BELGIAN SOCIETY FOR MICROBIOLOGY & BELGIAN SOCIETY FOR ANALYTICAL CYTOLOGY

NATIONAL COMMITTEE FOR MICROBIOLOGY
of
THE ROYAL ACADEMIES OF SCIENCE AND THE ARTS OF BELGIUM

Vlaams Kennis- en Cultuurforum

IMAGING TECHNOLOGY IN MICROBIOLOGY: CYTOMETRIC AND MOLECULAR APPROACHES

November 18th 2005

House of the Academies
Hertogsstraat, 1, Rue Ducale
Brussels

Programme

08.30 Registration desk open – Poster installation

09.00 Welcome & Opening by the Presidents of BSM & BSAC

09.15 **Hazel Davey** (Institute of Biological Sciences, University of Wales, Aberystwyth, UK): *Cytometry on mixed microbial populations*

10.00 **Sarah Baatout** (Laboratory of Radiobiology and Microbiology, Belgian Nuclear Research Centre, SCK•CEN, Mol): *Physiological responses in bacteria after stress*

10.20 *Selected poster 1:* **Laurent Gillet & A. Vanderplasschen** (Immunology-Vaccinology, Faculty of Veterinary Medicine, University of Liège) *Bovine herpesvirus 4 induces apoptosis of human carcinoma cell lines in vitro and in vivo*

10.35 Break

11.05 **Marcel Bruchez** (Quantum Dot, Invitrogen Corporation, Quantum Dot Corporation, Hayward, CA 94545, USA): *Quantum dots for sensitive multicolor detection in imaging and cytometry*

11.50 **Nico Boon** (Laboratory Microbial Ecology and Technology, University of Gent): *Flow cytometry as a tool in microbial ecology*

12.10 Selected Poster 2: **Nathalie Cools** (University of Antwerp, Faculty of Medicine, Laboratory of Experimental Hematology, Antwerp University Hospital) *Negative regulation of T cells by immature dendritic cells is mediated by TGF-β- and IL-10-producing T-helper cells*

12.30 Poster Viewing - Lunch - Poster Session

14.30 **Bernard E. Fuchs** (Max Planck Intsitut für Marine Mikrobiologie, Bremen): *The benefit of flow-cytometric sorting: access to 'unculterable' microorganisms*

15.15 **Christian Demanet**: (Laboratory of Haematology, University Hospital, University of Brussels -VUB): *NK cells in human viral diseases*

15.35 Selected Poster 3: **Sofie Barbé** (Lab. Bacteriology, Rega Institute, K.U.Leuven) *In vivo* evaluation of *Clostridium acetobutylicum*-directed IL2 production in combination with radiotherapy for tumour control using rat rhabdomyosarcoma as a model

15.55 **Luc Willems** (Molecular and Cellular Biology, FUSAG, Gembloux): *Dynamics of cell division and apoptosis in bovine leukaemia*

16.40 Poster Awards

16.50 End of session

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ABSTRACTS ORAL PRESENTATIONS

Flow cytometry of mixed microbial populations

Hazel M. Davey

Institute of Biological Sciences, University of Wales, Aberystwyth, Ceredigion, SY23 3DD, Wales, United Kingdom.

Flow cytometry is a technique, which allows one to analyse cells rapidly and individually, and permits the quantitative analysis of distributions of a property or properties in a population. It therefore offers many advantages over conventional measurements for the analysis of biological cells. Historically the technique has been widely applied for the study of mammalian cells, but its use in microbiology has been more limited; this is mainly a consequence of the smaller size of microbes, which results in the smaller optical signals that can be obtained from them. Developments in light sources and optics, together with brighter, spectrally-diverse dyes have reduced this barrier over recent years and the flow cytometer is now an essential tool in many microbiological research establishments.

The single-cell nature of flow cytometric analysis makes this approach particularly useful for the study of heterogeneity in microbial populations. Whilst microbial heterogeneity is well documented in both natural and laboratory environments, the underlying causes are less well understood. Flow cytometry will contribute to our understanding of the sources of microbial heterogeneity and their implication for pure and applied study of microorganisms.

