The cellular consequences are e.g. decrease CD4 and MHC-I surface expression levels. To identify the host proteins involved, a functional shRNA screening approach was applied. The host proteins identified illustrate that next to endocytic pathways, the Golgi-ER trafficking machinery is involved.

In line with previously published screens on host proteins essential for viral replication, our results suggest a complex interaction network used by an individual HIV protein to exert its function in the viral life cycle.
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Bacteriology
Metabolic adaptation of the intracellular pathogen *Brucella abortus* 2308.

T. BARBIER¹, C. NICOLAS¹, C.J. BOLLEN², L. PEYRIGA³, C. WITTMAN², J.-C. PORTAIS³ AND J.-J. LETESSON¹

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*Brucella* spp. are Gram negative coccobacilli, phylogenetically related to plant symbionts such as Rhizobiaceae. They are responsible for chronic infections of animals and humans. During their co-evolution with their host, *Brucella* adapted themselves to their preferential niches inside the organism and host cells. Despite the increasing knowledge on the molecular strategies used by this pathogen to perform its infectious cycle, basic knowledge on its metabolic capabilities, including its nutritional needs and how it adapts its metabolism to the intra-host conditions, are lacking to understand the pathogenesis. According to genomic data and biochemical information, *Brucella* disposes of a complete pentose-phosphate pathway and an incomplete Embden-Meyerhof-Parnas pathway, as it is lacking phosphofructokinase. Moreover, *Brucellae* have the two genes coding for the enzymes of the Entner-Doudoroff pathway (gluconate-6-phosphate dehydratase and 2-keto-3-deoxygluconate aldolase), but no in vitro activity was found for the first enzyme (ROBERTSON AND MCCULLOUGH, 1968). This is also true for several anaplerotic reactions.

This information is useful to define the potential “architecture” of the central metabolic network of *Brucellae*, but they do not provide any evidence neither on its functionality nor on its adaptation potential. A first objective was thus to assess which pathways are really active. To do so, the metabolome of *Brucella abortus* 2308 has been investigated after a growth on two distinct carbon sources: the erythritol (substrate assimilated in the central metabolism at the level of the trioses-phosphate) and the glutamic acid (substrate assimilated in the central metabolism at the level of the alpha-ketoglutarate). Most of the metabolites of the reconstructed central metabolic network have been detected suggesting an active status of the pathways. However no conclusion could be drawn for the Entner-Doudoroff pathway (gluconate-6-phosphate dehydratase and 2-keto-3-deoxygluconate aldolase), but no in vitro activity was found for the first enzyme (ROBERTSON AND MCCULLOUGH, 1968). This is also true for several anaplerotic reactions.

In parallel to these in vitro experiments, work has also been done in order to answer the question: « what are the carbon sources exploited when *Brucella* replicates into vacuoles within a eukaryotic cell? ». Up to now, first isotopic labeling experiments and GC/MS analyses of the amino acid labeling indicate that aspartate, serine, glutamate and alanine are provided by the host cell to the bacteria. However, a bacterial biosynthesis of alanine and glutamate seems to exist in parallel. Future work will allow the identification of other carbon sources provided by host cells to the bacteria.

Screening for leptospirosis in clinically suspected cases.

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Leptospirosis is a re-emerging zoonosis caused by a gram-negative aerobe spirochete of the genus Leptospira. The pathogenic species, who are (MAT-based) classified under L. interrogans sensu lato, can be ordered into 24 serogroups and into over 240 serovars. Humans and animals can be infected by contact with urine or with urine contaminated water. Rodents represent the main reservoir and are asymptomatic carriers, however, the disease was also well known as “milking fever”, indicating cattle as another reservoir. Dogs are mentioned as maintenance host for serogroup Canicola.

Infected animals often present renal and hepatic failure, anemia and reproductive disorders. Sometimes respiratory symptoms are seen, however, this is mainly in man. In the latter fever, headaches and rigidity are seen also. In severe cases, kidney and liver failure occurs as well.

In cats and dogs, leptospirosis may show itself as a mild case and the symptoms of leptospirosis will disappear, however, men, animals and environment may get contaminated. Leptospira can survive up till 4 years in the kidneys and are shed intermittently in the urine. Therefore we wanted to estimate the prevalence of Leptospira infections in dogs and cats, suspected of being infected based on biochemistry and hematology.

Serum samples of 95 dogs and 44 cats with liver and/or kidney failure were analyzed for antibodies against Leptospira using the Micro Agglutination Test. We tested against a panel of 12 serovars, with a threshold of 1/100. Student t and Wilcoxon tests were used to compare biochemical parameters results in cases (liver and/or kidney failure and positive for leptospirosis) to that in controls (liver and/or kidney failure and negative for leptospirosis).

Sera of 22 dogs (23.2%) and 1 cat (2.3%) were positive in the MAT-test. The dominant serogroup was Australis (40.7%). Antibody-titers higher or equal at 1/100 were also found for serogroups Autumnalis (3.7%), Ballum (3.7%), Canicola (7.4%), Grippotyphosa (11.1%), Icterohaemorrhagiae (7.4%), Javanica (7.4%), Manhao (3.7%) and Pomona (14.8%).

No significant differences (\(p<0.05\)) were found when comparing the hematological and biochemical results of the leptospirosis positive cases with the control group. Although, a substantial increase of leucocytes, urea, creatinine, TGO and TGP was noticed in the positive group.

In this study almost 1/5 of the Belgian dogs, presenting abnormal renal and/or hepatic parameters, showed antibodies against a Leptospira serovar not included in the current Belgian vaccines that contain only the serogroups Canicola and Icterohaemorrhagiae. As such the vaccine does not protect dogs against leptospirosis. Leptospirosis should therefore always be a part of the differential diagnose in animals with kidney and/or liver failure.

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Effect of quorum sensing inhibitors on antimicrobial susceptibility of bacterial biofilms
in vitro and in vivo

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Quorum sensing inhibitors (QSI) are considered promising anti-biofilm agents. However, little is known about the relationship between the anti-biofilm effect of QSI and the susceptibility towards antibiotics. These effects are often only investigated in microtiter plates, using high antibiotic concentrations and/or cytotoxic QSI. For this reason several QSI, targeting different bacterial QS systems, were evaluated for their effect on the antibiotic susceptibility of bacterial biofilms. Biofilms of B. cepacia complex bacteria, P. aeruginosa and S. aureus were formed on medical grade silicone disks, on RHE cells and in in vitro biofilm models mimicking chronic bacterial wound and soft tissue infections. The in vivo susceptibility was evaluated in the C. elegans and G. mellonella models.

Several QSI (e.g. hamamelitannin and cinnamaldehyde) affected the antibiotic susceptibility of preformed biofilms. QSI, added during biofilm formation, resulted in an increased antibiotic susceptibility of these biofilms. QSI alone or used in combination with antibiotics decreased bacterial virulence, while antibiotics alone often failed to increase C. elegans and G. mellonella survival after infection.

Our study provides evidence that a therapy with QSI alone or a combination of QSI and an antibiotic could increase the in vitro and in vivo susceptibility of sessile cells.
Q fever in Belgian cattle: Implication in abortions

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Coxiella burnetii, an obligate intracellular Gram-negative bacterium, is the causative agent of Q fever, a well known zoonosis. The clinical presentation of Q fever in most animals is non-specific, except in ruminants where Q fever is responsible for late term abortion, stillbirths and low birth weight. Domestic ruminants such as cattle, sheep and goats are identified as the major source of human Q fever cases. Most human infections occur due to inhalation of contaminated aerosols which are released during parturition but C. burnetii is also shed intermittently in milk, urine and feces. In humans, Q fever occurs in either an acute form (self-limiting febrile episode, pneumonia, hepatitis) or a severe chronic form (endocarditis).

C. burnetii is detected by means of PCR which is considered a useful test for screening large numbers and various types of samples.

The Federal Agency for the Safety of the Food Chain (FASFC) has implemented the “Abortion protocol” since December 2009. In this framework, 4495 bovine abortion samples were tested by real-time PCR, of which 388 were positive (8.6%), including 45 strong positive samples (1%). C. burnetii is an obligate intracellular pathogen and biochemical confirmation on isolated colonies is impossible. For determining the exact source of infections systematic genotyping is needed. Multiple Loci Variable Number of Tandem Repeats (VNTR) Analysis (MLVA) is a typing method (Arricau-Bouvery et al., 2006), which is gaining importance due to the availability of whole genome sequences (Seshadri et al., 2003). Advantages of this method are the high discriminatory power and the relatively low cost. The used MLVA is based on direct amplification of 13 specific loci. The PCR product sizes are estimated by electrophoresis and compared with the MLVA database (http://minisatellites.u-psud.fr/). Several samples from different locations in Belgium have been typed. The results show that all samples contain DNA of the same MLVA profile closely related to the French bovine strain CbB1.


Quantification of the spread of MRSA ST398 in piglets using transmission experiments

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Methicillin-resistant Staphylococcus aureus (MRSA) ST398 is worldwide colonizing and infecting humans and various animal species. Until now, the transmission characteristics of MRSA ST398 within and between species remain largely unknown. In this study we investigated the transmission of MRSA under experimental conditions among piglets during the nursery period.

Thirty-one 3-week-old MRSA negative piglets were randomly divided over three experimental groups (1-3) of eight animals and one control group of seven animals. After one week of acclimatisation, two piglets (seeders) from each experimental group were randomly selected for inoculation with MRSA strain C26 (spa-type t011, SCCmec-type V). Inoculation was done intranasally (both nares) and on the skin, with $2 \times 10^8$ CFU of the strain on each site. Two days post-inoculation (DPI) and after ensuring their MRSA positive status, the two seeders were returned in their pen. From all animals, swab samples were collected from the inoculation sites and the perineum every two days and this for a period of 6 weeks. Samples were inoculated in Muëller-Hinton Broth (Oxoid) supplemented with 6.5% NaCl and incubated aerobically for 24h at 37°C. Then, 1 µl of this broth was inoculated onto ChromID MRSA plates (BioMérieux) and incubated aerobically for 48 hours. Suspected colonies were purified on Columbia blood agar plates (BioRad), and thereafter, DNA was prepared for use in a multiplex PCR for identification of MRSA¹.

All piglets were negative for MRSA at the start of the trial. One day after introduction of the seeder pigs, all contact animals became positive for MRSA except for one piglet of group 2 that was positive one day later. During four weeks, all groups remained positive. However, in group 2, MRSA was detected only intermittently. From day 28 on, MRSA was detected only occasionally in group 1 and 2. In group 3, the prevalence also decreased 28 DPI but this restored and all animals were positive at the end of the trial. Using the maximum likelihood estimation of the basic reproduction ratio ($R_0$) the point estimate of the $R_0$ was $+\infty$ with the lower estimate of the 95% confidence interval of 2.15. Indicating that there is an efficient spread with an $R_0$ significantly larger then 1.

In conclusion, this study demonstrates that, under the presented experimental conditions, piglets colonized with MRSA will contaminate all pen mates very quickly in the first days of the nursery period. This model permits a better understanding of MRSA transmission and can be used for development of potential control measures of MRSA on pig farms.

Identification of Brucella melitensis proteins specifically interacting with human proteins involved in host vesicular trafficking

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Brucellae are facultative intracellular pathogenic bacteria able to hijack host cell vesicular trafficking to finally reach endoplasmic reticulum (ER), a compartment permissive for bacterial replication. Unlike other intracellular bacteria such as Legionella pneumophila or Salmonella spp., secreted effectors that may help to hijack vesicular trafficking are still unknown.

High-throughput yeast two hybrid system (Y2H) was applied between all predicted proteins from Brucella melitensis and human phagosomal proteins, Rab GTPases and ERES associated proteins. The specific interaction between human Rab2 and bacterial RicA (Rab2 interacting conserved protein A) was identified, as well as four other specific interactions between human and B. melitensis proteins. GST pull-down experiment confirmed this interaction and also suggested that RicA interacts preferentially with a GDP bound form of Rab2. We also showed that RicA is secreted in culture medium by Brucella abortus and this secretion is type IV secretion system independent. Furthermore, a ΔricA mutant proliferates faster inside HeLa cells and ΔricA containing vacuoles lose a lysosomal marker (LAMP1) faster than wild-type strain, suggesting that RicA function could be to slow down trafficking to its replication niche.

To the best of our knowledge, RicA could be the first reported effector of Brucella spp. involved in the control of vesicular trafficking. The four other potential effectors are currently under investigation.
Quorum sensing as an alternative biocontrol strategy for aquaculture: lessons learned from in vivo work with luminescent vibrios

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Vibrio harveyi and closely related species are important pathogens in aquaculture that can affect almost all types of cultured animals. The most important problems occur in shrimp larviculture, with mortalities as high as 100%. Due to large-scale use of antibiotics, many vibrios have acquired (multiple) resistance, which render antibiotic treatments ineffective. Therefore, alternative treatments to control aquaculture disease are urgently needed.

The disruption of quorum sensing, bacterial cell-to-cell communication, has recently been suggested as an alternative strategy to control infections caused by antibiotic-resistant bacteria in aquaculture. Quorum sensing has been shown to regulate virulence expression in many bacteria in vitro (i.e. in bacteria grown in synthetic growth media). However, microbiologists are becoming more and more aware of the fact that bacteria behave differently in different environments (VIRGIN, 2007). Hence, the question that arises is whether and how quorum sensing regulates virulence of pathogens where it really matters: in vivo during infection of a host.

In this presentation, we will discuss our current knowledge on the impact of quorum sensing and quorum sensing disruption on the virulence of Vibrio harveyi towards different host organisms in vivo. We found that quorum sensing regulates the virulence of Vibrio harveyi towards gnotobiotic brine shrimp larvae and rotifers. The use of gnotobiotic animals is quite important in this kind of studies in order to avoid bias caused by the micro-organisms that are naturally present in cultures of higher organisms. Very recently, we developed a method to monitor bacterial gene expression in vivo, during infection of gnotobiotic brine shrimp. Using this method, we found that there is a significant difference in the expression of quorum sensing-regulated virulence genes between virulent and non-virulent isolates. Finally, we found that quorum sensing also affects survival of turbot and giant river prawn larvae cultured in non-gnotobiotic conditions.

The most important quorum sensing-disrupting agents reported thus far include compounds that interfere with quorum sensing signal detection and signal transduction, and signal molecule-degrading bacteria. Quorum sensing-disrupting brominated furanones from the macro-alga Delisea pulchra increased survival of gnotobiotic shrimp infected with 12 different pathogenic vibrios belonging to three different (but closely related) species). Furthermore, we found that signal molecule-degrading bacteria isolated from aquaculture settings have a positive effect on survival of turbot and giant river prawn larvae cultured in non-gnotobiotic conditions. We are currently also studying the impact of metabolites produced by micro-algae that are frequently used in aquaculture on quorum sensing activity of Gram-negative bacteria.

In conclusion, the data we obtained thus far indicate that quorum sensing disruption is a valid alternative biocontrol strategy for aquaculture, that biocontrol agents with quorum sensing-disrupting activity can be obtained from the aquatic environment and that these agents have a beneficial effect on cultured organisms.

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Effect of vaccination, feed acidification and water acidification on the transmission of Salmonella Typhimurium in pigs.

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Despite current control measures, Salmonella Typhimurium in pigs still remains a major public health problem and causes large economic losses. Therefore the effect of 3 different intervention strategies on the transmission of Salmonella in pigs was evaluated in this study.

Sixty-nine Salmonella-negative piglets were randomly divided into 3 intervention and 2 control groups. For each group, except the negative control, the experiment was repeated. Group 1 (n=8x2) received feed supplemented with coated calcium-butyrate (Green-Cab-70®, Sanluc International), group 2 (n=8x2) received drinking water adjusted to pH 3,5-3,8 using a mixture of organic acids (Agrocid Super®, Agro2000), group 3 (n=8x2) was orally vaccinated (Salmoporc®, IDT), group 4 served as positive control (infected+untreated; n=8x2) and group 5 served as negative control (uninfected+untreated (n=5). All interventions were applied from weaning (3 weeks of age) until the end of the transmission experiment (14 weeks of age). At 8 weeks of age, two pigs of each group, except from the negative control, were challenged orally with 10^8 cfu of Salmonella Typhimurium strain 112910a and brought back in the group 24 hours later. Blood samples were collected once a week and examined for the presence of Salmonella-specific antibodies by an indirect ELISA (Idexx Laboratories). Faecal samples were examined twice a week for the presence of Salmonella spp. according ISO6579 annex D. All pigs were euthanized and necropsied at 38 days post infection. Ileum/ileum-content/caecum/caecum-content/ileocaecal lymph nodes/tonsils were taken for Salmonella isolation. Meat juice (diaphragm fluid) samples were collected for detection of Salmonella-specific antibiotics.

The mean Salmonella-specific antibody response was significant in the vaccination group 2 weeks after the booster vaccination and remained high during the whole experiment. Any serological response was observed in the negative control group. In the other groups a response was seen 2 weeks after challenge/transmission. Antibody levels were significantly higher in the group receiving acidified water compared to the positive control group and the Ca-butyrate supplemented feed group. Faecal sample analysis showed that significantly fewer pigs excreted Salmonella spp. in the vaccination and the Ca-butyrate group compared to the acidified water and positive control group. No Salmonella could be isolated from any of the samples of the negative control group. These finding were consistent with the number of infected contact animals during the transmission experiments. Out of the 96 tissue samples per group, taken at necropsy, the challenge strain was most present in the acidified water group (n=61) followed by the positive control group (n=39), the vaccination group (n=30) and the Ca-butyrate group (n=17). Remarkably, the vaccine strain was isolated in 13 out of the 30 tonsils samples of vaccinated pigs.

