NEMO

NON-MAMMALIAN **E**XPERIMENTAL **MO**DELS
FOR THE STUDY OF BACTERIAL INFECTIONS

Coordinator: Pr. P. Cosson (Pierre.Cosson@medecine.unige.ch)

Progress Report 2007

NON-MAMMALIAN **E**XPERIMENTAL **MO**DELS FOR THE STUDY OF BACTERIAL INFECTIONS

Coordinator: Pr. P. Cosson (Pierre.Cosson@medecine.unige.ch)

To test the ability of a bacteria to cause a disease it is usually necessary to infect a mammalian host and allow the disease to progress. These experiments inflict significant suffering to the animals. Our general aim is to stimulate the emergence of a community of scientists using alternative non-mammalian hosts for the study of bacterial infections. Our conviction is that many experiments currently carried out using mammalian hosts could be advantageously replaced by the use of alternative non-mammalian hosts.

Our network of laboratories was created in Feb 2005, initially as an informal gathering of research groups involved in similar subjects. Our specific goals are:

1 - To organize an annual meeting on the theme of Non-mammalian hosts for the study of bacterial infections, in order to stimulate exchanges among research groups.

2 - To strengthen our research in this field through a series of collaborative works, for which we hope to find Swiss and European financial support.

3 - To publicize the use of alternative non-mammalian hosts in the scientific community.

The five research groups currently implicated in this network are:

P. Cosson (Geneva, CH),
M.O. Fauvarque (Grenoble, FR)
G. Greub (Lausanne, CH)
H. Hilbi (Zurich, CH)
T. Soldati (Geneva, CH).

In this Progress Report we describe briefly the results obtained in 2007.

A summary of the 2007 NEMO meeting is also attached.

DICTYOSTELIUM, AN ALTERNATIVE HOST MODEL TO STUDY VIRULENCE OF *AEROMONAS* BACTERIA

Pierre Cosson, Centre Médical Universitaire, University of Geneva;
Pierre.Cosson@medecine.unige.ch

SUMMARY

Bacterial virulence designates the complex array of bacterial traits that allow pathogenic bacteria to cause a disease in an infected host. By definition, the virulence of a given bacterial strain can only be measured by confronting it with a host. The use of unicellular amoebae allows a very simple assessment of bacterial virulence in many different pathogens. In a typical experiment, *Dictyostelium* cells form a phagocytosis plaque on a lawn of nonpathogenic bacteria (Fig. 1A) but not on a lawn of pathogenic bacteria. The virulence of bacteria can thus be extrapolated from their ability to sustain *Dictyostelium* growth, as shown previously for *Klebsiella pneumoniae* or *Pseudomonas aeruginosa*. These previous studies also reported an excellent correlation between virulence as evaluated in a *Dictyostelium* host model and in a mouse infection model.

Assessing virulence of *Aeromonas* bacteria is challenging since different *Aeromonas* species (e.g., *A. salmonicida* and *A. hydrophila*) infect different hosts (fish, leeches, mice, and humans), have different growth requirements (e.g., low or high temperature), and cause very different diseases (furunculosis and septicemia in fish and wound infections, meningitis, pneumonia, gastroenteritis, and septicemia in humans). Fish can be used as hosts to evaluate virulence of *A. salmonicida* at low temperature, but this requires specific installations and poses significant practical problems, such as disposal of contaminated water.

In order to assess the virulence of *A. salmonicida* against *Dictyostelium*, we tested the ability of 1,000 *Dictyostelium* cells to grow at 17°C on a lawn of *A. salmonicida* (JF2267). This pathogenic strain was isolated from an arctic char with typical furunculosis and was able to establish a systemic and lethal infection in rainbow trout. This virulent strain (Table 1) did not allow growth of *Dictyostelium* amoebae (Fig. 1B). On the contrary, the JF2397 strain has lost its large pVirA virulence plasmid, is incapable of synthesizing type III secretion system (T3SS) components, and was permissive for *Dictyostelium* growth (Fig. 1B). Similarly, the mutant strain JF2747 was shown previously to be nonvirulent for trout, due to the deletion of the *ascV* gene encoding an inner membrane component of the T3SS. This deletion renders that bacterium incapable of secreting T3SS toxins and effector molecules. This strain was also permissive for *Dictyostelium* growth (Fig. 1B). The virulence against *Dictyostelium* was restored by complementation with a plasmid expressing AscV (strain JF3239), which restores secretion of T3SS proteins (Fig. 1B). Together, these results indicate that the T3SS-dependent virulence of *A. salmonicida* can be evaluated in a *Dictyostelium* host model. In the same study we also defined conditions where the virulence of *Aeromonas hydrophila* could be assessed. These results demonstrate that virulence of *Aeromonas* bacteria can be measured in Dictyostelium, and open the way to a systematic analysis of *Aeromonas* virulence in this alternative host model.

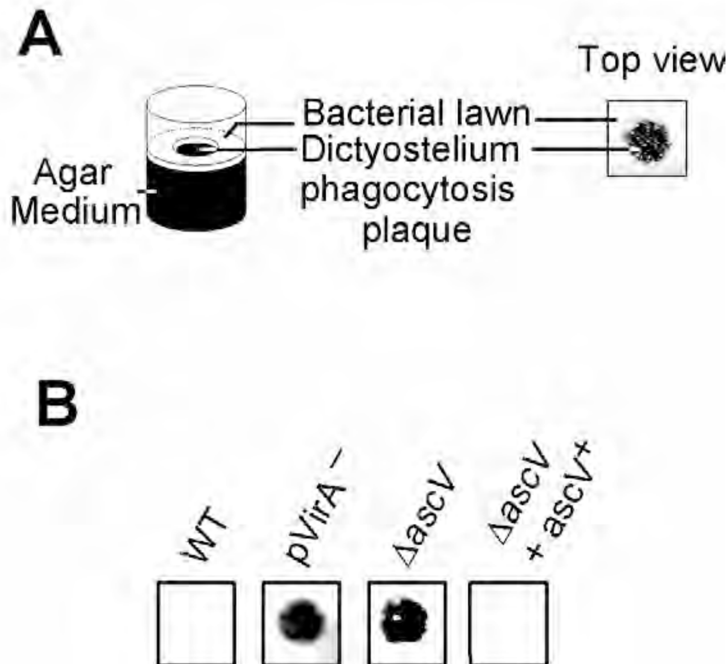


FIG. 1. Virulence of *Aeromonas salmonicida* against *Dictyostelium*. (A) The ability of *Dictyostelium* to grow on a bacterial lawn was assessed as described previously (2) by depositing 1,000 wild-type *Dictyostelium* DH1-10 cells on a lawn of bacteria grown on HL-5 agar medium. A phagocytosis plaque was observed 7 days later when bacteria were permissive (nonvirulent). (B) The wild-type virulent *A. salmonicida* strain (JF2267) did not allow growth of *Dictyostelium*, but T3SS-deficient strains (pVirA-negative strain JF2397 and *ascV* strain JF2747) did. Complementation of *ascV* mutant cells restored its virulent phenotype.