Physiological responses in bacteria after stress

Sarah Baatout
Laboratory of Radiobiology and Microbiology, Belgian Nuclear Research Center, SCK•CEN , Mol, Belgium

Flow cytometry provides a powerful means to measure a wide range of cell characteristics in microbiological research. Since bacteria may behave differently from eukaryotes in terms of their interaction with dyes, drugs and other reagents, it is therefore difficult to design multiparameter staining protocols that work, unmodified, across a range of bacterial species.

In our study, flow cytometry was employed to measure membrane integrity the presence or absence of an intact fully polarized cytoplasmic membrane, the transport systems across it and the cell viability. Using those protocols, it was possible to resolve a cells physiological state, beyond culturability under stress conditions. Importantly, results were available in real-time, a few minutes after a sample is take, enabling informed decisions to be taken about a process.

Flow cytometry has been extensively applied by us to monitor cell response for monitoring the stress responses to microorganisms in areas as bio-remediation and space biology that will be presented during the talk.

Quantum dots for sensitive multicolor detection in imaging and cytometry

Brunchez Marcel
Quantum Dot
Invitrogen Corporation

The unique spectral properties of Qdot nanocrystals allow simultaneous single wavelength excitation and simplified filter-based multiplexed detection at levels of sensitivity previously attained only with enzymatic amplification. Their photo- and chemical stability allows repeated analysis of samples for periods of many months to years. The application of commercially available Qdot Conjugates to a variety of biological problems, including single molecule tracking in live cells over long periods of time, protein trafficking in live cells, live animal imaging, and sensitive, multicolor biomarker detection in cells and tissues will be presented, along with examples of other applications of the Qdot Conjugate technology including cell tracking and flow cytometry.

Flow cytometry and fluorescent proteins in microbial ecology: what are the parameters influencing plasmid transfer?

Boon Nico, Depuydt Stephen and Verstraete Willy
Laboratory of Microbial Ecology and Technology (LabMET), Ghent University

Fluorescent proteins, like the green and red fluorescent proteins (GFP and RFP), have been used for *in situ* studies in microbial ecology. Analysis of the fluorescent-labeled bacteria in microbial communities is often performed by time-consuming microscopy. An alternative approach to examine the fate of fluorescent-labeled bacteria is the use of high-speed flow cytometry.
We used the combination of Flow Cytometry and the Green Fluorescent Protein to determine the optimal combination of environmental parameters on the transfer of the TOL plasmid from *Pseudomonas putida* BBC443 to *Escherichia coli* DH5alfa (Boon *et al*., 2006). This transferable plasmid was labeled with a gene encoding the green fluorescent protein (GFP) in order to make it a transfer reporter (Haagensen *et al.*, 2002). The *gfp* gene was under control of a *lac* promoter expression cassette and the *P. putida* donor contained a *lacI* repressor cassette. Thus, only in transconjugant *E. coli* cells, the *gfp* expression would lead to green fluorescent cells. A comparison of flow cytometry results with plating and microscopy showed that the majority of transconjugants were not culturable. Therefore, the amount of total cells (donor + acceptor) and transconjugants were determined by flow cytometry analysis, based on forward and sideward scattering, and green fluorescence, respectively. The number of transconjugants divided by the total number of cells represented the transconjugant ratio (TF). Five different parameters, i.e. temperature, carbon source, nitrogen source, antibiotics and chlorobenzoate, were combined by an evolutionary algorithm and the TF was considered as a measure of success. Based on the TF of each combination of parameters, the evolutionary algorithm showed that the optimal transfer conditions were obtained at 28°C and with the highest concentrations of carbon and nitrogen sources, resulting in a TF of 1.8×10^{-4}. Selective pressure was found to negatively influence the initial formation of transconjugants as such, but enhanced subsequent proliferation of the transconjugants. Future work will focus on using these new tools to be able to direct the generation of transconjugants in environmental samples.

Boon, N., S. Depuydt, and W. Verstraete. 2006. Evolutionary algorithms and flow cytometry to examine the parameters influencing transconjugant formation . FEMS Microbiology Ecology. In press.

Haagensen, J.A.J., S.K. Hansen, T. Johansen, and S. Molin. 2002. In situ detection of horizontal transfer of mobile genetic elements. FEMS Microbiology Ecology. 42:261-268.