In conclusion, these results show that vaccination against S. Typhimurium and feed supplemented with Ca-butyrate limit the transmission of Salmonella and might be efficient interventions in the control of Salmonella in pigs. Due to antibody response, vaccination however compromises current Salmonella surveillance programmes that are based on antibody detection. Further investigations on interventions which do not compromise diagnostics, or on new diagnostics which are not influenced by vaccination, are needed.

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New toxins homologous to ParE belonging to three-component toxin–antitoxin systems in Escherichia coli O157:H7

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Type II toxin–antitoxin (TA) systems are generally composed of 2 small open reading frames (ORFs) organized in an operon in which the antitoxin gene is located upstream of the toxin gene. The toxins characterized so far appear to interfere either with DNA replication or translation. The antitoxins antagonizes the toxin activity by protein–protein interaction. The antitoxin-toxin complex is also responsible for negative regulation of the operon. The ParE toxin (usually associated with the ParD⁰RK² antitoxin in the current classification) is known to target the DNA gyrase resulting in severe inhibition of replication and transcription, SOS induction and drastic reduction of viability. In E. coli O157:H7, we have identified a novel antitoxin family (PaaA) that is associated with homologues of the ParE toxin. The PaaA antitoxins do not have known DNA-binding domain but the paaA–parE gene pairs form an operon with a third component (paaR) encoding a transcriptional regulator. Two paralogous paaR–paaA–parE systems are found in E. coli O157:H7. For the paaR²–paaA²–parE² system, PaaR² and PaaA²–ParE² complex are able to regulate the operon expression and both are necessary to ensure complete repression. Deletions of the paaA–parE pairs in O157:H7 allowed us to show that these systems are expressed in their natural host and that PaaA antitoxins specifically counteract toxicity of their associated ParE toxin. The paaA antitoxin is degraded by ATP-dependent proteases in vivo and the paaR²–paaA²–parE² system mediates ClpXP-dependent post-segregational killing. The PaaR² regulator appears to be essential for this function. Ectopic overexpression of ParE² is bactericidal and is not resuscitated by PaaA² expression. ParE² colocalizes with the nucleoid, while it is diffusely distributed in the cytoplasm when PaaA² is coexpressed. This indicates that ParE² interacts with DNA-gyrase cycling on DNA and that coexpression of PaaA² antitoxin sequesters ParE² away from its target by protein–protein complex formation.
Diversity of type II bacterial toxin-antitoxin systems

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Type II toxin-antitoxin (TA) systems are in general composed of two proteins, a stable toxin that targets an essential cellular process and a labile antitoxin that inhibits the deleterious activity of the toxin. TA systems are organized in operon, in which the antitoxin gene precedes that of the toxin. Expression of the operon is regulated negatively by the antitoxin alone or in complex with the toxin. The characterized type II toxins target two cellular processes: DNA replication and translation of mRNAs.

Type II TA systems are currently divided into 10 families in which each family of toxins is associated to a specific family of antitoxins. However, few examples of hybrid systems composed of toxins and antitoxins belonging to different families have been recently described in the literature.

A bioinformatics approach developed in our lab led to the discovery of novel hybrid systems. We validated experimentally 6 of these novel hybrids in E. coli. These systems originated from various bacterial species, distantly related to E. coli. We are currently analysing the mode of toxicity of 6 toxins.

Our work shows that multiple associations between toxins and antitoxins do exist. Evolutionary aspects will be discussed.
Involvement of type IV secretion in biofilm formation

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For a long time, bacteria have been mainly considered as planktonic, freely suspended cells. However, they are rarely found in a free-living phase but rather found in association with surfaces in a structure known as a biofilm. Biofilms contains proteins and DNA that may be secreted by the bacterial cells to communicate and to survive. Secretion is universal and essential for the survival and the development of all living organisms. Up to now, 7 secretion systems have been identified in gram negative bacteria. Among them, the Type IV secretion system (T4SS) is known to be involved in various biological processes such as genetic material exchange and proteins secretion. Therefore, we decided to study the potential role of the T4SS in a biofilm formed by the aquatic model bacteria *Caulobacter crescentus*. We identified a set of 8 genes, predicted to encode a T4SS, on the *C. crescentus* chromosome. Quantitative RT-PCR showed that these genes are upregulated upon biofilm formation.

In order to characterize the T4SS, a deleted strain was created for all the genes encoding the T4SS. Morphology, motility and phages resistance were similar in both the wild type and the mutant strains suggesting that T4SS is not involved in cell morphogenesis. CB15 deleted for T4SS (henceforth referred to as *CB15Δt4ss*) was unable to form biofilms indicating a potential role of T4SS in the development of such bacterial communities.

Interestingly, *CB15Δt4ss* aggregates when grown in a conditioned medium from a CB15 biofilm, suggesting that type IV secretion is required for biofilm development.
New archaeal methyltransferases forming 1-methyladenosine or 1-methyladenosine and 1-methylguanosine at position 9 of tRNA

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Cellular RNAs possess numerous chemically modified nucleosides, but the largest number and the greatest variety are found in transfer RNA (tRNA). These modifications are introduced by many different enzymes during the complex process of RNA maturation. The functions of these modified nucleosides are not well known, but it seems that modifications in the anticodon region play a direct role in increasing translational efficiency and fidelity, while modifications outside the anticodon region are typically involved in the maintenance of the structural integrity of tRNA. Among naturally occurring nucleoside modifications, base and ribose methylations are by far the most frequently encountered (1). Most of them are formed by S-adenosyl-L-methionine (AdoMet)-dependent methyltransferases (MTases) (2).

There are only a few archaeal tRNA specific MTases functionally characterised to date. Those MTases can be very different in terms of structure, of target or in their mechanism. In order to improve the knowledge and understanding of these enzymes, we have undertaken a large-scale bio-informatical identification of ORFs encoding putative tRNA MTases in the genome of model archaeal species including the crenarchaeal hyperthermophilic organism Sulfolobus acidocaldarius. The only tRNA of a hyperthermophilic organism sequenced to date, tRNAiMet, is from this organism, and it contains 10 modified nucleosides, 9 of them bearing a methylation (3). The MTases responsible for these modifications are not known yet. One on the ORFs we identified in S. acidocaldarius genome was related to the Trm10p protein of Saccharomyces cerevisiae that acts as a 1-methylguanosine MTase at position 9 of tRNA. Interestingly, in the tRNAiMet of S. acidocaldarius, position 9 bears a modified nucleotide whose nature was unknown.

We showed that the Trm10p homolog we identified in S. acidocaldarius is the enzyme that acts at position 9 of tRNA, and forms 1-methyladenosine at this position. In the other major archaeal domain, the Euryarchaeota, we could also indentify a Trm10p homolog in Thermococcus kodakaraensis. Surprisingly, when we characterised the enzyme, we showed that it is the first protein possessing a broadened substrate recognition capability, acting at position N1 of both adenosine and guanosine (4). The characterisation of the enzymatic mechanism of both archaeal enzymes is in progress.

Yersinia pseudotuberculosis can intrude the parasitic life cycle of entomopathogenic nematodes.

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Entomopathogenic nematodes (EPNs) are able to invade and kill a wide range of insect hosts. The virulence of EPNs is associated with species-specific symbiotic bacteria belonging to the family Enterobacteriaceae (Xenorhabdus and Photorhabdus spp). In Steinernema nematodes, the symbiont is hosted in a specialized cell compartment called the symbiotic vesicle. Upon invasion of an insect prey, the symbiont is released out of the EPN and produces antibiotic compounds aimed at killing the prey and at inhibiting bacterial competitors. It further supplies degradative enzymes allowing EPNs to feed on the dead prey. The symbiont goes back to the digestive tract of new born EPNs before their exit from the dead prey. In an attempt to rationalize the long-term survival of Yersiniae in soil, we examined the capacity of \textit{Y. pseudotuberculosis} to colonise EPNs and to use them as temporary hosts to promote their own survival and / or dissemination. Our results show that \textit{Y. pseudotuberculosis} can take over some of the symbiont functions and be transmitted for at least 5 successive EPN generations fed continuously on moth larvae.
The two-component system PrlS/PrlR is implicated in EPS production and bacterial aggregation in response to hypersaline conditions in *Brucella melitensis* 16M

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*Brucella* spp., as facultative intracellular pathogens, encounter various environmental conditions during their virulent cycle. To survive in changing conditions, bacteria have to integrate different signals and trigger an appropriate response. This adaptative response requires fine-tuning regulatory systems like two-component systems (TCS), consisting of a sensor histidine kinase (HK) and a response regulator (RR). In a previous screening, we brought out a transpositional mutant (G4) attenuated for the *in vivo* pathogenesis in the mouse model of infection (LESTRATE et al., 2000). In this case, the mini-transposon was inserted between a sensor and a regulator of a putative TCS. Based on homology, this TCS was called *PrlS/PrlR* for probable proline sensor. The TCS *prlR/prlS* is well conserved among *Brucella* species and α-proteobacteria but the function of this system has not been investigated yet. In this aim, we constructed and characterized deletant strains for *PrlS/PrlR* partners in *B. melitensis* 16M.

We firstly showed that the virulence of the deletant strains ∆*prlR* and ∆*prlS* is attenuated in mice four weeks post-infection. Moreover, a *mscL* gene encoding a large-conductance mechanosensitive channel is usually found upstream the *prlS* gene. Considering that such channel serve probably as osmotic gauge within cells, we grew the ∆*prlR*, ∆*prlS* and the wild-type (WT) strains in various osmotic conditions. In this context, we observed that unlike the WT, deletant strains for either *prlS* or *prlR* do not aggregate when grown for 72h in 2YT medium supplemented with 400mM NaCl. Furthermore, we reported the existence of natural marine *Brucella* isolates mutated for *prlR* or *prlS* that do not aggregate in the hypersaline medium. These data reinforce our hypothesis for the implication of the PrlS/PrlR system in the clumping phenotype and most likely in EPS production. Indirectly, we identified for the first time a condition in which the WT strain produce EPS and form bacterial clumps. We showed that the clumping phenotype is ionic strength-dependent more than osmolarity-dependent. Finally, we analysed the EPS composition and showed that the EPS produced by the WT strain in this peculiar condition is a mannan as it was previously described for the EPS produced by a QS mutant (GODEFROID et al., 2010).

In conclusion, we suggest that in addition to being involved in the persistence, the TCS PrlS/R, is somehow involved in the mannan production in response to hypersaline conditions and in the resulting bacterial aggregation.

Identification of in vivo binding sites of an archaeal transcription regulator by ChIP on chip

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Archaea have a eukaryotic-like basal transcription machinery that seems to be regulated mostly by bacteria-like transcription regulators. Here, we study Ss-LrpB, a member of the bacterial/archaeal Lrp family (Leucine-responsive regulatory protein), of Sulfolobus solfataricus. S. solfataricus is a hyperthermoacidophilic model organism of the archaea, growing optimally at 80°C and pH 2-3. Only little is known about the physiological role of this regulator and about the identity of its target genes, and one approach to investigate this is by chromatin immunoprecipitation (ChIP) combined with microarray analysis (ChIP-chip). This allows a genome-wide identification of the DNA target sequences of the regulator.

A key component of the ChIP assay is the antibody which is critical to the efficiency and the specificity of the assay. Ss-LrpB-specific Nanobodies, derived from variable domain of Camelid heavy-chain-only antibodies, were generated and characterized. One of these Nanobodies, Nanobody 2RA1, and an irrelevant Nanobody (negative control) were used for ChIP. The ChIP materials obtained with these two Nanobodies were used for genome-wide microarray hybridization. These experiments resulted in the identification of 24 potential binding sites of Ss-LrpB on the entire genome of Sulfolobus solfataricus P2. The well-known binding site of Ss-LrpB, the control region of Sso2131 [1], was among the identified regions, validating our findings. Moreover, using qPCR on three biological replicates, we demonstrated that indeed the 24 identified binding sites are enriched by ChIP.

These results revealed previously unknown in vivo binding sites of Ss-LrpB. Some of these are located in control regions of genes, indicating a regulatory function, while others are located inside ORFs. Furthermore, our work introduces a new application of nanobodies, as being efficient and specific reagents for ChIP.

A model for the protein–DNA contacts of RutR to the carAB operator

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RutR (b1013, ycdC), a member of the commonly found TetR-family of transcriptional regulators, is originally identified to be involved in the expression of the rutABCDEFG operon (b1012), which encodes a novel pathway for pyrimidine utilization discovered in Escherichia coli. TetR transcriptional regulators, such as RutR, consist of two domains, a DNA binding domain with a helix-turn-helix motif, and an effector binding domain for diverse ligands that act as allosteric co-factors (1). A binding site for RutR, termed the RutR box, was indentified in the promoter P1 control region of the carAB operon, which encodes the unique carbamoylphosphate synthase of E. coli. The RutR box is located far upstream of the promoter and overlapping the binding site for aminopeptidase PepA (2,3), the key architectural element that plays a crucial role in pyrimidine and purine-specific regulation of carAB transcription through remodeling of the carP1 control region by DNA wrapping (4).

Previously, by means of a battery of footprinting and binding assays, we have established a high-resolution contact map of the RutR-carP1 operator, demonstrating that RutR binds to two successive major groove segments and the intervening minor groove all aligned on one face of the helix, and furthermore identifying key contacts for both backbone and base-specific groups (5). In continuation, we determined the sequence-specificity of RutR binding by saturation mutagenesis. The pattern obtained is reminiscent of the protein-DNA contacts of another TetR-family member from Staphylococcus aureus, QacR, whose DNA-bound co-crystal structure has been solved. On the basis of these elements, we built a structural model of the RutR-carP1 operator contacts. We then tested our model with the construction of several single amino acid substitution mutants of RutR and the analyses of their DNA-binding affinity and sequence-specificity. The results of this study are in full agreement with the proposed model.

In addition, our in vitro DNA-binding assays had indicated that uracil but not thymine is the physiologically relevant co-factor for RutR, unlike a previously published observation (3). This distinction in the effector potential of two rather similar molecules, differing only by one methyl-group, is in outstanding contrast with the multi-drug binding capacity of various TetR-family members, including QacR. The co-crystal structure of uracil-bound RutR provides a clue to this narrow ligand specificity, attributing this feature to the design of the ligand binding pocket. The involvement of various amino acids in the binding of uracil and the discrimination against thymine, apparently due to a steric clash, was tested with the construction of single amino acid substitution mutants.

Barp, a novel β-alanine-responsive regulatory protein from the hyperthermoacidophilic archaeon *Sulfolobus tokodaii*, is involved in coenzyme A metabolism

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Micro-organisms belonging to the archaeal domain have a eukaryotic-like basal transcription machinery, composed of a RNA polymerase II homologue and additional basal transcription factors. In contrast, transcription regulators share homology with their bacterial counterparts. *Sulfolobus tokodaii* is a hyperthermoacidophilic archaeon for which the genome has been entirely sequenced (KAWARABAYASI et al., 2001), allowing prediction of genes encoding transcription regulators based on homology with characterized regulators. *S. tokodaii* appears to contain several members of the Leucine-responsive Regulatory Protein (Lrp) family, a bacterial/archaeal family of allosteric regulators generally responding to amino acids and regulating genes involved in amino acid metabolism. However, in archaea only little information is available about the function of these regulators.

Here, we describe the characterization of a novel regulator in *S. tokodaii* belonging to the Lrp family, which we called **Beta-alanine-responsive Regulatory Protein** (Barp). We have shown that, *in vitro*, Barp binds to the control region of its own gene, suggestive of an autoregulation. This binding is completely abolished by β-alanine but is not responsive to any of the 20 natural amino acids. High resolution contact mapping experiments indicate that the Barp binding site is composed of two semi-palindromic subsites. Each subsite appears to be contacted by a dimeric part of a higher oligomeric (at least tetrameric) form of the protein. Although these two subsites border the promoter sidewise, binding of the basal transcription factors to the promoter is stimulated upon Barp binding, suggesting an auto-activation. We have further shown that Barp binds the control regions of its two neighbouring genes, encoding an aminotransferase and a seminaldehyde dehydrogenase. Although uncharacterized, genome annotations indicate that these two enzymes might be involved in the conversion between β-alanine and acetyl-coA. This is supported by the existence of a β-alanine-responsive regulation. β-alanine is an important metabolite as it is a precursor of coA. Other putative Barp targets, involved in β-alanine metabolism, are being investigated. This regulator is the first example of an Lrp-like regulator responding to a non-amino acid metabolite and indicates that archaeal members of this regulator family are also involved in regulating central metabolic pathways, besides amino acid metabolic pathways.

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Structure-activity relationships in the active site of dipeptidyl peptidase 4 of *Porphyromonas gingivalis*.

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Periodontal disease develops when bacteria of the dental plaque migrate from the dental root hard tissues to nearby soft tissues. This results in chronic inflammation leading to destruction of the periodontal ligament and the alveolar bone that supports the teeth. The process by which a generally benign biofilm transforms into pathologic periodontitis is complex and still poorly understood. However, the presence of one particular species, the gram-negative anaerobe *Porphyromonas gingivalis*, is considered to be an indicator of progression of chronic periodontitis (BYRNE et al., 2009).

A wide array of virulence factors have been described for *P. gingivalis*. They reflect the different processes by which the bacterium transforms from a commensal organism to a pathogen. *P. gingivalis* produces a unique set of proteases that are believed to be required for nutrient provision during its compulsory asaccharolytic growth, but may also contribute to evasion of the host immune system, adhesion and degradation of extracellular matrix. One of these proteases is *P. gingivalis* dipeptidyl peptidase 4 (pgDPP4). PgDPP4 was reported as a virulence factor (KUMAGAI et al., 2000, KUMAGAI et al., 2003).

pgDPP4 belongs to the same family of proteases (prolyl oligopeptidase family, S9clanB, MEROPS database) as the human DPP4, with which it shares about 25% amino acid identity. This is sufficient to deduce that it has a similar topology and enzymatic mechanism. Both enzymes likely share substrates present in the oral cavity, the gingival epithelium and sites of inflammation. Inhibition of human DPP4 activity on incretins (intestinal peptides regulating insulin secretion) proved to be successful for the treatment of type 2 diabetes. Over the last two decades numerous DPP4 inhibitors were synthesized, screened and tested for their performance in the diabetes setting. Other compounds were made to address selectivity and to investigate the roles of several related peptidases. Thus there is a sizable collection of DPP-inhibitors available for screening versus other targets.