In a separate study we also made use of the *Dictyostelium* host to evaluate the virulence of *Pseudomonas aeruginosa* and to discover new virulence factors. The results of this study demonstrated an excellent correlation between the role of virulence factors in our *Dictyostelium* assay and in infected mammals, further demonstrating the relevance of this alternative host model. These results are detailed in reference 4.

References (Year 2007)

1 Benghezal, M., Adam, E., Lucas, A., Burn, C., Orchard, M.G., Deuschel, C., Valentino, E., Braillard, S., Paccaud, J.P., Cosson, P. 2007. Inhibitors of bacterial virulence identified in a surrogate host model. **Cell. Microb.** 9:1336-42.

2 Charette, S., Cosson, P. 2007. A CHS/Beige homologue is involved in biogenesis of Dictyostelium secretory lysosomes. **J. Cell Sci.** 120:2338-43.

3 Froquet, R., Cherix, N., Burr, S., Frey, J., Vilches, S., Tomas, J.M., Cosson, P. 2007. An alternative host model to evaluate *Aeromonas* virulence. **Appl. Environmental Microb.** 73: 5657-9.

4 Alibaud, L., Köhler, T., Coudray, A., Prigent-Combaret, C., Bergeret, E., Perrin, J., Benghezal, M., Reimmann, C., Gauthier, Y., van Delden, C., Attree, I., Fauvarque, M.O., Cosson, P. 2007. Pseudomonas aeruginosa virulence genes identified in a Dictyostelium host model. **Cell. Microb.** In press

BACTERIAL VIRULENCE AND INNATE IMMUNE RESPONSE: *DROSOPHILA* AS A MODEL SYSTEM

Marie Odile Fauvarque, Laboratoire Transduction du Signal, iRTSV/LTS Inserm U873
CEA-Grenoble, 17 rue des Martyrs F-38054, France
marie-odile.fauvarque@cea.fr

1- Evolutive conservation of the innate immune response

We use the fruit fly *Drosophila melanogaster* as a model system for the study of bacterial virulence of host defence mechanisms. In particular, we developed in vivo models for the study of bacterial virulence factors that targets ancestral mechanisms of innate immunity, conserved throughout the animal kingdom. Innate immune response is essential for the elimination of pathogens and, in the case of Vertebrates, for the activation of the adaptative immune system allowing the production of specific antibodies. Pathogens recognition and engulfment relies mainly on epithelial cells and phagocytic cells from the blood cell lineage that also synthesise signalling molecules contributing to the production of inflammatory cytokines and of antimicrobial peptides by immune organs. The innate immune signalling pathways include NF-κB, Jun kinases- and p38-MAP Kinases-dependent pathways that are conserved in most live organisms, including *Drosophila*. Similarly as human macrophages, *Drosophila* blood cells of the hemocyte lineage can differentiate into phagocytes ensuring pathogens engulfment. During infection, pathogens synthesize a vast number of virulence factors targeting host defence mechanisms. In particular, the type III secretion system is a cell-to-cell contact-dependent apparatus that uses a needle-like structure to deliver toxic bacterial proteins either to the cell membrane or directly into the host cell cytoplasm. The type III secretion system is found in many Gram-negative pathogens including *Pseudomonas aeruginosa*, an opportunist pathogen that causes nosocomial infections and is the major cause of death in cystic fibrosis patient. Others and we have shown that virulence factors characterized previously in mammalian systems, including quorum-sensing and type III secretion system, also play a role in *P. aeruginosa* virulence against *D. melanogaster* and *D. discoideum*.

2 - Results

Various genetic approaches in *Drosophila* led us to identify new proteins involved in host defence against bacterial infections:

1) We developed a new transgenic approach that allowed us to analyze the contribution of the *P. aeruginosa* exotoxin S to bacterial virulence *in vivo*, and to identify new ExoS targets involved in inflammation and innate immune response (Avet-Rochex et al., 2005; Avet-Rochex et al., 2007).

2) A genetic screen in Drosophila allowed us to identify conserved proteins involved in bacterial resistance. Notably, we selected a deubiquitinating enzyme whose expression decreases flies resistance to infection. This enzyme possesses a human ortholog which expression is induced by proinflammatory pathways in activated lymphocytes. We further demonstrated that this protein is involved in the negative regulation of the Imd/NK-κB pathway in response to infection. It is also required to maintain the inactivated state of NF-κB in normal conditions, making this protein a putative biological target for the control of NF-κB-dependent inflammatory pathologies. We performed epistasis studies in vivo by the mean of transgenic flies to determine at which level this enzyme is acting on the Imd/NF-kB pathway. e identified one putative substrate by co-immunoprecipitation experiments in S2 cells. We are currently verifying that this putative target is regulated by ubiquitination upon infections.

3) We continued our collaboration with the laboratory of Pr. P. Cosson in order to analyse the interaction of pathogenic bacteria with non-mammalian hosts, respectively *Dictyostelium* amoebae and *Drosophila* flies.

- We have demonstrated that *Pseudomonas* mutants exhibiting decreased virulence in *Dictyostelium* also show decreased virulence in *Drosophila*. The mode of infection is crucial to determine the role of a given gene product: all mutants analyzed were still pathogenic following septic injury into the thorax or induced only slightly delayed mortality, while when fed to the flies, mutant bacteria lost their pathogenicity compared to wild type controls. This suggests that the virulence genes identified in this study play a crucial role in the early stages of a natural infection (such as the passage of the intestinal epithelium), but are dispensable at later stages (Alibaud et al., Cell microbiology, in press).
- *Dictyostelium* mutants with increased sensitivity to certain pathogenic bacteria (in particular *Klebsiella pneumoniae*) were obtained and characterized in the laboratory of Pierre Cosson including a gene called PHG1A. A mutant *Dictyostelium* strain defective for PHG1 exhibited a specific susceptibility to *Klebsiella pneumoniae* bacteria due to its essential role in bacterial intracellular killing (Benghezal et al. 2006). *PHG1* encodes a polytopic membrane protein with a N-terminal lumenal domain and nine potential transmembrane segments. The existence of *phg1* orthologs in mammals and *Drosophila* (*Dphg1*) suggests that it may play similar function in these organisms. To assess this question, a *Drosophila* strain where *Dphg1* is deleted was created in our laboratory. Remarkably we observed that Drosophila *phg1* mutants exhibited increased sensitivity to infection with *K. pneumoniae* (Benghezal et al., 2006). Its function in *Drosophila* cellular immunity has been characterized (Bergeret et al., in revision). We are currently creating mutation on the two others *Phg* genes in drosophila in order to get an integrated view of this new family of proteins in innate immunity and development.