The benefit of flow-cytometric sorting: access to 'unculterable' microorganisms

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Marine bacterioplankton is often pre-characterised by flow cytometric analysis. After staining the water samples with DNA stains, typically a few "operational" groups emerge in flow cytometric analysis, known as low nucleic acid (LNA) and high nucleic acid (HNA) containing populations. It was suggested that these populations have significantly different roles in the environment i.e. the LNA fraction being dormant and the HNA being the active part of the community. In parallel the diversity of bacterioplankton has been extensively explored in recent years by a set of methods called the full cycle rRNA approach. This approach is culture independent and therefore has the potential to monitor all microbial species in the marine environment. However from the phylogenetic affiliation alone little can be deduced about the role of a certain bacterioplankton group. Radio - labeled compounds are frequently used to elucidate the metabolic activity of the total bacterioplankton community. However it still remains a major challenge to link an identity of a population to a certain role. Here we report some case studies of the combination of flow cytometric sorting of radio labeled bacterioplankton populations and 16S rRNA gene cloning and fluorescence in situ hybridisation.

NK cells in human viral diseases

Demanet Christian
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Natural Killer (NK) cells are large granular lymphocytes and are very important in the innate host defence because of their capacity to lyse viral-infected and tumour cells spontaneously. The functional activity of NK cells is regulated by distinct cell surface receptors integrating a balance between positive and negative signals that either trigger or block NK cytotoxicity. Disruption of a dominant–negative signal by reduced expression of human leukocyte antigen (HLA) class I molecules on target cells triggers NK cell cytotoxicity mediated by activating receptors. Human NK cells express at least three types of receptors: the Killer cell Immunoglobulin-like receptors (KIRs), the CD94/NKG2 lectin-like receptors and the Natural Cytotoxicity receptors (NCRs). The former two are those that interact with HLA class I and signal inhibition or activation dependent on the amount of HLA class I molecules on the target cells.
The most important among these HLA class I-specific receptors are the KIRs that are encoded by a diverse and rapidly evolving family of 15 polymorphic genes located on chromosome 19. Within the human population, the variation of KIR haplotypes mostly depends on the gene number, the type of KIR genes and allelic polymorphism within individual KIR genes. As a result of the variable KIR gene content of NK cells, the innate immune response to control diseases, including against viral infections, may vary among humans. Additionally, it was recently shown that KIR and HLA together appear to affect outcome to viral infections, supporting a role for HLA class I in diversity in the innate immune response in addition to the acquired immune response.
In this presentation the basic immunology of NK cells will be presented and examples how HLA and KIR influence diseases such as HIV, Hepatitis C and CMV will be discussed.

Further reading:

- Parham P. MHC class I molecules and KIRs in human history, health and survival. Nature Reviews, vol. 5, 201-214; 2005.
- Martin M. et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nature Genetics, vol. 31, 429-433, 2002.
- Lodoen M. and Lanier L. Viral modulation of NK cell immunity. Nature Reviews, vol. 3, 59-69; 2005.
-Khakoo S. et al. HLA and NK cell inhibitory receptor genes in resolving Hepatitis C virus infection. Science, vol. 305, 872-874; 2004.
- Arase H. et al. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science, vol. 296, 1323-1326, 2002.
- Lopez-Botet M. et al. Natural Killer cell receptors for major histocompatibility complex class I and related molecules in cytomegalovirus infection. Review. Tissue Antigens, vol. 63, 195-203; 2004.

Gene activation therapy of retroviral diseases

Willems Luc
National Fund for Scientific Research, Molecular and Cellular Biology, Gembloux

Epigenetic modifications of chromatin may play a role in maintaining viral latent persistence of retroviruses like human T-lymphotropic virus type 1 (HTLV-1) and the related bovine leukemia virus (BLV). Indeed, HTLV and BLV presumably escape from immune surveillance by repression of viral transcription, conferring a partial advantage to the virus and leading to the progressive accumulation of infected cells. Conceptually, induction of viral gene expression could thus be used as a therapeutic strategy against retroviral associated diseases. We evaluated the potency of this concept aimed at activating viral gene expression in order to expose virus positive cells to the host immune response. We used valproate, a histone deacetylase inhibitor known since decades as a chronic and safe treatment for epileptic disorders. Valproate was efficient the treatment of BLV-induced leukemia in an ovine model system (Achachi et al 2005, Proc Natl Acad Sci USA, 102:10309-10314). Based on in vitro and in vivo data, we further provide evidence, as a proof of concept in human, that transient activation of the latent viral reservoir out of hiding causes its collapse, a process that might be curative for HTLV-associated diseases. In summary, we propose a mechanism of gene activation therapy tilting the host-pathogen balance in favor of an existing antiviral response.