We screened a collection of > 200 compounds, the majority of which were designed and synthesized at the university of Antwerp, for inhibition of pgDPP4 and established structure-function relationships in the active site. This may lead to the development of selective inhibitors and to an improved understanding of the differences between related enzymes on a molecular level.

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The presence and \textit{in vivo} expression levels of virulence genes in vibrios belonging to the \textit{Harveyi} clade in relation to virulence towards gnotobiotic brine shrimp

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Vibrios belonging to the \textit{Harveyi} clade are pathogenic marine bacteria affecting both vertebrates and invertebrates (AUSTIN and ZHANG, 2006). Intriguingly, there is a large variation between strains with respect to virulence. Many virulence factors have been identified in \textit{Harveyi} clade vibrios, including proteases, phospholipases, hemolysins, chitinase, bacteriophages, lipase etc (AUSTIN and ZHANG, 2006; DEFOIRDT \textit{et al.}, 2010). However, it is not clear which virulence factor(s) are most important for the virulence of these bacteria and whether it is merely the presence of a specific virulence factor or rather its \textit{(in vivo)} expression level that determines the virulence of a specific strain.

We investigated the presence of virulence genes in isolates belonging to the \textit{Harveyi} clade (more specifically the species \textit{V. harveyi} and \textit{V. campbellii}) and the expression level of the genes, both \textit{in vitro} and \textit{in vivo}, with the aim to link this information to the virulence of the isolates towards brine shrimp (\textit{Artemia franciscana}) larvae. The virulence genes were amplified by PCR with specific primers and the presence was further confirmed by dot blot hybridization. The quorum sensing master regulator gene \textit{luxR}, the transmembrane regulator gene \textit{toxR}, the \textit{vhh} hemolysin, \textit{chiA} chitinase, \textit{vhp} metalloprotease, and \textit{srp} serine protease were detected in all of the 48 isolates tested. Challenge tests with gnotobiotic brine shrimp larvae did not show any relation between the presence of a virulence gene and virulence of the isolates (RUWANDEEPIKA \textit{et al.}, 2010b).

We subsequently quantified the \textit{in vitro} expression level of \textit{luxR}, \textit{toxR}, \textit{vhp}, \textit{srp} and \textit{vhh} in 10 isolates by reverse transcriptase real-time PCR with specific primers. There was relatively low variation in the expression levels of \textit{luxR} (7-fold), whereas for the other genes, the difference in expression between the isolates showing lowest and highest expression levels was over 25-fold (RUWANDEEPIKA \textit{et al.}, 2010a). There was a significant correlation between expression levels of \textit{toxR} and \textit{luxR} and between the expression levels of these regulators and the protease genes. The expression levels of \textit{luxR}, \textit{toxR} and \textit{vhh} were negatively correlated with the survival of brine shrimp larvae challenged with the isolates, indicating that isolates that have higher production capacity of these virulence factors tend to be more virulent. Finally, virulence gene expression in one non-virulent, one moderately virulent and one strongly virulent isolate was monitored \textit{in vivo}, during infection of gnotobiotic brine shrimp larvae. There was a clear difference in virulence gene expression between both virulent isolates and the non-virulent isolate, with the virulent isolates showing a significantly higher expression level of all virulence genes tested. The virulent isolates showed a peak in expression after 24h challenge, whereas the non-virulent isolate showed a constantly low expression level of the genes.

In conclusion, we found that the \textit{(in vivo)} expression level rather than the presence of virulence genes is responsible for the virulence of vibrios belonging to the \textit{Harveyi} clade towards a shrimp host. The large variation in virulence between different isolates can be explained by differences in expression levels of specific virulence genes.

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Nosocomial Transmission of Necrotising Fasciitis from Patient to Health Care Worker

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Background: Severe streptococcal diseases, especially Toxic Shock Syndrome (TSS) and Necrotising Fasciitis (NF), have remarkably re-emerged since the mid-1980s.

Methods: The diagnosis of TSS and NF were based on consensus definitions. The emm-typing, multilocus sequence typing and the presence of 17 GAS virulence factors were detected by PCR and sequencing.

Findings: We describe here the first case of nosocomial transmission of necrotising fasciitis infection from a paediatric patient to a health care worker after sharp injury. The same strain infected both patients. This particular isolate carries four evolutionary divergent phage-encoded streptodornases and only one streptococcal superantigen exotoxin. Phage mediated horizontal gene transfer might explain the increased propensity of this particular strain to cause necrosis in two different hosts.

Conclusions: This particular isolates displayed a high propensity to cause necrosis in two different hosts. Further characterisations (bacterial genome sequencing, virulence factor deletions, host immune response and animal model) are being performed.
Functional analysis of the *Shigella* IpaD antigen and its potential use to prevent shigellosis

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*Shigella* is responsible for bacillary dysentery in human through host-mediated destruction of the colonic mucosa. It invades the epithelium and elicits an acute inflammation responsible for the disease. As for numerous Gram-negative pathogenic bacteria, *Shigella* possesses a type 3 secretion apparatus (T3SA) that spans and protrudes beyond the bacterial cell wall to form a molecular syringe that injects “effector” proteins from the bacterium directly into host cell cytoplasm, subverting cell physiology. To prevent early secretion of effectors, i.e. before host cell contact, the needle is plugged by IpaB and IpaD proteins (MENARD et al., 1994). IpaD is required for insertion at the cell membrane of a ‘translocation’ pore (made of IpaB and IpaC) through which effectors transit to promote *Shigella* internalization upon actin cytoskeleton remodelling (PICKING et al., 2005). This makes IpaD a “cornerstone” of *Shigella* virulence. The crystal structure of IpaD has been solved and it possesses two domains (N-terminal and central) separated by a long coiled-coil (JOHNSON et al., 2007).

Recently, we reported strong evidences about i/ IpaD localization at the T3SA needle tip and ii/ *in vitro* inhibition of the entry process using anti-IpaD (131-332) polyclonal antibodies (SANI et al., 2007).

In the present work, we report the phenotype of twenty -10 amino acids- truncated IpaD variants of its C-terminus and delineate two major functions; first, the coiled-coil domain (131-174 and 272-332) is important for the formation of a functional plug at the needle tip; and secondly, the central domain (175-271) is required in the pore formation. We generated eight anti-IpaD monoclonal antibodies (mAbs) that were found to recognize the first part of the coiled-coil and/or the central domain. We demonstrated that 3 among the 8 generated mAbs significantly impair the ability of *Shigella* to invade host cell. At present, we are currently (1) mapping their narrow epitope as well as (2) investigating their ability to bind IpaD at the needle tip and (3) testing whether their combination could have a synergistic effect against the invasion process.

Taken together, our results demonstrate that the central domain of IpaD plays a key role in *Shigella* virulence. Thus, as IpaD is a widely conserved antigen among the forty *Shigella* serotypes, generated antibodies against the central domain would be useful to prevent shigellosis.

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Global regulation by CsrA in *E. coli*

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Global regulators allow rapid and coordinated responses to environmental changes and/or modifications inside the bacterial cell. Global regulators act at each level of gene expression control (transcription, translation and protein stability). In *E. coli*, the global regulator CsrA (carbon storage regulator A) regulates central carbon metabolism, mobility and biofilm formation and acts at the post-transcriptional level either positively or negatively by affecting the stability of various mRNAs. It has recently been shown in our lab that the *csrA* gene is essential for growth on glycolytic carbon sources due to an imbalance of carbon fluxes.

We have shown that a *csrA* deletion mutant is affected in growth and viability, especially during stationary phase, and also strongly affected in central carbon metabolism. Indeed, expression of the small RNA *sgrS* as well as the universal stress protein A UspA is induced in this mutant, and PTS operons are down-regulated. This shows that the *csrA* deletion mutant suffers from the so-called ‘hexose-phosphate stress’ (accumulation of glucose-6-phosphate and/or fructose-6-phosphate). This is in accordance with reduced glycolysis and enhanced gluconeogenesis presented by the *csrA* deletion mutant.

Moreover, cAMP accumulates in the *csrA* deletion mutant (4-fold increase as compared to the wild-type strain). cAMP accumulation is independent of the hexose-phosphate stress, since cAMP also accumulates in a *sgrR csrA* double mutant. Molecular mechanism of cAMP overproduction is currently under investigation in our lab. The *csrA* deletion also causes a mucoid phenotype. Our preliminary data indicate that CsrA might regulate expression of capsular polysaccharides through the Rcs system.
Identification and characterization of binding sites for SaLysM, a Lrp-like regulator from Sulfolobus acidocaldarius

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Lysine is one of the ten essential amino acids for humans and animals. (HUTTON et al., 2007). Lysine biosynthesis occurs in two ways: one is the diaminopimelate (DAP) pathway found in bacteria, plants, and algae. The other one is the α-amino adipate (AAA) pathway which was supposed to be almost exclusive to fungi. However, thermophilic bacteria such as Thermus thermophilus utilize a modified AAA pathway (KOBASHI et al., 1999) that seems to exist also in hyperthermoacidophilic archaea belonging to the genus Sulfolobus. In Sulfolobus, there are two successive operons lysYZM and lysWXJK in which the latter one was bound by Lrp (Leucine-responsive Regulatory Protein) family member LysM from Sulfolobus solfataricus (BRINKMAN et al., 2002).

Here, we show the results of high resolution contact mapping experiments probing the binding of SaLysM from S. acidocaldarius, that has 74% sequence identity homology with SsLysM, to the control region of the lysWXJK operon, and analyse the effect on lysine on the binding affinity. SaLysM protects a 21 bp long stretch against DNase I cleavage and premodification binding interference experiments indicate that SaLysM interacts with two major groove segments and the intervening minor groove segment, all aligned on one face of the helix. Saturation mutagenesis by introduction of substitutions at positions 1 to 8 in one half site of the 17 bp consensus binding site show that G-C at position 3 and C-G at position 4 are crucial for the complex formation. Cofactor tests and mutation analysis of two amino acid residues of the lysine binding site of SaLysM demonstrate that L-lysine is the specific co-factor for SaLysM that reduces the affinity for the operator DNA.

Five SaLysM-specific nanobodies were purified by immobilized metal affinity chromatography and gel filtration. Enzyme-linked Immunosorbent Assay (ELISA) tests demonstrate that nanobody No.6 has the highest affinity for SaLysM. Eitpope mapping by ELISA indicates that different nanobodies are directed against different epitopes of SaLysM. Nanobody No.7 interferes with DNA binding and is, therefore, most likely directed against the N-terminal DNA-binding domain. Selected nanobodies will be used in the ChiP-chip assay to find other potential targets for SaLysM in the genome of Sulfolobus acidocaldarius.

Global regulators allow rapid and coordinated responses to environmental changes and/or modifications inside the bacterial cell. Global regulators act at each level of gene expression control (transcription, translation and protein stability). In *E. coli*, the global regulator CsrA (carbon storage regulator A) regulates central carbon fluxes, mobility and biofilm formation. CsrA acts at the post-transcriptional level either positively or negatively by affecting the stability of various mRNAs. In particular, CsrA regulates negatively glycogen synthesis by binding on the *glgCAP* mRNA and thereby promoting its degradation. Furthermore, we have shown that the *csrA* gene is essential for growth on glycolytic carbon sources due to an imbalance of carbon fluxes leading to a dramatic increase of glycogen content in the cell.

Scarce information is available in the literature regarding *csrA* regulation of expression. The CsrB and CsrC non-coding RNAs negatively regulate CsrA activity. These ncRNAs are composed of multiple CsrA binding sites and *in vitro* experiments showed that CsrA binds to these ncRNAs. Therefore, it was proposed that CsrB and CsrC sequester CsrA.

We have recently identified 2 proteins (TldD and TldE) that regulate CsrA stability. In mutant strains deleted of *tldD* and/or *tldE*, CsrA is highly unstable and degraded by the ATP-dependent protease Lon. Moreover, we showed that CsrA degradation is enhanced in condition of overexpression of CsrB and/or CsrC. Intriguingly, we observed the opposite effect when CsrA interacts with a target mRNA. Indeed, CsrA stability is increased when CsrA binds to *glgCAP* mRNA. Binding of CsrA on *glgCAP* mRNA and on CsrB and CsrC led to their rapid degradation. Molecular mechanisms underlying these different regulations are currently under investigation in our lab.
Adaptation of recombinase-based in vivo expression technology (RIVET) to avian pathogenic Escherichia coli (APEC): Construction of a functional reporter strain and test-screen of the APEC RIVET library in chickens

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APEC are group of E.coli strains causing systemic disease in poultry known as avian colibacillosis. The disease manifests itself initially with septicaemia then either sudden death or localized multiple organ inflammation. The disease is associated with major economic losses to the poultry industry worldwide. Host and bacterial factors influencing and/or responsible for carriage and systemic translocation of APEC inside the host are poorly understood. Identification of such factors could help in the understanding of its pathogenesis and subsequently development of control strategies.

RIVET strategy can be used to isolate host-induced APEC genes and is being developed to investigate APEC pathogenesis in chicken by identifying in vivo host-induced promoters. Random chromosomal DNA fragments from APEC genome are transcriptionally fused upstream to a promoterless cre gene to create APEC RIVET library in a plasmid containing ampicillin resistance marker. The reporter strain is constructed by integrating into APEC genome a cassette known as LoxP. This cassette consists of loxP sites in direct orientation flanking the neomycin resistance marker (neo) for positive selection and streptomycin sensitivity gene (rpsL) for negative selection (loxP-rpsL-neo-loxP). Expression of Cre due to the fused active promoter will cause recombination of the two loxP sites, deleting the cassette and permanently changing the bacterial phenotype such that can be detected after gene expression has ceased.

In vivo preliminary study was performed by first selecting APEC RIVET library on kanamycin and ampicillin antibiotics to eliminate in vitro active promoters. Then the bacteria were administered in chicken host via intra-tracheal route. The results showed that bacteria could be isolated from the liver, lungs, heart and kidney, 24-48 hours post infection, indicating that the mutant could survive inside the host. When screened for the host-induced promoters, the results showed that seven colonies were kanamycin sensitive and resistant to ampicillin and streptomycin. PCR analysis demonstrated a range of insert sizes suggesting that the screening is functional and the plasmid could stably be maintained in the bacteria even after infection. With these results APEC RIVET library could be adapted and the strategy showed to be functional for the screening of host-induced APEC promoters in chickens.

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Structure-function relationship in CopI, a blue copper protein of *Cupriavidus metallidurans*

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*Cupriavidus metallidurans* is the model bacterium for the study of metal resistance. Its two large plasmids bear genes conferring resistance to 9 heavy metals. Most of the “*cop*” copper resistance genes (21 ORF’s) are clustered on the pMOL30 plasmid. The precise function in the resistance mechanism of a majority of them is still unknown.

A survey of fully sequenced bacterial genomes for *cop* gene homologues indicate that 63 proteobacteria do contain a selection of *cop* genes, the most frequent ones being the *copF* P1-ATPase and the basic module *copSRABCD*. Another ubiquitous *cop* gene, *copI*, is predicted to encode a blue copper protein (or cupredoxin) and is therefore suspected to function as an electron shuttle protein.

We have expressed the *C. metallidurans* CopI protein in *E.coli* in its mature form, the native protein containing a signal peptide for periplasmic localization and could confirm that it is indeed a blue copper protein with a canonical type1 “blue” copper binding site. In addition, mass spectrometry experiments have pointed out two additional copper sites. In order to try and identify the residues involved in the copper ions coordination, several targeted substitutions were constructed, purified and tested for their copper binding capacity. On the other hand, we are currently growing protein crystals for X-rays diffraction structure determination.
Analysis of the biofilm-associated microflora on endotracheal tubes and their role in ventilator-associated pneumonia

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Patients receiving mechanical ventilation are at increased risk for ‘ventilator-associated pneumonia’ (VAP). The placement of an endotracheal tube (ET) is considered an important risk factor as it allows the entry of bacteria in the lungs. The ET itself is also colonized by bacteria, forming a biofilm in the ET lumen.

In the present study, we characterized the microbial populations of 20 samples of ET biofilms and surveillance cultures (throat, nose and sputum samples) obtained from 19 patients from the intensive care unit of the Ghent University Hospital by means of culture-dependent (differential growth media; Gram-staining; conventional microbiological tests; 16S rRNA gene sequencing) and culture-independent techniques (construction of clone libraries of the 16S rRNA genes). Finally, the antimicrobial resistance of isolated bacteria and consortia has been determined.

The results confirmed the presence of several potentially pathogenic bacteria (Pseudomonas aeruginosa, Staphylococcus aureus, Enterobacter aerogenes). Also, emerging nosocomial pathogens such as Staphylococcus epidermidis and Micrococcus luteus were found. The bacterial diversity of the samples was larger than initially thought, as e.g. Myroides odoratus, Photobacterium damselae, Bacillus simples, Rhodococcus sp. were detected (organisms not thought to be associated with VAP). In addition, many ET biofilms contained high numbers of antibiotic resistant organisms such as methicillin-resistant Staphylococcus aureus, methicillin-resistant Staphylococcus epidermidis, tobramycin-resistant Pseudomonas sp. and extended spectrum β-lactamase producing Enterobacteriaceae.
Presence of methicillin-resistant coagulase-negative staphylococci in Belgian pigs

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Increased attention has recently been directed towards methicillin-resistant staphylococci in animals. In food production animals, especially methicillin-resistant Staphylococcus aureus (MRSA) of clonal complex (CC) 398 may be a cause for concern. Questions have been raised on the origin and evolution of this clone. It is generally assumed that methicillin-resistant non-S. aureus staphylococci (MRNaS) may act as a reservoir for the genetic determinant of methicillin resistance (the meca gene, carried in a mobile genetic element, the staphylococcal cassette chromosome mec, SCCmec). However, few data are as yet available on carriage of MRNaS in food production animals.