3- Conclusion:

The general aim of the drosophila team is to identify new mechanisms of innate immunity by original approaches in *Drosophila* and to elucidate the contribution of bacterial virulence factors in inhibiting host defense mechanisms. We also analyze molecular and cellular dysfunction caused by a deregulation of several candidate genes *in vivo* during drosophila development and adult life.

4- References:

1. Alibaud L, Köhler T, Coudray A, Prigent-Combaret C, Bergeret E, Perrin J, Benghezal M, Reimman C, Gauthier Y, vanDelden C, Attree I, Fauvarque MO and Cosson P. *Pseudomonas aeruginosa* virulence gnees identified in a *Dictyostelium* host model. **Cell Microbiology**, in press

2. Avet-Rochex A, Perrin J, Bergeret E, Fauvarque MO. (2007) Rac2 is a major actor of *Drosophila* resistance to *Pseudomonas aeruginosa* acting in phagocytic cells. **Genes Cells**. 12:1193-204.

3. Benghezal, M., Fauvarque, MO., Tournebize, R., Froquet, R., Marchetti, A., Bergeret, E., Lardy, B., Klein, G., Sansonetti, P., Charrette, S.J., Cosson, P. (2006) Specific host genes required for killing bacteria by phagocytes. **Cell Microbiology** , 8: 139-148.

4. Avet-Rochex A, Bergeret E, Attrée I, Meister M and Fauvarque MO (2005). Suppression of *Drosophila* cellular immunity by directed expression of the ExoS toxin GAP domain of *Pseudomonas aeruginosa* **Cell. Microbiology** *7* :799-810

AMOEBAE AS A TOOL TO ISOLATE NEW BACTERIAL SPECIES, TO TEST BACTERIAL VIRULENCE AND TO STUDY THE BIOLOGY OF INTRACELLULAR AMOEBAE-RESISTING BACTERIA

Gilbert Greub, Institute of Microbiology, University of Lausanne
Gilbert.Greub@chuv.ch

Free-living amoebae feed on bacteria, fungi and algae. However, some microorganisms evolved to become resistant to these protists. These amoebae-resistant microorganisms include established pathogens, such as *Legionella* spp. *and Mycobacterium* spp., as well as *Chlamydia*-related emerging pathogens. Our group use amoebae as a tool for the culture of intracellular bacteria and new bacterial species that might be potential emerging pathogens (7). We are using amoebae as cells in a cell culture system to study the biodiversity of intracellular hosts of free-living amoebae in water (8) and since human are commonly exposed to water, the potential role played by these intracellular bacteria as agents of pneumonia. Our research mainly focus on *Mycobacteria* and on *Chlamydia*-like organisms, such as *Protochlamydia naegleriophila* (4)*, Waddlia chondrophila* (1, 2) and *Parachlamydia acanthamoebae* (3, 5).

We showed that pathogenic *Mycobacterium kansasii* strains are more virulent towards *Acanthamoeba castellanii* than non-pathogenic strains, with pathogenic strains being more cytopathic and growing faster in *A. castellanii* (Figure 1). Animal models are generally used to test bacterial virulence. This work provides evidence that amoebae may represent an alternative non-mammalian model for testing the virulence of *M. kansasii*, and suggests that it might also be used for other intracellular amoebae-resisting bacteria. Since this system based on amoebal co-culture is amenable to large screenings in 24- or 96-well plates, it may potentially be used to screen mutants for virulence factors. Although *Acanthamoeba* has been less well characterized genetically than *D. discoideum*, *Acanthamoeba* has the advantage over *Dictyostelium* of remaining viable and growing at temperatures above 25 °C. Thirty-two to 37 °C represents the temperature range at which bacteria encounter human macrophages during the process of infection and the Acanthamoeba model may thus more accurately reflect the in vivo setting of human infection (6).

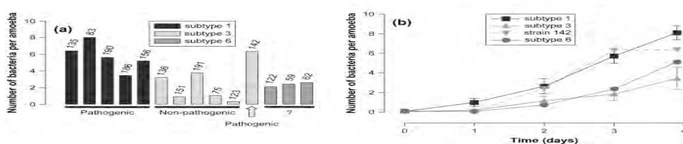


Figure 1.
(a) Mean number of bacteria per amoeba 3 days p.i. with each 14 *M. kansasii* strains studied. The number indicated above each bar is the strain number. (b) Mean number of bacteria per amoeba from day 0 to day 4 for pathogenic subtype 1 ($n = 5$), for non-pathogenic subtype 3 ($n = 5$), for pathogenic subtype 3 (strain $n = 142$) and for subtype 6 ($n = 3$). The data shown are the mean of three replicates.

More recently, we initiated a study that aim at investigating the interaction between the intracellular bacteria *Parachlamydia acanthamoebae* and the *Dictyostelium* amoeba. In particular, we intend to determine the molecular mechanisms that control their interactions. For this, we will determine which genes are important on the host side (*Dictyostelium*). As a preliminary to host genetic analysis, we already defined the intracellular transport of *Parachlamydia* in *Dictyostelium*. Preliminary results indicate that internalized *Parachlamydia* are located in the endocytic pathway in *Dictyostelium* (Fig. 2A). We also set up conditions to quantify the number of *Parachlamydia* by quantitative PCR (5), thus assessing its intracellular replication in *Dictyostelium* and other host cells (Fig. 2B). We are now ready to start analyzing the effect of various mutations in the host genome on the intracellular replication and trafficking of *Parachlamydia*.

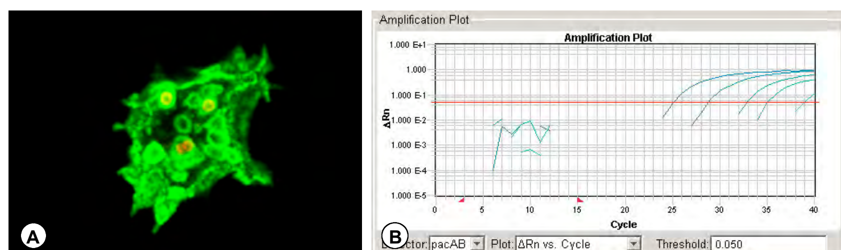


Figure 2.
A. *Parachlamydia* (red) traffic within an endocytic vacuole (green) in Dictyostelium;
B. Growth of *Parachlamydia* from day 0 to day 4 quantified using a taqMan quantitative PCR

We also studied the intracellular traffic of another *Chlamydia*-related bacteria, *Protochlamydia neagleriophila* strain KNIC*,* which role in lung infections has just been identified (4). This bacterial species was easily replicating within *Dicytostelium,* as confirmed by immunofluorescence and quantitative PCR (Figure 3).