POSTER ABSTRACTS

Validation of flow cytometry for the follow-up of *Trypanosoma* spp. infection

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The nowadays available methods for monitoring infection with *Trypanosoma* spp. are not satisfactory due to time-consuming manipulations that prevent the regular follow-up of large cohorts of animals. Here, we address the question of whether flow cytometry can yield values of parasitaemia that closely correlate with the established methods. Applied to peripheral blood drawn from *Trypanosoma evansi*-infected mice, a negative staining protocol combining erythrocytic glycophorin and nucleic acids (propidium iodide) tagging was found to allow very specific, accurate and reproducible countings. Furthermore, due to the small blood sample needed (1 µl), daily blood sampling in the laboratory mouse was possible without altering erythrocyte and reticulocyte countings. The results gathered here strongly plead for the use of flow cytometry in any situation where countings of circulating trypanosomes are needed. Moreover, the present labelling strategy allows the simultaneous quantitative study of the haemogram (erythrocytes, reticulocytes, granulocytes and nongranulocytes counts).

Flow Cytometry and Space Microbiology

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Laboratory of Radiobiology and Microbiology, Belgian Nuclear Research Center, SCK•CEN , Mol, Belgium

Bacteria exploit virtually every environmental niche on Earth ranging from the huge variety of habitats occupied by free-living organisms to those existing within food or within other organisms. Their success is due to their ability to deal with and adapt to the diverse stresses encountered during their varied interactions with the biotic and abiotic environment. Many bacteria are known to resist extremes of temperature, oxidation and reduction, acidity and alkalinity, pressure, osmolarity, radiation, high concentrations and a wide variety of toxic compounds, shortage of nutrients, competition and the transitory nature of the niche itself. Moreover it is known that many of the remarkable attributes of bacteria are only expressed under stress. Understanding how bacteria survive in such adverse conditions, both individually and at the population level, will contribute to their usefulness in the chemical and pharmaceutical industries as well as in bioremediation.

In this work, we have studied the effect of temperature stress (temperatures from -170°C to 70°C), oxidative stress (H_2O_2 concentrations from 0 to 880 mM), acid and alkaline stress (ranging from pH 2 to 12) and 'space flight' stress (10 days stay in the International Space Station) on various types of bacteria including *Cupriavidus metalidurans* and *Rhodospirillum rubrum* (tested for all stresses), and *Escherichia coli*, *Deinococcus radiodurans*, *Shewanella oneidensis* and *Arthrospira sp.* (tested for temperature and oxidative stresses only). Cell membrane permeability and potential, intracellular esterase activity, intracellular reactive oxygen species concentration and intracellular pH were assessed by flow cytometry to evaluate the physiological state and the overall fitness of individual bacterial cells under the different stress conditions.

We report that the bacterial strains exhibited varying responses in function of the type of stress applied and the type of bacteria. A moderate physiological change was observed at temperatures below or higher than the optimal culture temperature, at H_2O_2 concentrations above 13.25 mM, at two units pH under the optimal culture pH, and after a trip into Space. Membrane permeability and potential, esterase activity, intracellular pH and superoxide anion production were significantly modified at high or low temperatures, low pH and high H_2O_2 concentrations.

Our experiments show that a range of significant bacterial physiological alterations occurs under stress conditions and that fluorescent staining methods coupled with flow cytometry are useful for monitoring those changes.

Acknowledgements : This work is supported by the European Space Agency (ESA) and the Belgian Federal Science Policy Office (Belspo). The authors are grateful to Christel Paillé and Christophe Lasseur from ESA/ESTEC for their continuous support.