To assess the presence of MRNaS in pigs, we investigated ten MRSA CC398 positive Belgian pig farms. On each farm, nasal swab samples were collected from ten animals of each production group present (sows and/or piglets and/or fattening pigs) (n = 200 in total). Swabs were incubated overnight in Brain Heart Infusion broth supplemented with 7.5% NaCl, and subcultured on three different selective agars: home-made Columbia Agar plates containing 5% sheep blood and cefoxitin, ChromID S. aureus (Biomérieux, France) and Columbia colistine-aztreonam plates with sheep blood (Oxoid, France). After incubation, colonies showing a staphylococcal morphology were examined with a triplex PCR for 16S rRNA-meca-nuc, amplifying sequences specific for Staphylococcus, methicillin resistance and S. aureus, respectively. Strains confirmed as MRNaS were identified to the species level with tDNA intergenic spacer PCR and rpoB sequencing, after which their SCCmec type was determined by multiplex PCR following the method of Kondo et al. (2007).

MRNaS (n = 72) were isolated from 59 animals and were present on all ten farms. Seven different species were found: S. epidermidis (28 isolates), S. sciuri (13), S. pasteuri (13), S. rostri (9), S. warneri (6), S. haemolyticus (2) and S. hominis (1). A high diversity in SCCmec cassettes was found: 8 cassettes were nontypeable with the method used, 28 were type IV cassettes, 14 were type III cassettes and 4 type V.

Since SCCmec of type IVa and V are the most frequently detected cassettes in MRSA CC398, this study adds to the hypothesis that MRNaS could act as a reservoir for meca/SCCmec for MRSA CC398 in pigs. Moreover, important nosocomial species of MRNaS (S. epidermidis, S. haemolyticus) prevail in pigs, possibly posing new concerns. Further studies should reveal the genetic background of the MRNaS.

This study was funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment (contract RF-6189).

Localization and functional analysis of PdhS in *Brucella abortus* suggests the presence of differentiation states linked to cell cycle and infection

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*Brucella abortus* is a facultative intracellular pathogen responsible for a worldwilde zoonosis. *B. abortus* is passing through the endocytic pathway until late acidic compartments without replicating, and then reaches endoplasmic reticulum (ER), where it is able to proliferate massively. In bacteriological medium, this alpha-proteobacterium is dividing asymmetrically. Indeed, after each division, two sibling cells of different size are generated. The 1035 aa histidine kinase (HK) PdhS is localized at the old pole of *B. abortus*, and is thus differentially distributed between the sibling cells. Interestingly, the *pdhS* gene is essential, and overexpression of *pdhS* leads to multipolar cells as well as minicells, suggesting that PdhS is involved in cell division control.

Using allelic replacement of native *pdhS* by mutated *pdhS* alleles, we obtained one thermosensitive strain of *B. abortus* that are still able to grow at 36°C (permissive temperature) but not at 42°C (restrictive temperature). Complementation assay with wild type *pdhS* carried on a plasmid showed that the thermosensitive phenotype was indeed due to mutations in *pdhS* gene (*pdhS\(^{ts}\)* alleles). The growth rate of *pdhS\(^{ts}\)* strains is dramatically decreased at restrictive temperature, and bacteria do not display detectable morphological defects in these conditions. Viability of the thermosensitive strains was quickly decreased at restrictive temperature. Spontaneous suppressors (growing at restrictive temperature) were isolated. Genomic sequencing of three suppressors strains and the thermosensitive strain as control identified two mutations that were present in one suppressor strain, compared to the thermosensitive strain. These two mutated coding sequences are currently characterized.

The autophosphorylation of PdhS was not essential for polar localization in *B. abortus*, given that *PdhS\(^{H805A}\)*, where the conserved phosphorylatable histidine (His-805) is mutated in alanine, in fusion to YFP, is still able to be polarly localized. We showed that this mutated protein was not phosphorylated, while the native PdhS protein was phosphorylated. The N-terminal portion of PdhS (residues 1-434) was sufficient for polar localization in *B. abortus*. The expression of the first 638 aa was sufficient to give rise to aberrant morphologies in *B. abortus*. Indeed, minicells and « Y » shaped cells were observed, a phenotype comparable to the overexpression of the whole *pdhS* gene. Interestingly, the additional pole is appearing by an apparent duplication of the young pole, while the truncated PdhS protein (residues 1-638) fused to YFP is localized at the old pole. By time lapse microscopy suggests a unipolar growth mecanisme for *B. abortus*.

The fluorescent signal of the PdhS\(^{1-434}\)-YFP fusion is brighter compared to the complete PdhS fused to YFP. This observation enables us to clearly follow the localization pattern of PdhS (1-434) in an infection context. Interestingly, when PdhS localization is examined in *B. abortus* infecting HeLa cells and RAW macrophages, polar PdhS\(^{1-434}\)-YFP and PdhS-YFP localization is lost during the first 6-8 hours post-infection, a period at which *B. abortus* is transiting through the endocytic pathway. The polar localization of PdhS\(^{1-434}\)-YFP and PdhS-YFP is recovered when intracellular proliferation has started. These initial data suggest that *B. abortus* is blocked at a particular differentiation state during the early steps of its intracellular trafficking.

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IFN-γ-producing CD4+ T cells play a central role in the protective immune memory against *Brucella melitensis* in C57BL/6 mice

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*Brucella* organisms are facultative intracellular Gram-negative coccobacilli that cause brucellosis in humans and animals. Acute human brucellosis is characterized by undulating fever, which may result in chronic disease with serious clinical manifestations. No protective human vaccine exists and those developed for animals do not provide an optimum efficiency. Presently we don’t have a good comprehension of how protective immunity is acquired and what are the immune components implicated.

In the murine experimental model of infection, the primary immune response is mainly regulated by IFN-γ-producing CD4+ T cells that activate inflammatory dendritic cells (DC) to produce iNOS/NOS2. C57BL/6 mice displayed higher IFN-γ and iNOS production correlated to a better bacterial growth control when compared to BALB/c mice. The goal of the present study is to determine the implication of these cells in secondary immune response and if need arises, to identify new molecular and cellular effectors of memory response.

In a preliminary study, we have compared bacterial load of primo-infected C57BL/6 mice and C57BL/6 mice vaccinated with a live (wild type bacteria) or a heat-killed (HK) vaccine. Both protocols induced humoral specific response but sterilising protection is only observed in animals that received the live vaccine (almost 80%, 50 days post challenge). In contrast protection in HK vaccinated mice appears similar to the one observed with a primo-infection (around 20%). This demonstrates that the infectious cycle of *Brucella* is essential to provide a protective memory. The protection observed with the live vaccine is correlated to the presence of IFN-γ-producing CD4+ T cells in the peritoneal cavity (inoculation site) at 12 hours post challenge. In vivo, these cells are not detected in mice vaccinated with HK. Moreover, in vitro stimulation with heat-killed bacteria of peritoneal cells from mice vaccinated with live vaccine shows an important re-activation of IFN-γ-producing CD4+ T cells. This result demonstrates that IFN-γ-producing CD4+ T cells are present in the peritoneal cavity before challenge. Surprisingly, live vaccinated BALB/c mice present neither recruitment of IFN-γ-producing CD4+ T cells in peritoneal cavity nor protective memory.

Based on these results, we compared the ability of various deficient strain of C57BL/6 mice to mount protective memory following live vaccination. MHC-II deficient mice loose protection, confirming the central role of CD4+ T cells in protective secondary immune response. Mice deficient for IL-12p35, CD40 and CD28 (implicated in the activation of T cells to produce IFN-γ) displayed similar results. In contrast, iNOS-producing inflammatory DCs don’t seem implicated in the elimination of *Brucella* during secondary response since iNOS, TNF-α and CCR2 deficient mice appeared protected.

In conclusion protective memory response against *Brucella melitensis* seems to be mainly dependent on the IFN-γ-producing CD4+ T cells selected during the primary immune response.

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Multiplexed, single-tube test for the molecular identification of Brucella based on Single Nucleotide Polymorphisms

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Accurate identification of Brucella is a challenging issue for reference laboratories. Classical methods are time-consuming, hard to standardise, require high-level biosafety containment and well-trained personnel. Moreover, Brucella taxonomy is the matter of an ongoing debate and is almost continuously evolving. Although a large number of molecular tests have been developed during the last decade to overcome the drawbacks of classical methods, few of them are based on genetic markers with sound taxonomical value and usually do not cover the whole range of Brucella species described to date. This work describes a new laboratory test for the identification of any Brucella at species level and below species level for some of them. The test is a three-step, single-tube test that uses the Ligase Chain Reaction (LCR) procedure to determine simultaneously the identity of 16 Single Nucleotide Polymorphisms (SNPs) in the genome of the investigated strain. Most of these SNPs were selected from a compiled Multiple Locus Sequence database that has been extensively validated on all reference strains and on a large number of field strains. Robustness, simplicity, rapidity and customizability are the strong points of the proposed method.
ABSTRACTS OF PARTICIPANTS

Virology
Synergistic in vitro anti-HIV activity of tenofovir with carbohydrate binding agents (CBAs)

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The development of highly active antiretroviral therapy (HAART) in the mid-nineties improved the life expectancy of HIV-infected individuals significantly. At present, the main focus is the prevention of HIV transmission by topical microbicides such as vaginal and rectal gels. The nucleotide reverse transcriptase inhibitor tenofovir is the most prescribed anti-HIV drug and has potential microbicidal applications. Recently, a clinical trial in South-Africa (CAPRISA 004) showed that a 1% tenofovir gel was not only safe but also effective in reducing the HIV incidence (Abdool Karim et al., 2010). Here, we wanted to evaluate the combination of tenofovir with carbohydrate binding agents (CBAs). The CBAs are described as potent inhibitors of HIV replication and active in various cellular transmission models in vitro. They should be considered as potential candidates for microbicide application (Balzarini, 2007).

CD4+ T cells (MT-4) were infected with HIV-1 (strain NL4.3) and cultured with tenofovir and CBAs, alone and in combination. After 5 days of infection, EC₅₀ concentrations were determined for the various drugs in replicate assays by the MTS method. Analysis of the combined antiviral effects was performed using the median effect principle and software CalcuSyn specifically developed by Biosoft for these purposes.

Tenofovir was evaluated for its anti-HIV-1 activity resulting in EC₅₀-values between 5.2 and 6.7 µM. Next, tenofovir was tested in a two-drug combination assay with the CBAs Hippeastrum hybrid agglutinin (HHA), Galanthus nivalis agglutinin (GNA), Urtica dioica agglutinin (UDA), microvirin (MVN) and the anti-carbohydrate broad neutralizing monoclonal antibody mAb 2G12.

The strongest synergistic anti-HIV-1 effect was observed for tenofovir in combination with mAb 2G12 with a calculated combination index (CI) of 0.41. Also, synergistic anti-HIV activity was obtained when tenofovir was combined with the mannose-specific CBA GNA (CI of 0.47). A mild synergistic effect was found in the tenofovir /UDA and tenofovir /HHA combinations (CI of 0.68 and 0.70, respectively). Combination of tenofovir and MVN, a recently described mannose-specific lectin isolated from cyanobacterium M. Aeruginosa resulted in a slight synergism (CI of 0.86). In all combinations tested, a more than 3-fold decrease in EC₅₀-values for tenofovir was observed (EC₅₀ ranging between 0.66 and 1.99 µM). A comparable decrease in EC₅₀ was noted for all the combined CBAs.

We can conclude that tenofovir showed synergistic activity in vitro when combined with CBAs. In these drug combinations the overall effective concentrations of the anti-HIV agents can be lowered, which will reduce the risk on potential toxic side-effects. This study illustrates that tenofovir can be combined with other microbicidal agents (i.e. entry inhibitors such as CBAs) in order to further increase its antiviral potential.

Genital re-excretion of Murid gammaherpesvirus 4 following intranasal infection

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Gammaherpesviruses are the archetypes of persistent viruses that have been identified in a range of animals from mice to man. They are host-range specific and establish persistent, productive infections of immunocompetent hosts. Thus, infected individuals simultaneously both elicit antiviral protective immune response and secrete infectious virions. The best studied gammaherpesviruses are Human herpesvirus 4 and Human herpesvirus 8. As these viruses have no well-established in vivo infection model, related animal gammaherpesviruses are an important source of information. We are studying Murid herpesvirus 4 (MuHV-4), a virus that has originally been isolated from bank voles (Myodes glareolus). Although MuHV-4 has not been isolated from house mice (Mus musculus), infection of inbred laboratory mouse strains is commonly accepted as a good model for studying gammaherpesviruses in vivo. To date, it has however never been possible to monitor viral reexcretion and virus transmission in this species suggesting that this model could be imperfect. In order to identify potential re-excretion sites, intranasally infected mice were followed through global luciferase imaging for up to six months after infection. By this technique, we were able to detect appearance of viral replication in mice genital tract at various times post-infection. Typically, it firstly occurred between days 20 to 30 after infection, a period at which it is admitted that latency is established. Ex vivo imaging, quantitative PCR and immunohistochemistry helped us to determine that virus genomes were present in high quantity in the vaginal tissue and that viral replication occurred mainly at the vaginal external border. Finally, we highlighted the presence of free infectious viruses in the vaginal cavity at the moment of the observation of viral replication. In conclusion, we experimentally indentified for the first time a reexcretion site for MuHV-4 in mice that had been intranasaly infected. It therefore suggests potential genital transmission, either horizontal or vertical, of this virus in mice populations. In the future, these results could help us to better understand the biology of gammaherpesviruses but should also allow us to develop vaccinal strategies that could prevent the spread of these viruses in natural populations.

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Genetic diversity of porcine reproductive and respiratory syndrome virus isolates in Flanders

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Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped RNA virus that causes reproductive failure in sows and respiratory disease in pigs of all ages. In general, PRRSV isolates are categorized within the European genotype (prototype Lelystad virus) (Wensvoort et al., 1991) and the North-American (NA) genotype (prototype VR2332). Based on the nucleotide sequence variability, the European genotype is further divided into several subtypes (Stadejek et al, 2006 and 2008).

The aim of this study was to investigate the genetic diversity of PRRSV isolates present on Flemish farms, with a history of clinical signs that could be attributed to PRRSV (in breeding animals and growing pigs).

PRRSV-positive samples (aborted foetuses, lymph nodes, lungs or serum) were collected from 18 breeding-fattening farms over a period of 3 years (June 2007 to June 2010). At the moment of sampling, sows of 5 herds were vaccinated with the EU-genotype attenuated vaccine (Porcilis® PRRS). In 2 herds, the sows were vaccinated using an EU-genotype inactivated vaccine (Ingelvac® PRRS KV). In 8 herds, sows were vaccinated using the NA-genotype attenuated vaccine (Ingelvac® PRRS MLV). In 2 herds, the sows were vaccinated with both the attenuated NA-genotype vaccine and the EU-genotype vaccine, alternating in time. Sows in the remaining herd were not vaccinated. The genomic regions corresponding to ORF4, ORF5 and ORF7 were determined via RT-PCR and cycle sequencing. Sequence alignments and analysis were performed using CLC workbench 4 and Blastv2. For genetic relationship analysis, selected sequences from American and/or European genotype were enclosed. In total, 21 different PRRSV isolates were obtained. Seventeen strains belonged to the European genotype, subtype 1 and 4 strains belonged to the American genotype. A high genetic variability exists for PRRSV isolates on Flemish farms. In some cases herds harbour more than one isolate at the same time. As expected, the EU-genotype field isolates are genetically diverse, confirming previous reports from other European countries (Balka et al., 2008). The NA-genotype virus found in 4 of the 18 herds was almost identical to the vaccine strain. The genetic diversity of this RNA virus may hamper the efficacy of the current available vaccine strains.

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A trypsin-like serine protease is involved in PRV invasion through the basement membrane barrier in porcine nasal respiratory mucosa

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Different alphaherpesviruses, including pseudorabies virus (PRV), are able to cross the basement membrane barrier in nasal respiratory mucosa. The mechanism of this invasion process is still unknown. Local disassembly of the basement membrane, a part of the extracellular matrix, would enable virus to pass through in an efficient manner. Degradation of extracellular matrix components is almost always mediated by proteolytic enzymes. Inhibitors of the four major catalytic types, aspartic (pepstatin A), cysteine (E-64), metallo (phosphoramidon) and serine peptidases (AEBSF), were tested for their effect on 89V87 virus spread in porcine nasal respiratory mucosa explants. Using the software imaging system ImageJ, plaque latitudes and depths underneath the basement membrane were determined for plaques from mock-treated and protease inhibitor-treated explants at 20h post inoculation (pi), the timepoint at which PRV has crossed the basement membrane.