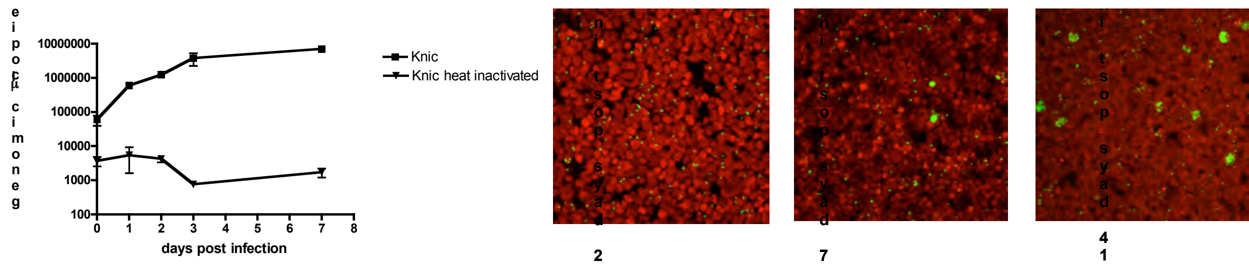


Figure 3. A specific quantitative PCR targeting the 16S rRNA gene of *P. neagleriophila* demonstrated the 2-log increase in the number of DNA copies in about 3 days when co-cultured with *Dictyostelium* (left panel); large inclusions were however mainly observed after 7 to 14 days of incubation (right panel).

Like *Parachlamydia acanthamoebae*, *Protochlamydia neagleriophila* strain KNIC co-localized mainly with markers of the endocytic pathway (p80) and to a lesser extend with markers of the contractile vacuole (Rh50, see Figure 4).

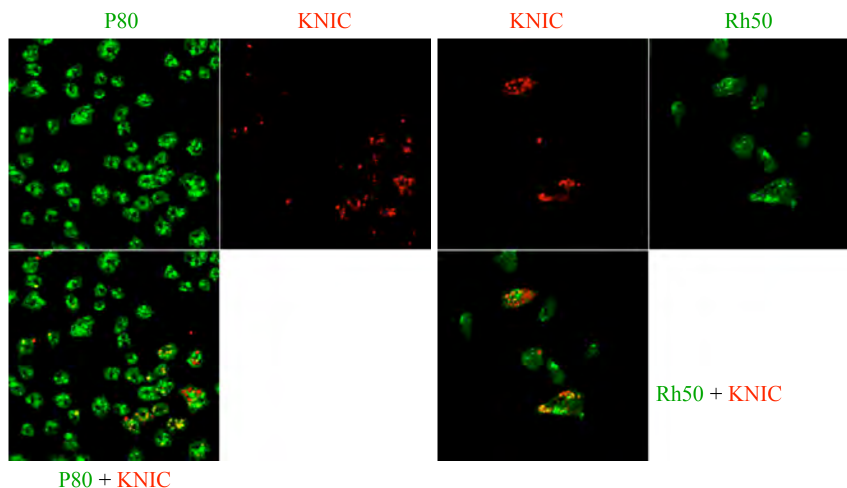


Figure 4. Co-localization of *P. neagleriophila* with markers of the endocytc pathway (p80, left panel) and with markers of the contractile vacuole (Rh50, right panel).

In conclusion, our works provide evidences on the usefulness of amoebae as tool to test bacterial virulence and to investigate the biology of intracellular bacteria resisting-amoebae. Amoebae appear thus to represent convincing alternative non-mammalian model to study bacterial virulence as well as the interactions of these bacteria with eucaryotic cells.

References.

1. Baud, D., L. Regan, and G. Greub. 2008. Emerging role of *Chlamydia* and *Chlamydia*-like organisms in adverse pregnancy outcomes. Curr Opin Infect Dis 21:70-76.
2. Baud, D., V. Thomas, A. Arafa, L. Regan, and G. Greub. 2007. *Waddlia chondrophila*, a potential agent of human fetal death. Emerg Infect Dis 13:1239-43.
3. Borel, N., S. Ruhl, N. Casson, C. Kaiser, A. Pospischil, and G. Greub. 2007. Parachlamydia spp. and related Chlamydia-like organisms and bovine abortion. Emerg Infect Dis 13:1904-7.
4. Casson, N., R. Michel, K. D. Muller, J. D. Aubert, and G. Greub. 2008. Protochlamydia naegleriophila as etiologic agent of pneumonia. Emerg Infect Dis 14:168-72.
5. Casson, N., K. M. Posfay-Barbe, A. Gervaix, and G. Greub. 2008. A new diagnostic real-time PCR for the specific detection of Parachlamydia acanthamoebae DNA in clinical samples. J Clin Microbiol.
6. Goy, G., V. Thomas, K. Rimann, K. Jaton, G. Prod'hom, and G. Greub. 2007. The Neff strain of *Acanthamoeba castellanii*, a tool for testing the virulence of *Mycobacterium kansasii*. Res Microbiol 158:393-7.
7. Thomas, V., N. Casson, and G. Greub. 2007. New *Afipia* and *Bosea* strains isolated from various water sources by amoebal co-culture. Syst Appl Microbiol 30:572-9.
8. Thomas, V. , Loret,J.F.;Greub,G.;. 2008. Biodiversity of amoebae and amoebae-resisting bacteria in a drinking water treatment plant. Appl Env Microbiol, submitted.