In vivo evaluation of *Clostridium acetobutylicum*-directed IL2 production in combination with radiotherapy for tumour control using rat rhabdomyosarcoma as a model

Barbé, S. [1], *Landuyt, W.* [2], *Verbeken, E.* [3], *Van Mellaert, L.* [1] *and Anné, J.* [1]
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Traditional anti-cancer therapies, such as surgical resection, radio- and chemotherapy, are effective in the treatment of many but not all patients. Hence, various alternative approaches for cancer treatment are being investigated such as bacterial vector systems, including *Clostridium* spp., acting as protein delivery vehicles. Upon administration to tumour-bearing organisms, spores of these strictly anaerobic, Gram-positive bacteria selectively germinate in the hypoxic/necrotic regions present in most solid tumours enabling the transfer of therapeutic proteins in the tumour microenvironment. Over the last few years, particular apathogenic *Clostridium* strains were successfully transformed with genes encoding the prodrug-converting enzymes cytosine deaminase and nitroreductase and the cytokine murine tumour necrosis factor alpha, resulting in intratumoural production of these therapeutic proteins.

The number of therapeutic proteins to be produced by *Clostridium* was recently extended with rat interleukin-2 (rIL2). This pluripotent cytokine was selected because it can enhance non-specific immune responses such as the activation of natural killer as well as lymphokine-activated killer cells, and major histocompatibility complex restricted T-cell responses resulting in neoplastic cell killing. Furthermore, IL2 (T-cell growth factor) was also reported to result in synergistic anti-tumour effects when combined with therapies that cause tumour cell death followed by an increased presence of tumour antigens, such as radiotherapy.

For the production of IL2 in *Clostridium acetobutylicum* DSM792, this strain was transformed with the plasmid pIMP1eglArIL2, in which rIL2 cDNA was translationally fused to the clostridial *eglA* signal sequence under control of the *eglA* promoter. rIL2 secreted by *C. acetobutylicum* (pIMP1eglArIL2) yielded *in vitro* up to 800 ng/ml growth medium and the secretion efficiency was up to 97 %. Intratumoural rIL2 amounts were assessed following injection of at least 10^7 spores of rIL2-secreting *C. acetobutylicum* into rhabdomyosarcomas transplanted in WAG/Rij rats. The measured rIL2 levels were on average 0.96 ng/g tumour tissue and were irrefutably higher than in control tumours. Furthermore, after immunohistochemical staining a clear increase in tumour-infiltrating $CD8^+$ T-lymphocytes was observed in tumours treated with rIL2-recombinant *C. acetobutylicum vs*. non-treated tumours. Finally, an experiment investigating the effect of small-scale fractionated radiotherapy (3 Gy/day for two days) combined with rIL2-recombinant clostridia on tumour growth, showed a significant growth delay compared to non-treated control tumours. This growth delay was larger than observed in tumours receiving radiotherapy alone or combined with intratumoural recombinant rIL2 injections. Interestingly, a small, significant growth delay was also observed in tumours receiving rIL2-recombinant bacteria alone.

The outcome of this experiment opens new perspectives to the clinical applicability of recombinant *C. acetobutylicum* DSM792 strains in combination with conventional therapies.

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The biosynthesis of branched-chain amino acids in *Mycobacterium tuberculosis* : cloning and characterization of the acetohydroxy-acid reductoisomerase

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Mycobacterium tuberculosis, the etiologic agent of tuberculosis, infects one-third of the world's population and kills 2 million people each year. There is an urgent need for new antimycobacterial agents and effective vaccines. Bacteria, fungi and plants, except animals, synthesize branched-chain amino acids (valine, isoleucine and leucine). Therefore, the corresponding biosynthetic enzymes are potential targets for specific herbicides, fungicides and antibiotics. Moreover, a leucine auxotroph mutant of *M. tuberculosis* was reported to show reduced growth in the host and is candidate for an auxotrophic vaccine. The acetohydroxy-acid reductoisomerase (ILVC) catalyzes the second common reaction in the biosynthesis of branched-chain amino acids. The reductoisomerase reaction is unusual and consists of two steps: isomerisation and reduction. The *M. tuberculosis* genome contains one copy of the *ILVC* gene and the gene product has not been characterized until now.