Pepstatin A, E-64 and phosphoramidon did not have a significant effect on the plaque dimensions. Using the serine protease inhibitor, 0.1 and 0.25mM AEBSF, the plaque depth underneath the basement membrane was reduced with respectively 58.7 and 88.1% compared to plaques from mock-treated explants. Further analysis using the Soybean Type I-S trypsin inhibitor for the serine protease subcategory of trypsin-like serine proteases resulted in a 92.0% reduction in plaque depth underneath the basement membrane compared to plaques from mock-treated explants. This revealed the involvement of a trypsin-like serine protease in the 89V87 PRV invasion process through the basement membrane. We suggest that this trypsin-like serine protease possibly degrades basement membrane components, thereby facilitating the PRV invasion process through the basement membrane barrier.
Microvirin, a novel α(1,2)-mannose-specific lectin isolated from Microcystis aeruginosa, has potent anti-HIV-1 activity

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The most effective approach to halt the HIV epidemic will be establishing effective prevention methods incorporating multiple types of intervention including among others anti-HIV microbicides.

Microvirin (MVN), a recently isolated lectin from the cyanobacterium Microcystis aeruginosa PCC7806, shares 33% identity with the potent anti-HIV protein cyanovirin-N (CV-N) isolated from Nostoc ellipsosporum and both carbohydrate binding agents (CBAs) bind to similar carbohydrate structures. MVN is able to inhibit infection by a wide variety of HIV-1 laboratory-adapted strains and various clinical isolates of different tropisms and subtypes in peripheral blood mononuclear cells (PBMCs) (IC₅₀: 2-167 nM). MVN also inhibits syncytium formation between persistently HIV-1-infected T cells and uninfected CD4⁺ T cells (IC₅₀: 124 nM). It also inhibits DC-SIGN-mediated HIV-1 binding (IC₅₀: 189 nM) and subsequent transmission to CD4⁺ T cells (IC₅₀: 168 nM). Long term passaging of HIV-1 exposed to dose-escalating concentrations of MVN resulted in the selection of a mutant virus with 4 deleted high-mannose-type glycans in the envelope gp120 (T297I, T341(T,I), N386(K,N), N392D). The MVN-resistant virus was still highly sensitive to various other CBAs (e.g. CV-N, HHA, GNA and UDA), but not anymore to the carbohydrate-specific 2G12 mAb. Importantly, MVN is more than 50-fold less cytotoxic than CV-N with CC₅₀ values of >35 μM and >7 μM in MT-4 cells and PBMCs, respectively. Also in sharp contrast to CV-N, MVN did not increase the level of the cellular activation markers CD25, CD69 and HLA-DR in freshly-isolated CD4⁺ T lymphocytes. MVN did not enhance viral replication in pre-treated PBMCs as was observed with several other CBAs.

Therefore, MVN may qualify as a useful lectin for potential microbicidal use based on its broad and potent antiviral activity and virtual lack of any stimulatory properties and cellular toxicity.

Bovine herpesvirus 4 glycoprotein L is non-essential for infectivity and is involved in virus entry.

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The core entry machinery of all the herpesviruses is composed of glycoproteins B, H and L (gB, gH and gL). It is commonly admitted that gH and gL associate to form a heterodimer that plays a central role in virus-driven membrane fusion. When archetypal alpha- or beta-herpesviruses lack gL, gH misfolds and progeny virions are noninfectious. However, a recent study showed that gL is not essential in the infection of Murid herpesvirus 4 (MuHV-4), a gamma-2 herpesvirus. MuHV-4 represents the first herpesvirus to date for which gL is not an essential glycoprotein. In order to define the role that gL plays in gamma-2 herpesvirus infections, we disrupted its coding sequence in bovine herpesvirus-4 (BoHV-4). BoHV-4 lacking gL remained infectious but exhibited a severe growth deficit. Binding experiments revealed that this deficit was mainly related to defect in entry. Finally, we investigated the consequences of this modification of the herpesviral core entry machinery on virions sensitivity to neutralization. All together, our results suggest that gL is non essential in gamma-2 herpesvirus although it is involved in viral entry. In the future, this study could help us to unravel new potential neutralization targets in order to develop vaccinal strategies that could prevent the spread of these viruses in natural populations.

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Bovine Herpesvirus-4 Bo10 gene encodes a non essential viral envelope protein that regulates viral tropism through both positive and negative effects.

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Besides the herpesvirus core entry proteins gB, gH and gL, all gammaherpesviruses encode an additional glycoprotein positionally homologous to Kaposi’s Sarcoma associated herpesvirus (KSHV) K8.1. Although these proteins are involved in cell binding, their precise role remains unclear. In this study, we characterized the Bovine Herpesvirus-4 (BoHV-4) Bo10 gene which encodes the BoHV-4 positional homolog of KSHV K8.1. Our results showed that Bo10 encodes a 180 kDa protein, hereafter called gp180, which is incorporated in viral particles. A Bo10 deleted strain was viable but showed a growth deficit that was associated with a reduction of binding to epithelial cells. This phenomenon was related to glycosaminoglycan (GAG) interaction as Bo10 deletion reduced the normal dependence of BoHV-4 on GAGs for efficient infection. However, Bo10 Del virions were paradoxically more infectious for GAG-deficient cells. By analogy to Murid Herpesvirus 4 gp150, we propose that BoHV-4 gp180 could regulate the attachment of virions to the cell surface by covering some high affinity receptor-binding protein until it has been displaced by GAGs. These proteins could therefore regulate viral tropism both positively and negatively in function of the presence or the absence of their receptor.

B. M., C. L., B.D. and L.G. are Research Fellows, Postdoctoral Researcher and Research Associate of the “Fonds de la Recherche Scientifique - Fonds National Belge de la Recherche Scientifique” (F.R.S. - FNRS), respectively. P. G. S. is a Wellcome Trust Senior Clinical Fellow (GR076956MA). This work was supported by the following grants: ARC “GLYVIR” and starting grant of the University of Liège (D-09/11) and scientific impulse grant of the F.R.S. – FNRS n° F.4510.10.
The A3 gene of *Alcelaphine herpesvirus 1* encodes a viral semaphorin that is non-essential for the induction of malignant catarrhal fever

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*Alcelaphine herpesvirus 1* (AlHV-1) is a γ-Herpesvirus carried by wildebeest asymptptomatically. AlHV-1 is however responsible for the development of malignant catarrhal fever (MCF) when cross-species transmitted to a variety of ruminant susceptible species. Wildebeest-derived (WD)-MCF is a frequently fatal lymphoproliferative and degenerative disease of ruminants. Experimentally, WD-MCF can be reproduced in rabbits. The A3 open reading frame (ORF) of the AlHV-1 encodes a putative semaphorin homolog protein, thereafter named AlHV-sema. Semaphorins are secreted and membrane-associated proteins characterized by a conserved amino-terminal ‘Sema’ domain. Initially identified as guidance factors that assist axons pathfinding during neural development, semaphorins have been shown over the last decade to have significant functions in various processes of immunoregulation. Bioinformatics analyses revealed that AlHV-sema has a high homology to the cellular Sema7A. Besides its roles in neural development, Sema7A has been shown to play pivotal functions in the regulation of cytokine secretion and as a tumor suppressor. In order to investigate whether AlHV-Sema could play a role in the pathogenesis of WD-MCF, we used the AlHV-1 BAC clone and produced a strain deleted for A3 and a revertant strain. The strain deleted for A3 replicated comparably to the wild-type parental strain in vitro. *In vivo*, rabbits infected with the strain deleted for A3 developed WD-MCF similarly to that observed with the parental strain with both severely increased CD8^+^ T cell frequencies and viral genomic charge over time in peripheral blood and in lymph nodes at time of death, as well as indistinguishable histopathological lesions in lymphoid organs and in liver, lung and kidney. In conclusion, this study demonstrates that AlHV-sema is not essential for the induction of WD-MCF in rabbits.
PhEVER: a database for the global exploration of virus-host evolutionary relationships

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Understanding how viruses interact with their hosts, and more specifically how the complex interactions between viruses and their host organisms are acquired and maintained throughout evolution, remains a major challenge. In order to assess this question, it is of prime importance to be able to detect and quantify the occurrence of lateral gene transfer events, and the impact of these events on viral-host co-evolution. Thus we developed a global approach aimed at providing accurate evolutionary and phylogenetic information to tackle these questions.

The major drawback of currently available databases of homologous families to the study of viral homologies and lateral gene transfer in viruses is their taxonomic compartmentalization. Indeed, current databases present families of homologies either restricted to viruses only (Protein Clusters, GeneTree) or to viral taxonomic groups (Viral Orthologous Cluster), some also not presenting viral information (HomoloGene). The few databases that do present viral and non-viral sequences, such as Pfam or the Conserved Domain Database do not provide complete phylogenetic trees. This translates into the fact that it is not currently possible to have a global view on viral-host lateral gene transfers due to the difficulty of obtaining global information on cross-taxon transfers at the viral level.

We present PhEVER (http://pbil.univ-lyon1.fr/databases/phever), a unique database of homologous gene families aiming to be a comprehensive tool for the analysis of virus-virus and virus-host relationships from an evolutionary point of view. It is the first open-access database to provide information at the cross-taxon scale for the analysis of virus-virus and virus-host protein transfers. PhEVER compiles information from all kingdoms of life, and handles data from the genomes of all completely sequenced viruses and prokaryotes and of a large range of eukaryotes: 2426 non-redundant viral genomes, 1007 non-redundant prokaryotic genomes, 43 eukaryotic genomes ranging from plants to vertebrates. All protein sequences from these genomes are clustered – without a priori and according to similarity criteria – into families containing either only viral sequences or both viral and cellular sequences. PhEVER therefore not only spans all known viral groups but also has the unique feature of presenting homologies with eukaryotic and prokaryotic sequences. Alignments and phylogenies for each family are built according to state-of-the-art phylogeny procedures and we provide tools to edit them and recompute them on the fly. We also provide the possibility for users to assign their sequence of interest to a family and to re-build the phylogeny accordingly through the HoSeqI tool. PhEVER thus constitutes a working tool to detect sequence homologies and possible gene transfer events. Public access and documentation is available through the database webpage and through all dedicated ACNUC retrieval systems.

The authors acknowledge the CC IN2P3 (Villeurbanne) for the computing resources and Pascal Calvat for his technical help as well as the computer department at PBIL-DOUA and LBBE for assistance and maintenance of the PhEVER server. This work was supported by the Interaubio project, granted by the Région Rhône-Alpes. L.P. was funded by the Région Rhône-Alpes and by the University of Liège.
Comparative analysis of replication characteristics of different BoHV-1 subtypes in bovine respiratory and genital mucosa explants: a phylogenetic enlightenment

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Bovine herpes virus 1 (BoHV-1) belongs to the subfamily of the Alphaherpesvirinae. In general, alphaherpesviruses use the epithelium of the upper respiratory and/or genital tract as a preferential site for primary replication. BoHV-1 may replicate at both sites and causes two major clinical entities designated infectious bovine rhinotracheïtis (IBR) and infectious pustular vulvovaginitis/balanoposthitis (IPV/IPB) in cattle. It has been hypothesized that subtype 1.1 invades preferentially the upper respiratory mucosa where subtype 1.2 favors replication at the peripheral genital tract. However, some in vivo data are in contrast with that hypothesis. A thorough study of primary replication at both mucosae could elucidate this question.

In the present study, we established a bovine respiratory and genital organ culture. The effect of in vitro culture on tissue viability and tissue morphometry was extensively assessed by means of TUNEL stainings, light microscopy, scanning electron microscopy and transmission electron microscopy. No significant changes in viability and morphometry were observed during in vitro culture. Next, bovine respiratory and genital mucosal explants of the same animals were inoculated with several BoHV-1 subtypes. A quantitative analysis of viral mucosal invasion was performed at 0, 24, 48 and 72 hours post inoculation (pi) by measuring plaque latitude and penetration depth underneath the basement membrane (BM). BoHV-1 spread plaquewise in the epithelium and penetrated the BM. Viral plaques were observed at 24hpi for all isolates in both respiratory and genital mucosa explants with the plaque latitude increasing over time. At 24hpi, all the different subtypes spread more efficiently lateral in the epithelium of the genital tract compared to the respiratory tract. On the contrary, all BoHV-1 subtypes exhibited a more profound invasion capacity in the respiratory tract at 24h pi compared to the genital tract. These findings take the edge of the belief of the existence of specific mucosa tropisms of different BoHV-1 subtypes.

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Role of p21-activated kinases 1 and 2 in pseudorabies virus US3-mediated effects on apoptosis and nuclear egress

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The pseudorabies virus (PRV) is a member of the alphaherpesvirus subfamily of the herpesviruses, and is often used as a model to study general aspects of this virus subfamily (Pomeranz et al., 2005, Microbiol Mol Biol Rev). The alphaherpesvirus subfamily contains closely related viruses of man and animal, including the human herpes simplex virus type 1 and type 2 and varicella zoster virus and different animal pathogens, including PRV in pigs, equine herpesvirus 1 in horses, bovine herpesvirus 1 in cattle, and Marek’s disease virus in poultry.

The alphaherpesvirus US3 kinase is a conserved multifunctional serine/threonine kinase that plays a role in several processes, including regulation of actin dynamics, egress of virus particles from the nucleus and apoptosis. Recently, we identified group A p21-activated kinases (PAKs) PAK1 and PAK2 as important effectors in the PRV US3-mediated rearrangements of the actin cytoskeleton (Van den Broeke et al., 2009, PNAS). Here, we investigated if PAKs are also involved in other functions mediated by US3. Like US3, PAKs have been reported to display anti-apoptotic properties (Rudel and Bokoch, 1997, Science; Schurmann et al., 2000, Mol Cell Biol). Using PAK1⁻/⁻ mouse embryonic fibroblasts (MEF) and PAK2⁻/⁻ MEF, we found that PAK1 plays a significant, yet relatively minor role in the US3-mediated protection of cells against apoptosis induced by PRV infection or staurosporine addition, while PAK2 appeared not to be involved.

On the other hand, we obtained indications that PAK2 may be an important protein in the US3-assisted export of progeny virus from the nucleus, which occurs via transfer of virus via the perinuclear space to the cytoplasm. Infection of cells with US3null PRV results in accumulation of primary enveloped virions in the perinuclear space (Wagenaar et al., 1995, J Gen Virol; Klupp et al., 2001, J Gen Virol). In PAK2⁻/⁻ MEF cells we found a similar amount of virus particle accumulation in the perinuclear space upon wt PRV infection as in control MEF cells infected with US3null PRV, while this was not the case in PAK1⁻/⁻ MEF cells. In addition, the perinuclear space showed dilations in PAK2⁻/⁻ MEF cells upon infection, while the perinuclear space was regular in control MEF and PAK1⁻/⁻ MEF cells.

Taken together, these data point at a significant yet minor role of PAK1 in the anti-apoptotic activity of PRV US3, and a possible role for PAK2 in structural integrity of the nuclear envelope and in nuclear export of PRV. These data further point at separate roles for PAK1 or PAK2, both in cell biology and in PRV infection.
Identification of the CD163 protein domains involved in infection of the porcine reproductive and respiratory syndrome virus.

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The porcine reproductive and respiratory syndrome virus (PRRSV) has a restricted tropism for cells of the monocyte/macrophage lineage. Two macrophage-specific molecules are essential during PRRSV infection, the siglec sialoadhesin mediating virus binding and internalization, and scavenger receptor CD163 most probably involved during virus uncoating (reviewed by Van Breedam, 2010). The interaction between PRRSV and sialoadhesin is well characterized, with the N-terminal domain of sialoadhesin being essential for PRRSV binding and internalization, and most recently identification of the M-GP5 complex as the viral ligand interacting with sialoadhesin. In contrast, much less is known about the interaction between PRRSV and CD163. CD163 is a member of the scavenger receptor cysteine-rich (SRCR) superfamily, and is composed of nine extracellular SRCR domains, a transmembrane domain, and a short cytoplasmic tail (reviewed by Van Gorp, 2010). In this study we wanted to determine the CD163 protein domains that are required for PRRSV infection. To this end, CD163 deletion mutants were created, and domain swapping was performed between porcine CD163 and human CD163-L1, which does not sustain PRRSV infection. Infection experiments revealed that SRCR domain 5 is essential for PRRSV infection, while the four N-terminal SRCR domains and the intracellular cytoplasmic tail of CD163 are not required. Remaining CD163 protein domains need to be present, but can be replaced by similar ones resulting in reduced (SRCR6 and interdomains) or unchanged infection efficiency (SRCR 7-9). In addition to the infection experiments, a CD163-specific polyclonal antibody was selectively depleted on different chimeric receptors. The depleted antibodies were used to inhibit PRRSV infection in primary alveolar macrophages, confirming that the blocking effect of the pAb is mainly due to the antibodies recognizing an epitope present in SRCR 5.

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The α-2,3-sialyltransferase encoded by Myxoma Virus is not essential for virus replication in vitro but contributes to virulence in vivo.

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Myxoma virus is a pathogenic Poxvirus that induces a lethal disease called Myxomatosis in European rabbits. Myxomatosis is characterized by fulminating lesions at the primary site of inoculation, followed by rapid dissemination to internal organs, production of external secondary lesions and bacterial superinfection. Myxoma virus is one of the very rare viruses that encodes an α-2,3-sialyltransferase that transfers sialic acid from CMP-Sia to glycoproteins and glycolipids. Very little information is available about the role played by this glycosyltransferase in the biology of the infection and in the pathogenesis. Here, we report the characterization of two recombinant Myxoma virus strains deleted for the M138L gene encoding the α-2,3-sialyltransferase and a derived revertant strain. After a classical in vitro characterization that did not reveal major differences between the recombinant strains and the wild-type strain, the virulence of the three recombinant strains was compared to the parental strain by intradermal inoculation of rabbits. Our data show that the M138L deleted strains are highly attenuated in vivo in comparison with the revertant and the parental strains. All but one rabbits inoculated with the deleted strains survived the infection while all rabbits infected with the parental and the revertant strains died within 9 days post-infection. Moreover, rabbits infected with the deleted strains survived to a subsequent challenge with the parental strain. Histological analysis of tissue materials from the primary site of infection revealed that by opposition to wild-type parental and revertant strains, an intense heterophils infiltration was observed all over the dermis at day 4 post-infection in deleted strains infected rabbits and a widespread mononuclear cell infiltrate was present at day 9 post-infection. Finally, two-dimensional differential gel electrophoresis (2D-DIGE) identified some virulence factors that are post-translationally modified by the viral α-2,3-sialyltransferase. The absence of these modifications could be responsible of the in vivo attenuation observed in this study. All together, these results demonstrate therefore that, although non essential, the α-2,3-sialyltransferase is a virulence factor for Myxoma virus pathogenesis in the European rabbit.