AMOEBAE: A CELLULAR PATHOGENESIS MODEL FOR THE LEGIONNAIRES' DISEASE AGENT *LEGIONELLA PNEUMOPHILA*

Hubert Hilbi, Institute of Microbiology, ETH Zürich, Switzerland
hilbi@micro.biol.ethz.ch

SUMMARY

Legionella pneumophila is an opportunistic human pathogen that replicates within environmental amoebae including *Acanthamoeba castellanii* and the social amoeba *Dictyostelium discoideum*. The Icm/Dot type IV secretion system promotes phagocytosis and intracellular replication of *L. pneumophila* in an endoplasmic reticulum-derived "*Legionella*-containing vacuole" (LCV). *L. pneumophila* adopts a biphasic life cycle consisting of a replicative growth phase and a transmissive (stationary) phase, the latter of which is characterized by the preferential expression of genes required for motility and virulence. A bioinformatic analysis of the *L. pneumophila* genome revealed a gene cluster homologous to the *Vibrio cholerae cqsAS* genes, encoding a putative quorum sensing autoinducer synthase (*lqsA*) and a sensor kinase (*lqsS*), which flank a novel response regulator (*lqsR*). We found that an *L. pneumophila lqsR* deletion mutant grew in broth with the same rate as wild-type bacteria, but entered the replicative growth phase earlier. The *lqsR* mutant strain was impaired for phagocytosis by *A. castellanii*, as well as for intracellular growth within and cytotoxicity against the amoebae (Fig. 1; Tiaden *et al*., 2007).

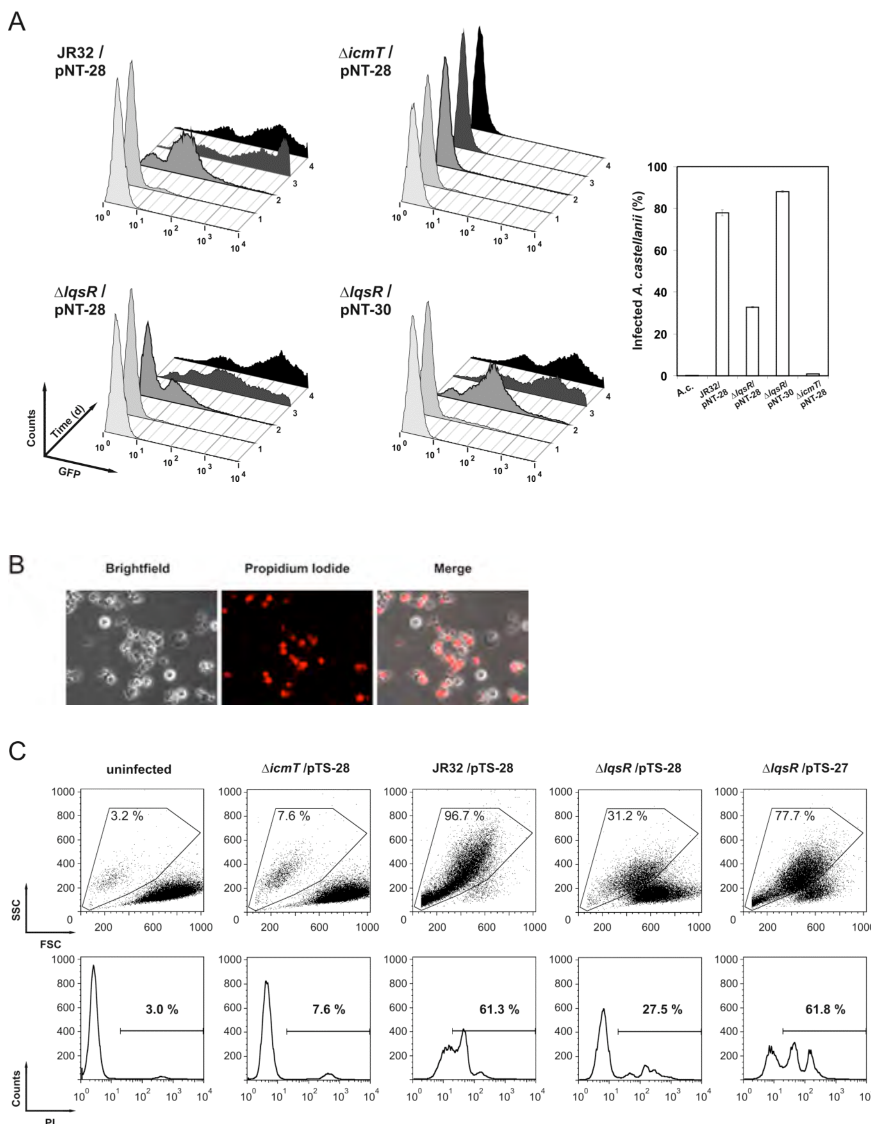


Fig. 1. *L. pneumophila* lacking *lqsR* is impaired for growth in *A. castellanii* and cytotoxicity. (**A**) Intracellular replication of GFP-expres-sing *L. pneumophila* strains within *A. castellanii* was assayed by flow cytometry using the wild-type strain JR32, an *icmT* mutant, or an *lqsR* mutant harboring either an empty GFP-expression plasmid (pNT-28) or the corresponding complement-ting plasmid expressing *lqsR* under control of its native promoter (pNT-30). Inset: quantification of GFP-positive *A. castellanii* 2 d post infection. (**B**) Cytotoxi-city of *L. pneumophila* against *A. castellanii* was assayed by propidium iodide staining and (**C**) quantified by flow cytometry 24 h post infection using the wild-type strain JR32, an *icmT* or an *lqsR* mutant harboring either an empty plasmid or a com-plementing plasmid expres-sing *lqsR* under control of its native promoter (pTS-27).

L. pneumophila lacking *lqsR* was also phagocytosed less efficiently by *D. discoideum*, and intracellular replication and the formation of LCV was defective (Tiaden *et al*., 2007). The expression of *lqsR* was dependent on the alternative sigma factor RpoS and, to a lesser extent, on the two-component response regulator LetA. Finally, DNA microarray experiments revealed that *lqsR* regulates the expression of genes involved in virulence, motility and cell division, consistent with a role for LqsR in the transition from the replicative to the transmissive (virulent) phase. Our findings indicate that LqsR is a novel pleiotropic regulator involved in RpoS- and LetA-controlled interactions of *L. pneumophila* with phagocytes (Fig. 2; Tiaden *et al*., 2007).