The *ILVC* gene of *M. tuberculosis* was cloned into the pRSETc vector and overexpressed in *E. coli.* The gene product was purified to homogeneity by Ni-NTA Sepharose affinity chromatography and gave on SDS-PAGE a 39 kDa band, as expected from its sequence. The enzymatic assay was developed based on that described for the plant reductoisomerase. The enzyme obeys the Michaelis-Menten kinetics. The K_M values for acetolactate, NADPH and Mg^{2+} were calculated from Lineweaver-Burk plots. The optimum conditions (stability, buffer, pH, temperature, divalent ions) for the reductoisomerase reaction were determined. The enzyme is inhibited at high concentrations of both the NADPH substrate and the $NADP^+$ product. Ascorbic acid slightly inhibits the ILVC activity. Known inhibitors of plant reductoisomerase (2-dimethylphosphinoyl-2-hydroxyacetic acid, Hoe 704 and *N*-hydroxy-*N*-isopropyloxamate, IpOHA) were effective also with the mycobacterial enzyme, and their relative IC_{50} were determined. However, the enzyme inhibition by Hoe704 and IpOHA was fully reversible. The antimycobacterial activity of both compounds was tested *in vivo* by following the growth of *M. tuberculosis* at the presence of the inhibitors. IpOHA had a bacteriostatic effect, and at 1 mM concentration reduced the growth of *M. tuberculosis* by 50%.

Altogether, our results indicate that the *M. tuberculosis* reductoisomerase differs significantly from the plant ILVC and could represent a good target for new drugs. Recent reports on transposon mutants of *M. tuberculosis* suggest that the *ILVC* gene could be non-essential at least in certain stages of the parasite's life. Consequently, *M. tuberculosis* must have other means for the acquisition of branched-chain amino acids. To address this intriguing possibility, we are currently preparing a knock-out mutant of *M. tuberculosis* with an inactivated *ILVC* gene and trying to identify transporters of branched-chain amino acids in *M. tuberculosis.*

CD8 expression in B-cell chronic lymphocytic leukemia : case report

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B-cell chronic lymphocytic leukemia (B-CLL) is one of the most commonly diagnosed leukemias in the western world and affects mainly elderly individuals. Immunophenotype of B-CLL is characterized by expression of pan-B-cell antigens (CD19, CD20, HLA-DR), of CD23 and co-expression of CD5, an antigen found on T lymphocytes. We described a case of B-CLL with aberrant CD8 expression and we analysed the genetic and immunophenotypic prognosis factors.

Case report: A 80 year old man was referred to our hospital for hyperlymphocytosis discovered in a preoperative evaluation prior to coronary angioplasty. Clinical examination revealed no adenopathy, hepatosplenomegaly or oedema. Peripheral blood evaluation showed a haemoglobin of 12.7 g/dl, platelets of 194000/mm³ and white blood cell count of 9620/mm³ with 4980 lymphocytes/mm³. There were no elevation of LDH or hepatic enzymes levels and no hypergammaglobulinemia. All others parameters were strictly within the normal limits. On peripheral blood smear, we found typic small lymphocytes with clumped chromatine and the bone marrow aspiration confirmed the medullar invasion of 50% by these lymphocytes. The patient presented thus a B-CLL with a stable and indolent disease course (Binet A).
Analysis of cell surface antigens: The immunophenotype of B-CLL was established by flow cytometry. All the B lymphocytes both in peripheral blood and in bone marrow expressed CD19, CD20, CD23, light chain Kappa and CD5 (Catovsky score = 5/5). Expression of CD38, which was found to correlate with the presence of unmutated VH genes and an unfavorable clinical outcome in different studies [1, 3], was negative in our case. Almost all of the B-CLL cells were positive for CD8 without detection of other T-cell marker, including CD2, CD3, CD4, CD7 and TCR $\alpha\beta$ or $\gamma\delta$.
Cytogenetic analysis by fluorescent *in situ* hybridization (FISH): Clonal genomic aberrations can be identified in approximately 80% of CLL patients by FISH [1-2]. The most frequent changes are deletion in 13q, deletion in 11q, trisomy of 12q and deletion of 17p. 11q deletion and 17p deletion are unfavourable aberrations whereas 13q deletion is associated with favourable outcome. Analyses of the patient' B-CLL cells by FISH did not show any of these chromosomic aberrations.
Mutational status of immunoglobulin heavy-chain variable region genes in the leukemic cells : One of the most important molecular genetic parameter is the mutation status of VH genes defining pathogenic and prognosis subgroups in CLL [3]. While CLL with unmutated VH shows an unfafourable course with rapid progression, CLL with mutated VH often shows slow progression and long survival. In our case, the patient had a mutated VH gene (VH4-34).