In the future, this work should help us to understand how the α-2,3-sialyltransferase could regulate the host’s immune response to infection.

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Interferon alpha suppresses alphaherpesvirus immediate early protein levels in sensory neurons, leading to the establishment of a latent infection in vitro

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Alphaherpesviruses contain closely related human and animal pathogens, including human herpes simplex virus (HSV-1) and porcine pseudorabies virus (PRV).

Cycles of latency and reactivation are a crucial characteristic of these alphaherpesviruses, e.g. leading to recurrent episodes of cold sores and genital lesions in HSV. Neurons of the trigeminal ganglion (TG) are the predominant site of latency in both HSV-1 and PRV. Establishment of latency is the result of a delicate balance between virus, neuron, and unidentified immune effectors.

Using an in vitro system based on porcine TG cultures, we have identified interferon alpha (IFN) as an immune effector that is capable to direct both HSV-1 and PRV in a latency-like state in vitro.

The IFN-induced establishment of in vitro latency was found to correlate with IFN-mediated suppression of the immediate early (IE) protein ICP4 in HSV-1 and its orthologue IE180 in PRV. IFN-mediated IE suppression was more efficient and rapid in HSV-1 than in PRV, correlating with a more efficient establishment of in vitro latency using HSV-1 versus PRV.

To further investigate the mechanism of IFN-mediated IE suppression and the differences in its efficiency between HSV-1 and PRV, rat dorsal root ganglion neuronal cells (50B11) (Chen et al., 2007) were used.

At 4h post inoculation (hpi), for HSV-1, ICP4 protein expression was strongly (75%) reduced in IFN-treated samples while for PRV, IFN treatment only slightly affected IE180 protein levels (15% reduction). At 8hpi and 12hpi, IE protein levels were strongly suppressed for both viruses. Using qRT-PCR, mRNA levels of either HSV-1 ICP4 or PRV IE180 at 4hpi were not significantly different in IFN-treated samples versus control samples, whereas they were strongly reduced at 8hpi and 12hpi (76.5 to 96%). This indicates that IFN induces a rapid translational block of ICP4 in HSV-1 but not of IE180 in PRV, and a slower transcriptional block of IE genes in both viruses.

To investigate the lack of rapid IE translation inhibition during PRV infection, we analyzed IFN-mediated phosphorylation and thereby inactivation of the translation initiation factor eIF2α. Treatment of cells with IFN and subsequent infection with HSV-1 resulted in a strong increase in phosphorylation of eIF2α. This increase was entirely absent in PRV-infected cells, showing that PRV circumvents IFN-mediated translation inhibition by interfering with phosphorylation of eIF2α.

In summary, IFN-mediated suppression of viral IE proteins may be a key step in establishment of alphaherpesvirus latency. IFN acts at two stages to suppress IE protein levels: first at the translational level and later at the transcriptional level. However, PRV (but not HSV-1) is able to avoid IFN-mediated translational control of IE levels by phosphorylation of the translation initiation factor eIF2α.
Anti-HIV-1 activity and pharmaceutical availability in biorelevant media of gel-formulated saquinavir as a vaginal microbicide.

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The HIV protease inhibitor saquinavir is an interesting microbicide candidate for its potent anti-HIV activity and favorable resistance profile. Here, we wanted to evaluate the formulateability of this poorly water-soluble compound in vaginal microbicide gels in terms of anti-HIV-1 activity and pharmaceutical availability in biorelevant media.

Saquinavir mesylate was dissolved in an aqueous hydroxyethylcellulose gel (pH 4.6) at a concentration up to 2 mg/g (10⁵-fold in vitro anti-HIV-1 IC₅₀ values). The in vitro anti-HIV-1 activity of saquinavir was preserved in the formulation: comparable mean IC₅₀ values were obtained for formulated and native saquinavir mesylate (10 and 13 ng/ml, respectively). In the vaginal environment, potential precipitation of saquinavir in vaginal fluid or semen may reduce its availability. Therefore, saquinavir concentrations were monitored upon adding the formulation to vaginal fluid simulant (VFS) or semen simulant (SS) (1:1 dilution). Dilution of the saquinavir mesylate solution with VFS resulted in preservation of its antiviral activity, both at low (0.1 mg/g) or high (1 mg/g) dose, without noticeable precipitation. Comparable data were obtained for low dose saquinavir in combination with VFS and SS. However, dilution of high dose saquinavir (1 mg/g) with VFS and SS caused immediate precipitation of saquinavir with subsequent reduction in anti-HIV-1 activity (5-fold increase in IC₅₀ value).

Inclusion of the solubilizing excipients polyethylene glycol 1000 (12%) and hydroxypropyl-β-cyclodextrin (2.5%) to the formulation reduced the extent of precipitation of saquinavir and restored its antiviral potency.

In conclusion, while the mesylate salt of saquinavir allows the formulation of a relatively high concentration (1 mg/g) in vaginal gels, the present study suggests precipitation of the active ingredient in the vaginal environment. Thus, solubilizing excipients are required to avoid precipitation and to preserve the antiviral activity of saquinavir. This study illustrates the importance of evaluating the pharmaceutical availability of poorly water-soluble microbicide candidates in biorelevant media.
Feline cell-mediated immune response against viral pathogens using 5-bromo-2’-deoxyuridine (BrdU) labeling

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The feline immune response against viruses consists of a humoral and cellular immune response. At present, four immune parameters can be monitored by flow cytometry in order to evaluate the CMI: activation-proliferation, cytokine-production, cytotoxicity and receptor-detection.

In this study, the lymphocytic proliferative response to infectious and inactivated virus (feline calicivirus (FCV), feline parvovirus (FPV) and feline herpesvirus 1 (FHV)) was investigated in vitro by using 5-bromo-2’-deoxyuridine (BrdU) labeling in combination with a double staining (CD3 - CD8) and subsequent flow cytometrical analysis to specifically identify the subset of proliferating T cells. Macrophages (MΦ) and monocyte derived dendritic cells (mDC) were used as antigen presenting cells (APC). Additionally, the production of interferon-γ, the signature molecule of Th1 cells, was evaluated in cell culture supernatant using an enzyme-linked immune sorbent assay (ELISA). Briefly, feline MΦ and mDC from FCV/FPV/FHV-vaccinated cats were incubated with several infectious viruses (FCV, FHV and FPV) and with equivalent amounts of inactivated virus. After washing, the cells were further incubated with 1.10^5 ml^-1 autologous lymphocytes for 4 days at 37°C, 5% CO₂. Cells were then stained for proliferation with BrdU and against CD3 and CD8 with specific monoclonal antibodies to identify the proliferating cell population. With mDC as APC, significant proliferation signals (comparable to mitogen treated lymphocytes) were seen when adding inactivated FCV, FHV and FPV. When adding infectious virus, severe proliferation suppression was seen with FPV and FHV in comparison with inactivated virus. Infectious FCV induced a proliferation equivalent to that of inactivated virus. The distribution of all signals was comparable between the CD8+ and CD8- lymphocyte population. When MΦ were used as APC, both CD8+ and CD8- lymphocyte populations did not show any significant proliferation when incubated with infectious and inactivated virus, with the exception of inactivated calicivirus which gave a 12.2% proliferation signal in the CD8-lymphocyte population. Additionally, the IFN-γ concentrations found in the culture supernatant concurred well with the amount of proliferation of the DC-stimulated lymphocytes, validating this technique as a tool for studying the feline CMI against viruses.

To the author’s knowledge this is the first time that the BrdU technique was used in combination with MΦ and mDC as APC and both infectious and inactivated virus in order to evaluate the CMI of individual cats upon virus vaccination and subsequent viral infection in vitro.

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A genome-wide genetic screen for host factors required for gammaherpesvirus infection

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Gammaherpesviruses are archetypes of persistent viruses that have been identified in a range of animals from mice to man. They are host-range specific and establish persistent, productive infections of immunocompetent hosts without immunosuppression, viral expression products tolerance or significant antigenic switch. Most of the gammaherpesvirinae members are associated with neoplastic disease. For example, the best studied gammaherpesviruses are Human herpesvirus 4 and 8 that are respectively associated with Burkitt’s lymphoma and Kaposi’s Sarcoma. Although gammaherpesvirus genomes encode numerous proteins, these viruses depend heavily on host factors for propagation. Each of these host factors dependencies is a potential therapeutic target. To find critical host factors required by gammaherpesviruses, we are completing a genome-wide small interfering RNA (siRNA) screen using Murid herpesvirus 4 (MuHV-4) as model. We first transduced murine NIH 3T3 cells with a commercially available lentiviral vector-based shRNA expression library. This library contains about 150,000 shRNAs predicted to target 39,000 murine mRNAs. Relatively low multiplicities of infection were used in order to avoid expression of multiple different shRNAs in individual cells. Untransduced cells were eliminated by antibiotic treatment and surviving cells were pooled and challenged with an eGFP expressing MuHV-4 strain. The amount of MuHV-4 eGFP virus used was adjusted so that about 100% of the parental cells would be infected. eGFP-negative cells (hence resistant to MuHV-4 infection) were sorted by flow cytometry and cloned. These clones were then expanded and superinfected with MuHV-4 eGFP virus in order to allow classification in three groups: weakly, moderately and highly resistant. Finally, in order to specify the nature of the resistance, these clones were subjected to binding assay with MuHV-4. This revealed a decreased binding of MuHV-4 to nine of these clones. In the near future, amplification of shRNAs sequences should allow us to indentify the cellular genes that are targeted in these individual clones. The identification of host factors participating in the complete MuHV-4 cycle will not only help us to better understand the biology of gammaherpesviruses, but should also reveal new therapeutic targets to fight these viruses.

S.V. and L.G. are Research Fellow and Research Associate of the “Fonds de la Recherche Scientifique - Fonds National Belge de la Recherche Scientifique” (F.R.S. - FNRS), respectively. S.F. is a Research Fellow of the Belgian ‘Fonds pour la formation à la Recherche dans l’Industrie et dans l’Agriculture’. P. G. S. is a Wellcome Trust Senior Clinical Fellow (GR076956MA). This work was supported by the following grants: starting grant of the University of Liège (D-09/11) and scientific impulse grant of the F.R.S. – FNRS n° F.4510.10.
ABSTRACTS OF PARTICIPANTS

Parasitology
Trypanosomes adapt their energy metabolism to the available carbon sources, which differ in their mammalian and insect hosts. This requires alternative metabolic pathways depending on the environment. Oxidative stress plays an important role in the parasite’s life cycle, as does NADPH, which contributes to the trypanothione system and the parasite’s oxidative stress defence. Glucose-6-phosphate dehydrogenase (G6PDH) is the main NADPH source in the cytoplasm and the glycosomes. But as the G6PDH uses glucose-6-phosphate as substrate, there have to be redundant NADPH sources which are independent of glucose as available carbon source. We postulate that the glycosomal isocitrate dehydrogenase (IDHg) and the cytoplasmic malic enzyme (MEc) are the alternative NADPH sources in the absence of glucose in the fly.

Here we present the results of our studies on the IDHg and the MEc as NADPH sources. To analyse the role of the MEc, we used the Alamar Blue Assay in a 96-well format. To investigate the role of the IDHg in vivo, Tsetse flies were transfected with the TbΔIDHg cell line. First results indicate that the IDHg is involved in the maturation of the parasite in the fly.

Additionally a polyclonal anti-TbIDHg antibody was generated to investigate stage-specific expression levels and to verify the localisation of the IDHg in T. brucei.
A role of *T. brucei* PKA-like kinases in cold-induced stage differentiation and motility

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Cyclic AMP is an important second messenger in a broad spectrum of organisms. The *Trypanosoma brucei* genome harbours a large number of adenylate cyclase and phosphodiesterase genes, yet the target of cAMP has been elusive. We have identified a small family of PKA-related kinases that are not activated by cAMP. With reverse genetic manipulation of intracellular cAMP levels, targeted deletion of kinase subunits and a transgenic reporter kinase assay, we demonstrate that PKA-like kinases are not regulated by cAMP *in vivo*. Sequence deviation of the cNMP binding sites of the R subunit is consistent with the absence of cAMP binding. Here we show that the kinase is specifically activated in the bloodstream stage of the parasite by cold shock, an established differentiation trigger in the parasite’s life cycle (Engstler and Boshart, 2004). We suggest that PKA-like kinase gives insight into the evolution of PKA by recruitment of an alternative activation mechanism in an early branching group of protozoa. High resolution fluorescence microscopy, colocalisation analysis, and electron microscopy revealed that the regulatory subunit (PKAR) is associated with an axoneme proximal substructure of the flagellum, the paraflagellar rod. Targeted disruption of PKAR gave a flagellar motility phenotype that was fully rescued by ectopic wild type level expression of the R subunit. Detailed analysis suggests that PKA-like kinase is important for phase synchronisation of the emerging daughter flagellum with the parental flagellum.

Molecular mechanisms involved in the controlled degradation of glycosomes in trypanosomes and a determination of the importance of these processes.

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Trypanosoma brucei is the parasite responsible for sleeping sickness that affects people and livestock in the sub-Saharan region of Africa. Trypanosomatids possess peroxisome-like organelles called “glycosomes”. These are unique in the sense that they enclose most enzymes of the glycolytic pathway. This compartmentalisation has been shown to be essential for the stage of the parasite living in the mammalian bloodstream, where glycolytic enzymes make up 90% of the glycosomal protein content. In contrast, other enzymes are more prominent in glycosomes of the procyclic stage (representative of parasites in the tsetse fly) with the glycolytic enzymes comprising only 40%. This difference reflects the parasite’s dependence on glucose in the bloodstream stage and its more flexible metabolism in the procyclic stage, with amino acids as major energy source.

Different enzymatic content has also been reported for yeasts growing in different media. Pexophagy (autophagy of peroxisomes) participates in the adaptation of yeasts to changes in nutrient source, with old peroxisomes being targeted for recycling, while new ones, still protein-import competent, are spared.

Previous morphological studies have shown the presence of autophagy in different trypanosomatids, and genetic studies showed its crucial role in the differentiation from one life-cycle stage to the next for organisms closely related to T. brucei. Our previous morphological analysis indicated that glycosome turnover in T. brucei also involves pexophagy (HERMAN et al., 2008). An in silico study by our laboratory has identified in the trypanosomatids around 20 genes that are homologous to yeast genes involved in autophagy (AuTophaGy-related genes or ATGs) (HERMAN et al., 2006). We are now targeting two of these genes (ATG24 and VAC8) that may be involved in pexophagy, as well as ATG8 which participates in general autophagy, to determine their importance in the parasite’s adaptation and specifically glycosome turnover during differentiation. We follow the parasites during starvation, adaptation to changes in nutrient source and differentiation while using the RNAi approach as well as performing morphologic and localisation studies of these proteins.

Our preliminary results indicate that, although the RNAi was never able to completely knock down the expression of these proteins, they do not seem to be essential under normal growth conditions in vitro, where there is excess of nutrients and autophagy is not expected to be important. Their subcellular localisation is in agreement with that observed for their yeast counterparts. We are currently studying the effects of compounds and conditions that will block or induce autophagy during the change from one nutrient source to another as well as during in vitro differentiation.

In conclusion, we are trying to prove the importance of the recycling of glycosomes during the parasite’s differentiation by targeting putative pexophagy-specific proteins. Our RNAi approach has not been suitable to completely knock down the target proteins, but compounds that, in various organisms, have been shown to interfere with different stages of autophagy, are now being used during adaptation to changes in nutrient source and in vitro differentiation to further characterise our target proteins as well as autophagy in T. brucei.

A. Brennand is recipient of a FRIA fellowship.

Expression of trypanosome-development inhibitory proteins into the periplasm of *Sodalis glossinidius*, a bacterial symbiont of the tsetse fly.

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Paratransgenesis focuses on the use of genetically modified symbionts to express molecules within the vector that are deleterious to the pathogens they transmit. *Sodalis glossinidius* is a gram-negative bacterial endosymbiont of the obligate blood feeding tsetse fly, the insect vector of trypanosome parasites that cause African trypanosomiasis.

To eliminate parasites in the tsetse fly midgut, it is imperative that the effector molecules are delivered in an active form to the midgut lumen. Secretion of recombinant proteins to the extracellular environment or periplasm has several advantages over cytoplasmic production in terms of enhanced biological activity, higher product stability and solubility. However, our current knowledge on the functional secretion pathways to the periplasmatic and/or extracellular environment of *Sodalis* is very limited.

The objective of this work was to develop a periplasmic expression system for which we analysed the functionality of the twin-arginine translocase (Tat) pathway in *Sodalis*. For that purpose, *S. glossinidius* was transformed with a plasmid containing the green fluorescent protein (GFP) reporter gene fused to the signal peptide of *E. coli* TMAO reductase (TorA) which is known to be transported across the cytoplasmatic membrane via the Tat translocation system. Fractionation studies and fluorescence measurements demonstrated that GFP is exported to the periplasm where it is fully active. Imaging studies on transformed cells further illustrated that GFP-fluorescence is confined to the periplasmic space. These data also show for the first time that heterologous proteins can be exported to the periplasm of *S. glossinidius* in an active form by the Tat pathway.