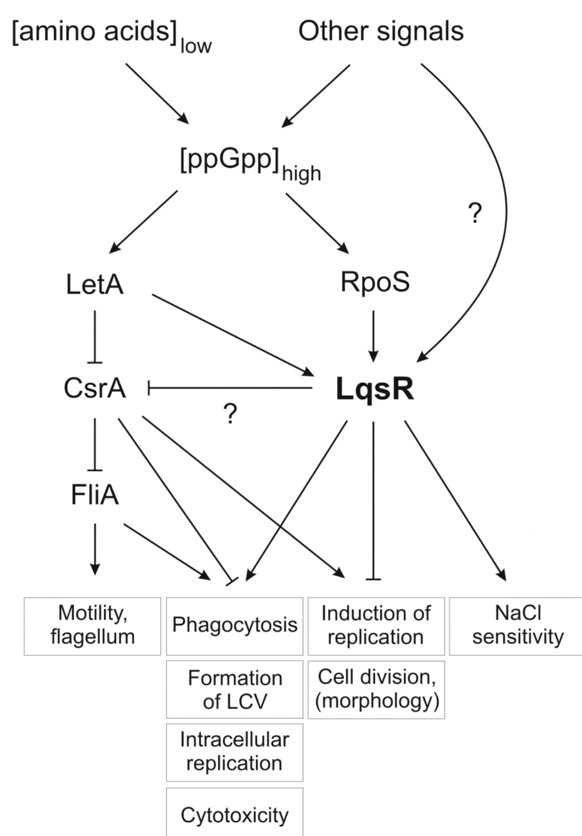


Fig. 2. Model of the hierarchical position of LqsR in the regulatory network of *L. pneumophila* in stationary growth phase. *L. pneumophila* mutually exclusively expresses replicative or transmissive traits. Upon nutrient (amino acid) starvation, the cellular level of the "alarmone" ppGpp increases, which stimulates the two-component system LetA/LetS, as well as the stationary phase sigma factor RpoS and leads to an induction of transmissive traits, including motility and virulence. RpoS and LetA promote the expression of the response regulator LqsR, which controls the induction of virulence traits and suppresses the transition from transmissive (stationary) to replicative phase. LqsR might antagonize CsrA, a RNA-binding global repressor of transmission traits and essential activator of replication. In addition to the pathways involving RpoS or LetA, other signals such as an autoinducer might converge on LqsR and result in its expression or activation.

References (Year 2007)

1. Hilbi, H., Weber, S. S., Ragaz, C., Nyfeler, Y. & Urwyler, S. (2007) Environmental predators as models for bacterial pathogenesis. **Env. Microbiol.** *9*: 563-575.
2. Tiaden, A., Spirig, T., Weber, S.S., Brüggemann, H., Bosshard, R., Buchrieser, C. & Hilbi, H. (2007) The *Legionella pneumophila* response regulator LqsR promotes host cell interactions as an element of the virulence regulatory network controlled by RpoS and LetA. **Cell. Microbiol**. *9*: 2903-2920.
3. Albers, U., Tiaden, A., Spirig, T., Al Alam, D., Goyert, S.M., Gangloff, S.C. & Hilbi, H. (2007) Expression of *Legionella pneumophila* paralogous lipid A biosynthesis genes under different growth conditions. **Microbiology** *153*: 3817-3829.
4. Tiaden, A., Spirig, T., Carranza, P., Brüggemann, H., Riedel, K., Eberl, L., Buchrieser, C. & Hilbi, H. Synergistic contribution of *Legionella pneumophila* *lqs* genes to pathogen-host interactions. Submitted.

THE AMOEBA *DICTYOSTELIUM DISCOIDEUM* AS A MODEL HOST TO STUDY MYCOBACTERIUM MARINUM INFECTION

Thierry Soldati, Department of Biochemistry, University of Geneva
thierry.soldati@biochem.unige.ch

1. Establishment of *Dictyostelium* as a host model to study mycobacteria infection

Mammalian host model systems, whole organisms as well as cell lines, are essential to understand the pathogenicity of mycobacteria. However, over the past decades the complexity of these systems has hampered advancement to unravel the dynamic host-pathogen interactions. Pathogenic mycobacteria exploit phagocytosis to enter the macrophages of the innate immune system, in which they proliferate. To further understand the mechanism of establishment and maintenance of infection, we succeeded in establishing *Dictyostelium* as a host system for *M. marinum* (Hagedorn & Soldati, 2007). *Dictyostelium* is a professional phagocyte that naturally lives on bacteria. Combining this genetically and biochemical tractable host with a pathogen, whose genome sequencing has just been completed, provides a powerful synergism and ensures rapid progress.

We developed an infection protocol that allows for synchronous and efficient uptake of *M. marinum* and confirmed that the establishment and course of infection in *Dictyostelium* are similar to those observed for pathogenic mycobacteria in other host systems. The non-pathogenic strain *M. smegm*atis is efficiently killed by *Dictyostelium* and an avriulent strain is rapidly exocytosed after uptake, because it is less efficient at establishing a replication niche. Our data suggest that the pathogen does not induce a complete maturation arrest, but rather bifurcates from it, leading to a near-neutral compartment that is not bactericidal (e.g. no delivery of lysosomal enzymes). In addition, strong accumulation of the *Dictyostelium* flotillin at the replication niche allowed us for the first time to directly observe the growth and rupture of this vacuole and the release of bacteria into the cytosol. In addition, we were able to demonstrate that these steps and strategies are conserved in evolution, because flotillin accumulation and vacuole rupture were also observed during infection of blood derived monocytes and other mammalian macrophage cell lines.

2. Identification of novel host factors that modulate cell-intrinsic immunity

To characterize the course of infection at the single cell, population and functional levels, we applied a combination of approaches, which together showed that the lack of one of the flotillin isoform, Vacuolin B, but not Vacuolin A renders the host more immune against infection. To our surprise, a host strain lacking the RacH GTPase showed contrasting behaviour, being more susceptible to *M. marinum* proliferation (Fig 2). Absence of RacH results in disorganization of the compartments decorated with vacuolin and almost complete lack of phagosomal acidification.

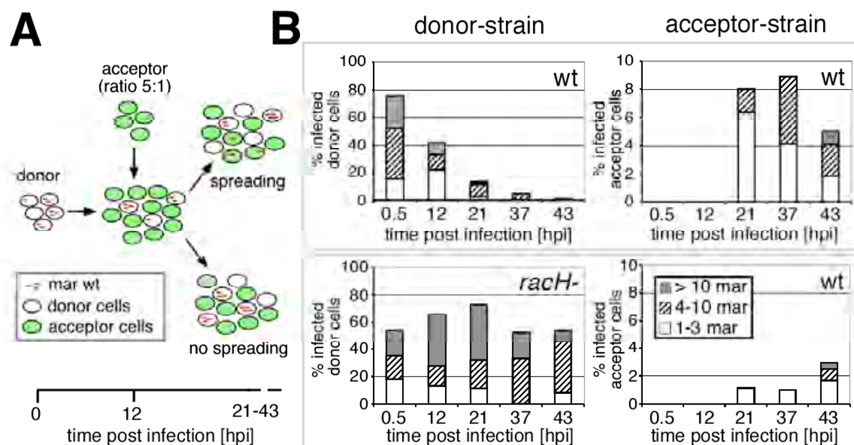
3. The ejectosome: a novel, nonlytic mechanism of pathogen release from its cell host

Pathogen release from its host cell is an important virulence determinant and an aspect of host-pathogen interactions that has long suffered from neglect. It is known that pathogenic mycobacteria can spread from cell to cell, as monitored using a microplaque assay. The exact mechanism is not known, however, it has been proposed that bacteria-containing filopodia are engulfed by neighbouring cells, resulting in cell-to-cell transmission.