Conclusion: This case report describes a patient presenting a CD8+ B-CLL with a stable and indolent disease course (Binet A). This suggests that CD8 expression might be a favourable prognostic marker in CLL as reported in other cases described in the litterature [4-5]. Other prognosis markers were in accordance with the clinical features.

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Pasteurization of Milk Abolishes Bovine Herpesvirus 4 Infectivity

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Bovine herpesvirus 4 (BoHV-4) is a gammaherpesvirus highly prevalent in the cattle population that has been isolated from the milk and the serum of healthy infected cows. Several studies reported the sensitivity and the permissiveness of some human cells to BoHV-4 infection. Moreover, our recent study demonstrated that some human cells sensitive but not permissive to BoHV-4 support a persistent infection protecting them from tumor necrosis factor-α–induced apoptosis. Together, these observations suggested that BoHV-4 could represent a danger for public health. To evaluate the risk of human infection by BoHV-4 through milk or serum derivatives, we investigated the resistance of BoHV-4 to the mildest thermal treatments usually applied to these products. The results demonstrated that milk pasteurization and thermal decomplementation of serum abolish BoHV-4 infectivity by inactivation of its property to enter permissive cells. Consequently, our results demonstrate that these treatments drastically reduce the risk of human infection by BoHV-4 through treated milk or serum derivatives.

Structural organization in a multimember linuron degrading bacterial biofilm visualized by Confocal Laser Scanning Microscopy

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Phenyl urea herbicides are among the most widely used herbicides in agriculture, resulting in important contamination of both surface water and groundwater. Recently, several multimember microbial communities mineralizing phenyl urea herbicides such as linuron have been isolated from long-term treated agricultural soils, indicating that bacteria adapted to the contamination.
The topsoil is considered as an important biological filter which determines the extent of pesticide degradation and hence, the extent of groundwater contamination. So far, little attention has been paid to the role of the solid surface on the structure, spatial organization, stability and activity of commensal pollutant degrading communities of natural origin. In order to study these parameters, we developed a method to differentiate between the three members of a linuron mineralizing bacterial consortium using reporter genes and nucleic acid staining. *Variovorax* sp. WDL1 is the key member of the consortium as it degrades linuron to 3,4-dichloroaniline (3,4-DCA) and *N,O*-dimethylhydroxylamine. WDL1 is able to mineralize 3,4-DCA but at a low rate leading to leakage of 3,4-DCA. Residual 3,4-DCA is mineralized by *Comamonas testosteroni* WDL7. *Hyphomicrobium sulfonivorans* WDL6 degrades the intermediate *N,O*-dimethylhydroxylamine.

Comamonas testosteroni WDL7 was labeled with a reporter gene encoding for the fluorescent protein Rfp, while *Hyphomicrobium sulfonivorans* WDL6 was equipped with an Yfp encoding gene cassette. A fluorescent nucleic acid stain (DAPI) was used to distinguish *Variovorax* sp. WDL1 from the specifically labeled strains.
The structural interactions between the community members grown as mixed biofilms were studied in flow chambers by means of Confocal Laser Scanning Microscopy (CLSM). Under selective nutrient conditions with linuron as sole source of carbon and nitrogen (0,2 mM), the consortium members formed an heterogeneous biofilm with a single layer base and randomly positioned mushroom-like structures. These structures consisted mainly of associated *Comamonas* and *Variovorax* cells with very few *Hyphomicrobia*. However, more downstream some of the mushroom-like structures consisted almost exclusively of *Hyphomicrobium* cells.
On the other hand, when the consortium was fed with a non-selective carbon source, i.e. citrate, both structure and organization of the biofilm was altered. The synergistic association between the bacteria was lost and *Comamonas testosteroni* WDL7 became the dominant member forming a more homogeneous multilayered biofilm.