Next, a battery of trypanosome-interacting monoclonal antibodies (mABs) are being expressed in the periplasm of *Sodalis*. After re-introducing recombinant *Sodalis* in the tsetse fly, the influence of mAB delivery will be evaluated in the context of trypanosome development in the tsetse fly midgut.

This research was supported by the Belgian Co-operation (DGOS), SOFI-B grant, InterUniversity Attraction Pole programme (IAP) and FWO (1.5.147.09) for funding a part of the equipment.
Glycosomes of trypanosomatid parasites; biogenesis and function offer perspectives for drug development against African sleeping sickness


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Trypanosomatids are parasitic protists which contain peroxisome-like organelles called glycosomes, in which important metabolic pathways such as glycolysis are compartmentalized in addition to regular peroxisomal enzymes. *Trypanosoma brucei* is responsible for human African sleeping sickness, a disease for which no adequate treatment is available. Currently available treatment has limited efficacy, may have serious side effects and drug resistance is spreading. The development of new efficacious drugs is badly needed. *T. brucei*, living in the mammalian bloodstream, is entirely dependent on glycolysis for its ATP supply. The parasite’s glycolytic enzymes are considerably different with respect to their function and structure from their human counterparts. The differences can in part be attributed to the long evolutionary distance between parasites and humans, but also to the unique compartmentalization of glycolysis in the trypanosomatids. These differences, and the essentiality of glycolysis for the parasites render glycolytic enzymes potentially promising targets for new drugs to be developed. Such compounds should inhibit the activity of the *T. brucei* glycolytic enzymes without having any affinity for the corresponding (or any other) enzyme of the human host. Furthermore, we have shown for the majority of the glycolytic enzymes that their depletion from the cell by RNA interference-mediated knockdown of their expression, to reduce the glycolytic flux by 50%, is already sufficient to kill the parasites. Biogenesis of glycosomes occurs via similar routes as that of human and yeast peroxisomes. Peroxisomal/glycosomal matrix enzymes are synthesized in the cytosol and, upon recognition of their peroxisome-targeting signal (PTS), post-translationally imported, usually without processing. Partial mislocalization of a glycosomal enzyme in the cytosol, by the expression of an additional transgene coding for a matrix enzyme without PTS, is detrimental. Import of enzymes into the organellar matrix is mediated by proteins called peroxins (acronym PEX) that exert their functions through a cascade of protein-protein interactions. In yeasts and other eukaryotic organisms together, 33 peroxins have been characterized; for *T. brucei* 12 orthologues have been identified and their function in glycosome biogenesis confirmed. The PEX sequences are poorly conserved; the amino-acid sequence identity between the peroxins of *T. brucei* and the human host is typically between < 10% and approximately 35%. Expression knockdown of trypanosomatid peroxins leads to partial relocation of some glycosomal enzymes to the cytosol and subsequent growth arrest followed by death of the parasite. The essentiality of peroxins for the correct compartmentalization of glycosomal matrix enzymes and consequently for the viability of the trypanosomes, together with the low sequence conservation, make also the peroxins promising candidates for drugs. It might be feasible to develop compounds interfering with the interactions between trypanosome peroxins or between peroxins and the matrix proteins to be imported, without doing so in host cells. We present here some results of our studies of essentiality and ‘druggability’ of the trypanosomatid proteins, glycolytic enzymes and peroxins, and the efforts to develop compounds that interfere with their function and affect the viability of trypanosomes, without inhibiting the corresponding protein in the human host and growth of human cells.

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Anomeric forms of sugars and sugar-phosphates in glycosomal carbohydrate metabolism of trypanosomatids

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Trypanosoma brucei is a flagellated parasite of the order Kinetoplastida causing sleeping sickness in human beings and a similar disease called ‘nagana’ in cattle. Trypanosomes contain unique peroxisome-like organelles designated as glycosomes, which contain enzymes involved in a variety of metabolic processes such as the glycolytic and pentosephosphate pathways and routes of lipid anabolism and catabolism.

Trypanosomatids possess a hexokinase (HK) inside the glycosomes. In addition, a glucokinase (GlcK) was found in glycosomes of Trypanosoma cruzi and Leishmania species, but not in those of Trypanosoma brucei (CACERES et al., 2007). The crystal structure of TcGlcK with bound glucose shows that the sugar is present in its beta-anomeric form (CORDEIRO et al., 2007). In contrast, all known HK-glucose structures possess alpha-glucose, in line with results from kinetic studies that HKs have a preference for this latter anomer.

The enzymes following the sugar-kinases in the glycolytic and pentosephosphate pathways, phosphoglucone isomerase and glucose-6-phosphate dehydrogenase (G6PDH), are highly specific for respectively the alpha- and beta-form of glucose 6-phosphate. Although both forms can spontaneously anomerize, the rate of this interconversion may be too low if a high activity of the pentosephosphate pathway is required (for NADPH production) and only a HK is present that produces essentially the alpha-anomer. In T. brucei, where GlcK is absent, a glucose-6-phosphate-1-epimerase (G6PE) (also known as hexose-6-phosphate mutarotase) may therefore be required for the anomerization.

A candidate G6PE gene was found in the trypanosomatid genome databases. The predicted amino-acid sequence contains a typical peroxisome-targeting signal (PTS1) that suggests a glycosomal localization of the enzyme. The G6PE activity of the enzyme was confirmed after its expression in Escherichia coli as a recombinant protein. Knocking down its expression in bloodstream-form trypanosomes has no effect on the growth of the parasites in regular growth medium. However, the RNAi-induced cells display an increased susceptibility to oxidative stress in non-reducing medium, similar to results obtained for trypanosomes in which G6PDH has been depleted. These results suggest a role of glucose-6-phosphate-epimerase in the interconversion of alpha- and beta-anomers inside glycosomes of Trypanosoma brucei making glucose 6-phosphate available for the pentosephosphate pathway and consequent NADPH production.


Leishmania major infection modulates human macrophage miRNA expression to counteract early host cell apoptosis.

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Leishmania major is the causative agent of cutaneous leishmaniasis, which is characterized by the development of lesions at sand fly bite sites. This obligate intracellular parasite inhabits the phagolysosomes of macrophages. Manipulation of host cell signalling pathways and gene expression is critical for the parasite survival. L. major is highly adept at manipulating the macrophages’ apoptosis pathway by inhibiting it during the early times of primary human macrophage infection(1). Here, we present the results of our study on the modulation of miRNAs expression and apoptosis in primary human macrophages during an infection with L. major.

During this work, miRNAs expression was studied by using microfluidics cards that allow simultaneously the detection of 384 human miRNAs. The experimental conditions are: non-infected (NI) and infected (IF) human primary macrophages after 3h, 6h, 12h and 24 hours post-infection with L. major. This analysis allowed us to highlight two miRNAs (mir133b and mir210) that are involved in apoptosis regulation and which expression is modified during infection. More precisely, infection-induced miR210 overexpression could contribute to caspase-3 downregulation, which was observed by Western Blot analysis in infected samples.

On the other hand, since it is the first time that miR210 induction is shown in leishmania-infected macrophages, we searched for a pathway that could be triggered by the parasite infection and lead to miR210 induction. In hypoxic conditions, miR-210 induction is known to be controlled transcriptionally by HIF-1α, and we showed an activation of this transcription factor in leishmania-infected samples. In addition, HIF-1α stabilisation and activation is correlated to an increased phosphorylation of AKT.

To validate our hypothesis of a p-AKT/HIF-1α/mir210/caspase pathway, we plan to use primary human macrophages transfected with anti-HIF-1α siRNA. These cells will be infected with L. major and different actors of apoptosis will be analysed. Moreover, addition of etoposide (a topoisomerase II inhibitor used to induce apoptosis in mammalian cells) during infection of transfected cells will determine if the parasite is still able to inhibit this induced apoptosis.

The molecular events that drive parasite survival and replication inside the macrophage are under active investigations, but the informations are yet fragmentary. Altogether, our results could highlight the role of miRNA through a pAKT/HIF-1α pathway to impair the apoptotic response of the macrophage during an infection with L. major.

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Trypanosoma brucei glycosomal ABC transporters potentially involved in fatty acid transport

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Trypanosoma brucei is a flagellated protozoan of the order Kinetoplastida which causes sleeping sickness in humans. The parasite is transmitted between mammalian hosts by tse-tse flies. The life cycle of T. brucei involves two main forms of the parasite: the procyclic form, present in the intestine of the insect, and the bloodstream form living in the mammalian host. Trypanosomes contain unique peroxisome-like organelles designated glycosomes which contain enzymes involved in a variety of metabolic processes. Glycolytic enzymes make up 90% of the protein content of glycosomes in bloodstream forms. In contrast, in procyclic insect forms, the glycolytic enzymes comprise only 40-50% of the organelles’ protein content. Glycosomes of cultured procyclics have increased levels or activities of enzymes of both catabolism, such as the hexose-monophosphate pathway and β-oxidation of fatty acids, and anabolic processes such as purine salvage, pyrimidine biosynthesis and ether-lipid biosynthesis.

Despite good knowledge of the metabolic processes occurring within glycosomes, there is very little knowledge about the transport of metabolites through the boundary membrane of these organelles. Previously, we identified three homologues of peroxisomal ABC transporters in T. brucei (YERNAUX et al., 2006). These proteins, which belong to subgroup D of the ABC transporter family, were shown to be associated with the glycosomal membrane and were designated GAT1-3 for Glycosomal ABC Transporters. Moreover, the region containing the glycosome targeting determinant was determined.

We started to undertake an investigation of the function of these transporters, addressing the question if they are involved in uptake of proteins, or if they have primarily a function in metabolism by import or export of metabolites. First, we observed that GAT1 and GAT3 are expressed in both the procyclic and the bloodstream form, whereas GAT2 is mainly expressed in bloodstream-form. The topology of the transporters in the membrane was determined by protease protection experiments which showed that the ATP-binding domain is at the glycosomal membrane’s cytosolic face, making it likely that the transporters function as importers. Depletion of GAT1 and GAT3 by RNAi in procyclic cells cultured in glucose-containing medium did not affect growth. However, knockdown of GAT1, but not GAT3, in procyclics cultured in glucose-free medium was lethal. These experiments suggest a possible role of this transporter in the energy metabolism under conditions of low or zero glucose levels.

To determine if these transporters were involved in fatty-acid metabolism, we compared the fatty-acid composition of wild-type trypanosomes and GAT1 depleted cells. Upon GAT1 silencing, only minor changes were observed in the levels of most fatty acids, including oleate (C18:1), yet the linoleate (C18:2) abundance was significantly increased. Furthermore, glycosomes purified from procyclic wild-type cells incorporate oleoyl-CoA in a concentration- and ATP-dependent manner, while this incorporation was severely reduced in glycosomes from cells with decreased GAT1 levels. Together, these results strongly suggest that GAT1 serves to transport primarily oleoyl-CoA into the glycosomal lumen. The increased level of linoleate rather than oleate, in the GAT1-depleted cells may be attributed to the high activity of an oleate desaturase observed in procyclic trypanosomes.

IL-10 limits the pathogenic TNF production by liver M1 myeloid cells through the induction of nuclear p50 NF-kB during parasitic infection

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We use parasitic infections as models to unravel the contribution of myeloid cells to liver injury, aiming to identify new therapeutic leads to treat hepatic inflammation in general and parasite-induced hepatic injury in particular. The development of classically activated myeloid cells (M1) is a prerequisite for effective elimination of African trypanosomes. However, persistent activation of M1 and associated production of TNF and NO cause tissue injury including liver cell necrosis, hereby negatively affecting survival of the infected host. In relatively tolerant models of trypanosome infection, such as Trypanosoma congolense infection in C57BL/6 mice, we observed an accumulation of bone marrow-derived CD11b+ Ly6G- Ly6C+ inflammatory myeloid cells in the liver of infected mice. These myeloid cells were characterized as a main pathogenic M1 subpopulation that produces TNF and iNOS. Using in vivo anti-IL-10R antibody treatment we show that IL-10 reduces liver injury and increases survival during T. congolense infection by limiting both recruitment and M1-type activation of CD11b+ Ly6G- Ly6C+ inflammatory myeloid cells. Indeed, in absence of IL-10R signaling, CCL2/CCR2 dependent accumulation of liver CD11b+ Ly6G- Ly6C+ myeloid cells and their TNF and iNOS expression level is drastically increased. In addition, by using myeloid cell-specific IL-10 KO mice, we show that IL-10 derived from myeloid cells is involved in limiting TNF production by CD11b+ Ly6G- Ly6C+ inflammatory myeloid cells during T. congolense infection. Moreover, we provide evidence that this IL-10-dependent suppression of TNF production is regulated via the NF-kB family of transcription factors. Indeed, a preferential nuclear accumulation of the p50 NF-kB subunit was observed that could block M1 activation in liver myeloid cells in an IL-10 dependent manner, providing a possible downstream mechanism for the anti-inflammatory role of IL-10 on liver M1 activation.
Protective vaccination against histomonosis in turkeys using *in vivo* passaged *Histomonas meleagridis* protozoa with reduced pathogenicity

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Histomonosis (blackhead disease or infectious enterohepatitis) caused by the extracellular protozoon parasite *Histomonas meleagridis* is an important disease of turkeys and a serious threat to the poultry industry. Currently, no effective therapeutic or prophylactic measures against histomonosis are available in the European Union due to recent restrictions in drug legislation. Research for new alternatives is hence an urgent matter. Therefore we attempted to attenuate a virulent strain for use as a vaccine against the disease. The *in vivo* attenuated strain originated from a diseased turkey naturally infected with *H. meleagridis* from a backyard breeder in Belgium. This virulent MCL-strain has been repeatedly used for experimental *H. meleagridis* infections with high morbidity and mortality rates. After having been consecutively cloacally passaged 10 times at 7 days interval, the MCL.C1.L10-strain seemed to have reduced pathogenicity as it induced not any liver or caecal lesions on subsequent inoculations. Only, dilated caeca with a yellow and foamy content could be noticed. The pathogenicity of the attenuated MCL.C1.L10-strain was then compared with that of a virulent *H. meleagridis* strain from an *in vitro* culture (HNA.C2.L1) by cloacal inoculation of birds. No mortality (0%) nor predominant caecal or liver lesions could be recorded in the groups inoculated with $10^3$, $10^4$ or $10^5$ histomonads per bird of the attenuated strain. The groups inoculated with similar doses of the virulent strain, displayed a dose related pathology and mortality up to 94%. Apparently, severe attenuation is associated with a reduced capacity to penetrate through the caecal wall and hence a blocked migration to the liver. The vaccinating protective capacity of the attenuated strain could be demonstrated as none of the birds died upon challenge with the virulent strain. Fourteen-day-old birds were cloacally vaccinated with $10^3$, $10^4$ or $10^5$ histomonads. Following challenge with $10^5$ virulent *H. meleagridis*, the birds withstood the challenge. Hereby, no mortality (0%) was observed whereas 79% of the unvaccinated birds died. Interestingly, no or very minor pathological lesions in the caeca and liver could be detected after challenge of the vaccinated birds. In conclusion, vaccination by cloacal application of an *in vivo* attenuated *H. meleagridis* strain was able to induce full protection of turkeys against histomonosis.

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ABSTRACTS OF PARTICIPANTS

Late abstracts
Study of surfactant effect on staphylococci biofilm and identification of NRPS genes in clinical *Staphylococcus* strains.

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The ability to adhere to artificial surfaces and to form biofilms is considered as a virulence factor of *Staphylococcus epidermidis* and other coagulase-negative staphylococci (CoNS), one of the major causes of the medical implant-related infections. At present, no medical treatment is described to efficiently prevent bacterial adhesion involved in these infections. Nevertheless bioactive molecules synthesized by the NRPS (non ribosomal peptide synthetases) are newly tested in vitro for their potential antibiotic or antibiofilm effect. We have constructed a representative library of *Staphylococcus* strains involved in infections. It consists out of 120 clinical isolates collected from French hospitals. Almost all strains were isolated from infected implanted devices.

Our main goal is to test the effect of some bioactive molecules from *Bacillus* sp. on degradation of staphylococci biofilm and to screen our strain collection for NRPS genes encoding bioactive secondary metabolites.

First, to identify the strains a phylogenetic tree based on partial 16S rDNA and *rpoB* gene sequences was constructed by the neighbor-joining method using the program MEGA. The most frequently isolated species were *Staphylococcus epidermidis* and CoN *Staphylococcus aureus*, followed by *Staphylococcus lugdunensis*, *Staphylococcus capitis* and *Staphylococcus warneri*.

Second, the surfactin, a lipopeptide from *Bacillus* sp. was tested to hydrolyze a preformed biofilm or to inhibit bacterial adhesion in vitro. In the former case, no biofilm detachment is observed in our conditions. In the latter case, decreasing in biofilm formation is only observed in few strains, *S. epidermidis* or *S. lugdunensis* but in a minor extent.

Then, our clinical collection of *Staphylococcus* strains was screened for NRPS genes by PCR. This screening has focused on surfactin, iturin and fengycine, the best known NRPS lipopeptides in *Bacillus*. The results show that about 30% of the isolates gave an amplicon during the PCR screening, indicating that they have putative NRPS genes coding for lipopeptides. Some of the fragments were cloned and sequenced. mRNA expression is currently being verified. The highest similarity was found to surfactin synthetase, as annotated in the GenBank sequence database.