FACS analysis of the *racH* phenotype at late stages of infection showed two interesting characteristics. First, the almost complete absence of extracellular bacteria and, second, the absence of lowly infected (presumably newly infected) host cells, suggesting an inhibition in cell-to-cell transmission. To test this, we adapted the widely used microplaque assay to motile cells, by adding green fluorescent acceptor cells (Fig 1A). Quantitative comparison of spreading efficiency (Fig 1B) between wild-type and *racH* null

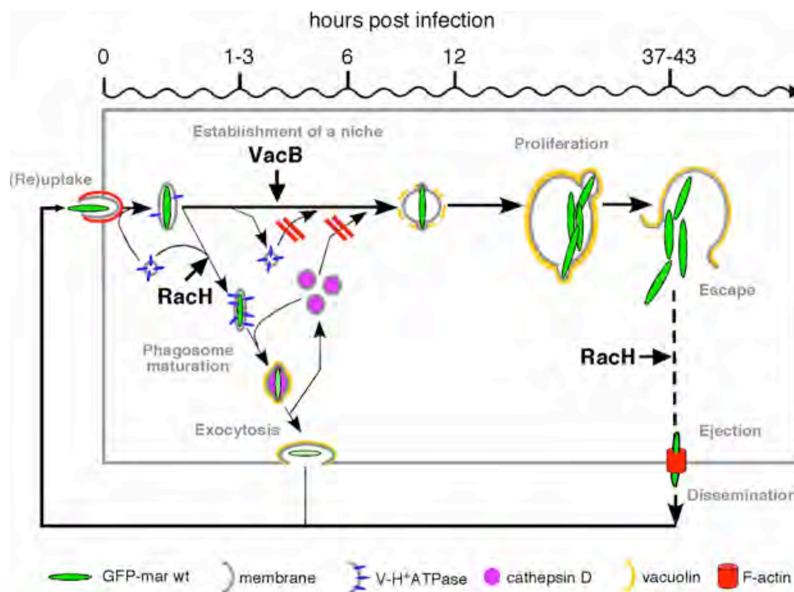
Dictyostelium confirmed, that the *racH* null strain retains the bacteria and is deficient in cell-to-cell transmission. To our knowledge this is the first time, a host mutant has been identified that is deficient in the cell-to-cell spreading of mycobacteria.

Figure 1



To elucidate the mechanism of cell-to-cell transmission we monitored infected cells by live microscopy. This approach led us to identify a novel structure by which bacteria are released from their host cell in a non-lytic fashion. We termed the structure “ejectosome” (Fig 2). It is defined by a barrel-shaped region of strong F-actin-accumulation, through which the bacterium exits the cell. The ejectosome is morphologically distinct from a phagocytic cup and the relative topological distribution of plasma-membrane markers confirms that the bacterium is exiting the cell through the ejectosome.

Figure 2



4. Publications for the year 2007

1. Hagedorn, M., and **Soldati**, T. Nonlytic ejection of pathogenic mycobacteria from its host is crucial for cell-to-cell spreading. (2008) **submitted**
2. Dieckmann, R., Gopaldass, N., Escalera, C., and **Soldati**, T. Monitoring time-dependent maturation changes in purified phagosomes from *Dictyostelium discoideum*. (2007) **Methods Mol. Biol.** in press.
3. Pino, P., Foth, B.J., Kwok, L., Sheiner, L., Schepers, R., **Soldati, T**., and Soldati, D. Dual targeting of antioxidant and metabolic enzymes to the mitochondrion and the apicoplast of *Toxoplasma gondii* (2007) **PLOS Pathogens.** 3(8):e115.
4. Hagedorn, M., and **Soldati**, T. Flotillin and the RacH GTPase modulate intracellular immunity of *Dictyostelium* to *Mycobacterium marinum* infection. (2007) **Cell Microbiol.** 9, 2716-33

NEMO Meeting 2007

You all are welcome to take part to this meeting
including dinner on 6th Feb and COST meeting on 7th Feb., i.e. from 6th Feb at 18.30 to 7th Feb 18h

Tuesday 06th February

16h30-18h NEMO committee (room 502)
1. annual report
2. review paper
3. main axe and options for the COST grant
4. varia

18h30 Dinner at the "Lausanne-Moudon" restaurant (paid by NEMO)

Wednesday 07th February

08h	Welcome in room 502, load presentation on PC (USB stick or CD-rom)
08h20	Pierre Cosson; Geneva (CH): The Dictyostelium amoebae
08h40	M.O. Fauvarque, Grenoble (FR): A RNAi screen of the Ubiquitin Specific Proteases in Drosophila cells identified 3 novel regulators of the NF-kB pathway in response to bacterial infection
09h00	Gilbert Greub, Lausanne (CH): Amoebae as a tool to discover new agents of pneumonia
09h20	H. Hilbi, Zürich (CH): Subversion of Dictyostelium phosphoinositide metabolism by Legionella
09h40	Thierry Soldati, Geneva (CH): Identification of Dictyostelium proteins that modulate intracellular immunity to Mycobacterium marinum infection
10h	break
10h20	Maria Leptin: The analysis of the genetics of pathogen resistance in the zebrafish
10h45	Margaret Dallman: Immunity, Inflammation and Infection in the zebrafish
11h10	Vincent Thomas, Paris (FR) – Amoeabe: a reservoir for Mycobacteria and a tool to test virulence of these intracellular bacteria
11h35	H. Faix: Growth of Legionella within defined *Dictyostelium* mutants
12h00	lunch
13h20	Ludovic Wiszniewski, Epithelix
13h45	Robin May: Bacterial and fungal immunity in C. elegans and other nematodes
14h10	Salvatore Bozzaro: Proteomics and transcriptomics of phagocytosis in *Dictyostelium*
14h35	break
15h00-17h00	Brainstorming for COST funding

Practical informations

Hotel reservation: please book the hotel yourself; your expenses will be covered with NEMO money for up to one night. We suggest the following hotel that is ideally located near to IMUl and to the restaurant where we will have dinner on 6th Feb.