We have shown the presence of NRPS genes in clinical *Staphylococcus* strains which provide evidence that different species of clinical *Staphylococcus* could produce natural products through NRPS pathways. Further analysis of these sequences may reveal a functional role in virulence in these staphylococci.
Evaluation of MALDI-TOF MS as a tool for high-throughput dereplication

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BACKGROUND. High-throughput dereplication tools are of utmost importance in any cultivation-dependent diversity study. Dereplication is a rather ambiguous term, originally referring to the grouping of isolates at the strain level, although nowadays used generally for the grouping of isolates at any taxonomic level in the first steps of an identification process. MALDI-TOF MS is a recent tool in microbiological research, with high taxonomic resolution, high speed and simplicity, low cost, making it an interesting tool for dereplication. Here, the applicability of MALDI-TOF MS for dereplication of an unidentified microbial diversity was assessed through comparison with rep-PCR, widely applied for dereplication at subspecies-to-strain level.

METHODS. 250 isolates were obtained from the rhizosphere of potato plants from the Central Andean Highlands. MALDI-TOF MS was performed on 4800 Plus MALDI TOF/TOF™ Analyzer (Applied Biosystems). Rep-PCR fingerprints were generated using the (GTG)₅ primers. Data generated was analyzed using BioNumerics 5.1 (Applied Maths).

RESULTS. Bacterial cell extracts were prepared from bacterial cells grown on 10-fold diluted TSA for 48h at 28°C. Mass spectra from 250 unidentified bacterial isolates were generated using the CHCA matrix. Also, rep-PCR fingerprints of alkaline lyses of all isolates were obtained. Reproducibility for both methods was determined by analyzing 10% of the isolates in triplicate. Clustering was done with the Pearson correlation coefficient. Clusters were delineated based on overall mean similarities between replicate profiles. All members of all clusters obtained from both techniques were identified through partial 16S rRNA gene sequence analysis for verification of cluster delineation.

CONCLUSIONS. This study is the first to evaluate the applicability of MALDI-TOF MS for dereplication of an unidentified microbial diversity, through comparison with rep-PCR. From our results it is clear that MALDI-TOF MS can be used as a cheap high-throughput dereplication tool with a taxonomic resolution situated at species to strain level.

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Challenging “The Great Plate Count Anomaly”: Isolation of Methanotrophic Bacteria

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BACKGROUND & AIMS. Methane oxidizing bacteria (MOB) are ubiquitous and play a vital role in reducing emissions of the greenhouse gas methane. The isolation of these fastidious organisms into pure culture has proven difficult and laborious. The majority are so far not-yet-cultured. Therefore, next to traditional agar-plating, non-conventional and/or miniaturized high throughput cultivation techniques have been developed recently. This study compares a miniaturized high-throughput extinction culturing approach with plating for the retrieval of novel methanotrophic bacteria.

METHODS. Samples were taken from a wastewater treatment plant, cow slurry pit, wetland and biofilter soil. Methane oxidation activity was measured with gas chromatography. MOB were enumerated with the MPN technique using gas-tight vials with methane added to the headspace. From these MPN enrichments, isolation was performed by miniaturized extinction culturing and plating (gellan gum as solidifying agent). Strains were screened via microscopy, GC and gene sequence analysis.

RESULTS. MOB were present in all four samples. Prolonged incubation of the MPN enrichments, up to 5 weeks at 20°C, was necessary for the higher dilutions to oxidize methane effectively. No MOB were isolated using the dilution plating technique. In contrast, MOB from all four samples could be isolated from the MPN enrichments using the extinction culturing technique performed in microtiter plates. Moreover, purity could be reached during extinction culturing, making purification steps on plates, often stated as problematic for MOB, redundant. The extinction approach was less laborious and increased capacity, few gas-tight jars required, compared with conventional plate isolation. Preliminary identification showed that several isolates will have to be assigned to novel methanotrophic species.

CONCLUSIONS. A miniaturized, high-throughput two-step liquid culturing protocol for rapid isolation of methanotrophic bacteria was successfully validated with three anthropogenic and one natural site. This procedure can be readily applied, in large scale, to other environmental samples.

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The who, where, when and why of endosymbiotic bacteria within the marine green alga 
Bryopsis (Bryopsidales, Chlorophyta)

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Endobiotic associations between marine macroalgal hosts and bacteria have been reported over the past 40 years. Only in the siphonous (single celled, multinucleate) chlorophyte Bryopsis, however, endogenous bacteria have been electron microscopically visualized in the cytoplasm at every stage of development, including the gametes, suggesting vertical transmission of the endosymbionts. This indicates a more stable and specific relationship between the algal host and its symbionts in which both partners may provide mutualistic ecological benefits. In this study, the presence of bacteria inside Bryopsis algae was verified by in situ hybridization and the endophytic bacterial diversity within Bryopsis thalli from diverse geographical regions was analyzed by means of DGGE and clone libraries. Sequencing results covered Rhizobiales, Rickettsiales, Rhodobacterales, Xanthomonadales and Bacteroidetes species with well-known symbiotic features. Moreover, when the identified endobacterial diversity was analyzed in regard to the Bryopsis samples’ phylogeny and geographic distribution, some patterns could be observed: Rhizobiales and Rhodobacterales species seem widespread in Bryopsis plants, whereas Rickettsiales, Xanthomonadales and Bacteroidetes species are restricted to Bryopsis algae from, respectively, temperate and tropical seas. Also the phylogenies of the bacterial partners themselves indicate a more facultative life style of the Rhizobiales and Rhodobacterales species in contrast to a more obligate nature of the Bacteroidetes, Xanthomonadales and Rickettsiales symbionts. These and additional results on the potential function and the host-, time- and site-specificity of the bacterial partners show Bryopsis algae harbor host-specific endobacterial communities which (i) are distinct from the epiphytic and surrounding seawater populations, (ii) differ with geographic distribution of the algal samples, (iii) have the potential to fix nitrogen in situ and (iv) can vary over time indicating the presence of both facultative and obligate endosymbiotic bacteria. Future investigations will include quantitative PCR and fluorescent in situ hybridization experiments with species-specific probes.
Transduction of RNA interference to discover pathways involved in HIV infection and replication

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Infection by Human Immunodeficiency Virus is difficult to treat thanks to its persistent viral reservoir and to its high rate of mutation that allows appearance of resistance to the available treatment. A new approach could be the identification of cellular partner interacting with the virus during its life cycle as new drugable targets.

HIV and other lentiviruses interact with the target cell cytoskeleton during several steps of their life cycle. In order to establish an effective infection, cytoskeleton is either an obstacle to be overcome or a support to be hijacked. HIV interaction with cytoskeleton components and its regulators has already been shown over the years but all the cofactors and the pathway involved are far from being identified.

We used RNAi technology to identify new HIV partners in the interaction with the host cytoskeleton. By transducing the cells with lentiviral vectors carrying shRNA sequences, we silence different target genes, members of pathways involved in actin rearrangement. After infection with HIV-NL4.3-eGFP reporter virus we evaluate any alteration in HIV replication rate compared to wild type HIV infected cells. To date, we could identify “co-factors” and “restriction factors” for the establishment and the progression of HIV infection in different T cell lines, such as SupT1, Jurkat CD4-CCR5 and Jurkat E6-1. However, since some discrepancy in the results, we decided to apply our system in primary cells, establishing an optimal protocol for lentiviral transduction and HIV infection in peripheral CD4 T Lymphocytes. Therefore, the next step will be the identification of cytoskeletal proteins having a role during HIV infection directly in the most relevant target cells for the infection.
Escape of intracellular Shigella from autophagy requires binding to cholesterol through the type III effector, IcsB


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Type III secretion systems are present in many pathogenic bacteria and mediate the translocation of bacterial effectors into host cells (Parsot et al., 2009). Identification of host targets of these effectors is crucial for understanding bacterial virulence. IcsB, a type III secretion effector, helps Shigella to evade the host autophagy defense system by binding to the autophagy protein, Atg5 (Ogawa et al., 2005). Here, we show that IcsB is able to interact specifically with cholesterol. The cholesterol binding domain (CBD) of IcsB is located between residues 288 and 351. Specific mutations of single tyrosine residues Y297 or Y340 of IcsB by phenylalanine (F) slightly reduced cholesterol binding, whereas deletion of the entire CBD or double mutation Y297F-Y340F strongly abolished interactions with cholesterol. To determine whether Shigella expressing IcsB variants could evade autophagy as effectively as the wild-type Shigella, we infected MDAMC cells stably expressing the autophagy marker LC3 fused to GFP and bacterial autophagosome formation was quantified using fluorescence microscopy. Mutation Y297F or Y340F slightly impaired IcsB function, whereas complete removal of CBD or mutation Y297F-Y340F significantly impaired autophagy evasion. Furthermore, we report that BopA, the counterpart of IcsB in Burkholderia pseudomallei with similar autophagy-evading properties, contains the CBD domain and is also able to bind cholesterol.


Evaluation of Pathways and new host proteins involved in CD4 down-modulation during HIV-1 infection

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Downmodulation of CD4 receptor is one of the hallmarks of HIV infection because it confers to the virus a selective replicative advantage in vivo. HIV has evolved redundant mechanisms to remove the receptor from the cell surface and accelerate its degradation, mainly mediated by three viral proteins: Vpu, Env and Nef. Up to date, the mechanisms that lead to CD4 depletion from the surface of CD4+ T lymphocytes, the natural targets of HIV, are still poorly understood and only partially characterized. We are interested in the discovery of pathways and human proteins involved in the process, in order to eventually find potential new drug targets. To pursue our aim, we first performed a functional screening on HeLa CD4+ cells expressing Nef, using a shRNA lentiviral interference delivery system targeting the whole human genome compatible with an Affymetrix GeneChip Microarray. The read out was the rescue of the CD4 high phenotype. The results were analyzed with Affymetrix program Expression Console 1.1 and the web-based bioinformatic resource DAVID. After four different screens we obtained a final list of 75 genes enriched in the cells sorted for high surface CD4. These genes appear to code for proteins involved in endocytic trafficking, trans-Golgi trafficking and lysosomal degradation pathways. To validate these results we first evaluated the possibility to carry the experiment for each single shRNA lentiviral vector in Nef-expressing HeLa CD4+, SupT1 cells and Primary Blood CD4+ T cells. We will then test all the hits on Nef-, Env- and Vpu-expressing Primary Blood CD4+ T cells and validate the actual downregulation of the genes expression via qRT PCR and Western Blot.
The absence of surface-exposed loops is responsible for the lysosomal targeting of SRA, the *Trypanosoma brucei rhodesiense* antidote to the trypanolytic factor of human serum.

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In Eastern Africa human sleeping sickness is caused by *Trypanosoma brucei rhodesiense*. Human infection by this parasite is due to a protein termed SRA (for Serum Resistance Associated), because SRA inhibits the trypanolytic activity of the human-specific serum protein apolipoprotein L1 (apoL1) [1]. From sequence analysis SRA appears to be a Variant Surface Glycoprotein (VSG) devoid of antigenic loops [2,3]. Contrary to regular VSGs, SRA is not targeted to the cellular surface, but is directed to the endocytic pathway where this protein meets and neutralize apoL1 through direct coil-coiling interaction [4,5].

In the *T. brucei* TREU 927 genome we have identified a VSG gene from which SRA could have derived following the deletion of the sequence encoding the surface-exposed epitopes [3]. We termed this gene SRA BC, for SRA basic copy. As opposed to SRA, this gene did not confer resistance to apoL1 when transfected into *T. brucei* [3].

We hypothesized that:

- the particular endosomal trafficking of SRA is key to its ability to neutralize apoL1;
- this trafficking results from the sequence truncation of SRA.

In order to evaluate these hypotheses we constructed a repaired SRA gene containing the region encoding the surface-exposed loops of SRA BC (mutSRABC), and conversely we deleted this region from SRA BC (SRABC ∆AL). In both cases we investigated the ability of these genes to confer resistance of *T. brucei* to apoL1.

Bloodstream *T. b. brucei* parasites were transfected with either plasmid (pTSARib mutSRABC or pTSARib SRABC ∆AL). The expression and subcellular localization of the proteins were checked by Western blotting and immunofluorescence respectively, and the resistance phenotype to human serum was measured. It was found that when containing the surface-exposed loops of SRA BC, SRA was no longer targeted to the endocytic system, and this resulted in the complete absence of parasite protection against human serum. However, in SRA BC the deletion of the region encoding the surface loops was insufficient to confer the resistance phenotype.

We conclude that the sequence truncation characteristic of SRA is necessary and sufficient to both target this protein to the endocytic system and confer the resistance phenotype. In order to strengthen this conclusion we are presently inserting the sequence encoding the surface loops within the SRA gene in situ. Regarding SRA BC we are currently investigating if the lack of resistance results from improper subcellular targeting or lack of interaction with apoL1.

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Denitrification, a common feature in members of *Bacillus*

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Although several Gram-positive denitrifiers have been characterized in the past, there is still uncertainty about the occurrence of the denitrification trait among these bacteria. A terrestrial ecosystem (luvisol soil) was found to harbor *Bacillus* spp. as the most abundant cultivable denitrifying bacteria next to members of the *Rhizobiaceae* family and the genus *Cupriavidus*. Subsequent screening of 180 representatives of the genus *Bacillus* (encompassing more than half of the currently known described diversity in *Bacillus*) was performed. The screening demonstrated the potential for dissimilatory reduction of nitrogen compounds in 45 of the 87 investigated species, with 19 species containing denitrifying members. The influence of several electron donors and acceptors was tested. The use of more than one electron acceptor, e.g. nitrate and nitrite, was required to detect the denitrification potential of the reference strains. Complex electron donors (most suitable for aerobic growth) were ideal for denitrification testing, while retrieval of denitrifiers from the environment was facilitated by the use of defined electron donors due to less interference of other anaerobic growers. The screening also indicated that a considerable fraction of the representative strains of the genus *Bacillus* were able to denitrify in every included ecosystem from which strains were selected. The results of the isolation campaign and the screening of the reference strain set suggest that bacilli may possibly be more significant to environmental N-cycling than previously assumed in terrestrial and possibly other ecosystems.

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Characterization of a LTTR regulator (PA4203) and its regulated genes in *Pseudomonas aeruginosa*

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*Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen, which causes a lot of problems for immune-compromised persons, such as AIDS patients and people with severe burn wounds. *P. aeruginosa* is also the predominant pathogen found in the airways of cystic fibrosis (CF) patients. When infecting the lungs of the CF patients, this bacterium can lead to their death. A good treatment is still not available, because the bacterium is able to protect itself against antibiotics and shearing stress. This is due to the transition of single colony life to a more aggregated form of life, called biofilm. Biofilm formation is caused by the adherence of cells to each other or to a surface, and the production of extracellular polymeric substances (EPS) which affects the cells mode of life.

The majority of transcription regulators in bacteria, and also in *P. aeruginosa*, are LysR-type transcriptional regulators (LTTRs). These regulators are known as transcriptional activators of a single gene of operon, that negatively autoregulate their own expression. Most of the time, they use one or more effector molecules, often a product of an intermediate of a given metabolic pathway, itself activated by the LTTR. LTTRs regulate a broad range of functional genes, among others virulence.

One of the LTTRs in *P. aeruginosa* is PA4203. Until now, not much is known about this particular regulator and its regulon. We now that PA4203 is surrounded by three other genes from which two are upstream and transcribed in the opposite orientation (D-alanine-D-alanine ligase (*ddl*, PA4201) and one gene encoding a probable 3-nitropropane dioxygenase (PA4202)), and one downstream gene which is transcribed in the same orientation (encoding a periplasmic gluconolactonase [*ppgL*, PA4204]).

We hypothesized that PA4203 regulates these 3 genes, including itself. Indeed, negative auto-regulation was confirmed using a PA4203 *lux*-fusion, which was repressed by the presence of PA4203 in multi-copy. On the other hand, both PA4202 and PA4204, but not *ddl*, are repressed by PA4203.

Phenotypical characterization of a PAO1Δ4203 revealed an increased swarming (pili) and swimming (flagellae) motility and increased autolysis, accompanied by release of DNA in the medium. The autolysis can be explained by the repression of PA4204, which is needed to break down the toxic gluconolactone, an intermediate in the oxidative glucose metabolism.
GyrB sequence analysis and MALDI-TOF MS as identification tools for plant pathogenic Clavibacter

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The genus Clavibacter includes five subspecies, most of them belong to quarantine or q-alert organisms and cause a variety of plant diseases and serious crop losses. To limit economic losses and avoid dissemination of the pathogen to pathogen-free areas, rapid and reliable identification is required. In the frame project QBOL (Quarantine Barcoding of Life), we focus on the development of an accurate DNA-based identification of Clavibacter and close relatives. In addition, quick analysis through matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) was also evaluated.

A total of 180 strains from 5 Clavibacter michiganensis subspecies (C. michiganensis subsp. michiganensis (Cmm), C. michiganensis subsp. sepedonicus (Cms), C. michiganensis subsp. nebraskensis (Cmn), C. michiganensis subsp. insidiosus (Cmi), C. michiganensis subsp. tesselarius (Cmt)) and some outgroups were included. Firstly, 16S rRNA gene analysis was performed to check the reliability of the collected strains and to separate Clavibacter from closely related bacteria. In order to select suitable targets for barcoding the two completely sequenced Clavibacter genomes namely, Cmm and Cms and one from outgroup Leifsonia xyli subsp. xyli together with already published MLSA data were searched to select shared single copy genes. Several housekeeping genes were preliminary tested: gyrB, dnaK, Cox1, CTP synthetase, GluRS and rpoB. Only gyrB and Cox1 gave amplicons in all tested groups. Sequence analysis of 500 bp region of gyrB gene showed high discriminatory power, making it a suitable barcode for adequate Clavibacter subspecies identification. In parallel, manual analysis of MALDI-TOF MS peak profiles allowed us to select subspecies specific biomarkers that can be used for correct Clavibacter strains assignment. In conclusion, two quick and reliable identification tools for the plant-pathogen Clavibacter were found, a DNA-based barcoding region and specific MALDI-TOF MS biomarkers, able to differentiate between different subspecies of the genus.