Hôtel de l'Ours	phone 00 41 21 321 49 49
rue du Bugnon 2	fax 00 41 21 1 320 49 73
1005 **Lausanne**	

2007 annual NEMO meeting

The 2007 annual meeting of the NEMO network was held at the Institute of Microbiology of the University of Lausanne in Lausanne (Switzerland) on 7th February 2007. Members of the NEMO network (Non-mammalian experimental models for the study of bacterial infections), invited speakers. Moreover, during the meeting postdocs and PhD students exchanged new data on different non-mammalian systems used to study bacterial pathogens. A total of 11 different group leaders were present, and the audience totalled to 22 persons from 5 different European countries (Germany, Italy, Spain, United Kingdom, France and Switzerland).

One aim of the meeting was to promote in the scientific community the use of alternative models to study bacterial infections, thus allowing the replacement of animals by non-mammalian hosts in experiments. The other aim was to exchange ideas and communicate on advantages, limits and pitfalls of these alternative models.

Host cells/organisms studied by some of the groups included *Dictyostelium, Acanthamoeba, Drosophila,* and zebra fish. These hosts were used to analyze interactions with new or established bacterial pathogens such as *Pseudomonas, Legionella, Parachlamydia* and *Mycobacterium*.

Pierre Cosson (Geneva, CH) opened the meeting by a talk entitled "The *Dictyostelium* amoebae". His group is studying both the host determinants and the bacterial virulence traits that determine the outcome of the encounter with bacterial pathogens such as *Klebsiella pneumoniae.* They are specifically looking at early steps involved in bacterial internalizations.

Marie-Odile Fauvarque (Grenoble, FR) then demonstrated the usefulness of the *Drosophila* fly to study innate immunity. Her talk entitled "A RNAi screen of the ubiquitin specific proteases in *Drosophila* cells identified 3 novel regulators of the NF-κB pathway in response to bacterial infection" clearly demonstrated a role for dUSP36 in the regulation of immune response *in vivo*, in cell survival and in *Listeria monocytogenes* killing.

Gilbert Greub (Lausanne, Switzerland) gave a talk entitled "Amoebae as a tool to discover new agents of pneumonia". He showed how his group use amoebae to isolate new bacterial species, including obligate intracellular bacteria. This method allows isolation of fastidious microorganisms even from complex microbial environments. Isolated new species might represent new pathogens since being resistant to the phagocytic amoebae, they might have adapted to also resist to human macrophages. This and other approaches were suitable to isolate *Legionella* spp, *Mycobacterium* spp., as well as *Chlamydia*-related bacteria. Amoebae might thus be used in a cell culture system as an alternative to other approaches (such as inoculation of animals) to identify agents involved in diseases outbreaks of unknown etiology.

The talk by **Hubert Hilbi** (Zürich, CH) entitled "Subversion of *Dictyostelium* phosphoinositide metabolism by *Legionella*" demonstrated the usefulness of *Dictyostelium* amoebae as a model to study on a molecular and cellular level the biology of bacterial pathogens such as *Legionella* that naturally infect amoebae. He outlined how host cell phosphoinositide lipids participate in membrane fusions and hence may modify the fate of the *Legionella*-containing vacuole. Specifically, he presented data establishing a role for phosphoinositide-3 kinases in intracellular trafficking of *Legionella.* Moreover, he demonstrated that the secreted *Legionella* protein SidC anchors to the *Legionella* vacuole via a specific phosphoinositide.

Then, **Thierry Soldati** (Geneva, CH) gave a talk entitled "Identification of *Dictyostelium* proteins that modulate intracellular immunity to *Mycobacterium marinum* infection". This work highlighted the usefulness of amoebae to identify bacterial proteins involved in the subversion of immune control.

The two next talks were on zebrafish (*Danio rerio*) models. First, **Maria Leptin** (Cologne, D) showed that genetics of pathogen resistance may be analysed in zebrafish. Indeed, zebrafish is amenable to various genetic approaches including random mutagenesis, transgenesis, gene knockdown and Notch null mutants.

Then, **Margaret Dallman** (London, UK) gave a talk entitled "Immunity, inflammation and infection in the zebrafish" that allowed the audience to familiarize with the advantage of this non-mammalian model. Zebrafish is genetically tractable and possess an immune system similar to other vertebrates, with both the innate and adaptive branch. Moreover, zebrafish are easy to maintain, breed frequently and in large numbers. Zebrafish embryos develop rapidly *ex utero* and are optically transparent, allowing an easy follow-up of the fate of pathogens labelled with fluorescent compounds.

Vincent Thomas, who is currently working in Paris with the company Steris, presented the work he did on Mycobacteria in G. Greub's group (Lausanne, CH). His talk entitled "Amoeabe: a reservoir for Mycobacteria and a tool to test virulence of these intracellular bacteria" first showed that amoebae may represent a reservoir for Mycobacteria. Indeed, Mycobacteria were more likely recovered from samples from which amoebae were also recovered. Then, he showed that pathogenic *Mycobacterium kansasii* strains are more virulent towards *Acanthamoeba castellanii* than non-pathogenic strains, with pathogenic strains being more cytopathic and growing faster in *A. castellanii*. Animal models are generally used to test bacterial virulence. This work provides evidence that amoebae may represent an alternative non-mammalian model for testing the virulence of *M. kansasii*, and suggests that it might also be used for other intracellular amoebae-resisting bacteria.

Hans Faix (Hannover, D) then give a talk entitled "Growth of *Legionella* within defined *Dictyostelium* mutants". His work done in close collaboration with H. Hilbi focussed on the construction of a *Dictyostelium* phosphoinositide-4 kinase mutant strain and on how this mutant will be used to further study the role of phophoinositides during *Legionella* infection.

Ludovic Wiszniewski (Geneva, CH), who is working at Epithelix corporation, showed that various epithelia are commercially available and discussed advantages and limits of these non-mamalian system to study bacterial virulence.

Robin May (Birmingham, UK) then gave a talk entitled "Bacterial and fungal immunity in *C. elegans* and other nematodes". This talk gave the audience the opportunity to discover advantages and limits of this additional non-mammalian system to study bacterial virulence.

Salvatore Bozzaro (Torino, IT) gave a talk entitled "Proteomics and transcriptomics of phagocytosis in *Dictyostelium*" in which he described recent advance to understand at a genome-wide level the adaptations of *Dictyostelium* to growth conditions and to the specialisation to bind, engulf, kill and digest bacteria. They report profound changes in gene regulation as well as post-translational modifications of cytoskeletal proteins, digestive enzymes and a few genes homologous to genes implicated in human diseases.

Finally, general discussions and a brainstorming session completed this fruitful 2007 NEMO meeting kindly supported by the 3R foundation. Special thanks for the organization of the meeting to Sébastien Aebi and other members of G. Greub's group.