Detection and isolation method for bacterial lysozyme inhibitors by use of lysozyme affinity chromatography

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Lysozymes are hydrolytic enzymes that cleave the β-(1,4) glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycan, the major bacterial cell wall polymer. Although lysozymes have since long been the subject of intense study, the first proteinaceous lysozyme inhibitor, named Ivy (*I*nhibitor of *v*ertebrate l*y*sozyme), was only recently discovered in *Escherichia coli* (Monchois *et al.,* 2001). Interestingly, homologues of Ivy have thus far only been found in the genera *Shigella, Erwinia, Yersinia, Gluconobacter, Pseudomonas, Chromobacterium* and *Burkholderia* on a total of more than 200 completely finished bacterial genome sequences to date. Nevertheless, in view of the widespread occurrence of lysozymes and their role in bactericidal systems, a general role for bacterial lysozyme inhibitors to counter these potentially lethal enzyme attacks seems likely and such inhibitors are thus expected to be more widespread than the homologous sequences of the Ivy protein suggest. In the search for new types lysozyme inhibitors, an adequate and function based method to detect and isolate these proteins is required.

Recently, we successfully developed a method to detect and isolate the lysozyme inhibitor Ivy from *E. coli* (Callewaert *et al*., 2005). To examine its efficiency in isolating additional lysozyme inhibitors from different bacterial sources, in this study the procedure was applied to wild type *E. coli* and *Shigella flexneri,* a bacterial strain predicted to contain an open reading frame in its genome with homology (298 bits) to the *E. coli ivy* gene.

Cells were grown to stationary phase, harvested by centrifugation and subjected to a cold osmotic shock to isolate the periplasmic proteins (Callewaert *et al*., 2005). These extracts were loaded on a Hen Egg White Lysozyme (HEWL)-affinity column. After washing with 2 column volumes 0.1 M Tris-HCl pH 7.0 the column was eluted with a linear gradient from 0 to 2.0 M KCl in 0.1 M Tris buffer, adjusted to pH 12.0. After neutralisation to pH 8 and stabilisation with BSA, eluted fractions were dialysed against potassium phosphate buffer (10 mM, pH 7) and inhibitory activity against HEWL was measured. To analyse the degree of purity, the fractions with inhibitory activity of the two different bacteria were subjected to SDS-PAGE, followed by silver staining. As expected, we found a single band on SDS-PAGE in the purified chromatography fraction of *E. coli* corresponding to the molecular weight of Ivy (14.103 kDa). For *S. flexneri*, also a single band, with similar molecular weight as Ivy from *E. coli* was detected. Tandem mass spectrometry analysis subsequently identified these proteins as Ivy of *E. coli* and the hypothetical protein YkfE of *S. flexneri*, which corresponds to the Ivy-homologous sequence respectively.

The method described in this work was successful to detect and isolate bacterial HEWL-inhibitors from *E. coli* and *S. flexneri.* Since even small amounts of inhibitor were detected and pure fractions which showed no activity loss were gathered, this method was proven to be adequate in the search for new HEWL-inhibitors. Moreover, the existence and the inhibitory activity of the *S. flexneri* Ivy homologue, as well as its periplasmic location, is demonstrated in this study.

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Resistance of *Propionibacterium acnes* biofilms against antimicrobial agents commonly used in the treatment of acne

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The development of the common skin disorder *acne vulgaris* is related to changes in hormone levels, e.g. an increase in androgen levels with the onset of puberty. This increase in androgens is related to abnormal keratinisation of cells in the pilosebaceous duct, an increase in sebum production, accelerated growth of *Propionibacterium acnes* and inflammation. *P. acnes* plays an important role in the pathogenesis of acne. By producing extracellular lipases, *P. acnes* accelerates the breakdown of sebum into free fatty acids, leading to inflammatory acne lesions and local tissue destruction.

Recently it was suggested that *P. acnes* residing within the pilosebaceous follicles grows as a biofilm. Sessile cells (i.e. cells that grow in a biofilm) in general show marked phenotypic and physiological differences compared to planktonic cells, including a drastically increased resistance to commonly used antimicrobial agents.

In the present study we evaluated the biofilm-forming ability of *P. acnes* in a microtiter plate model. We also evaluated the resistance of biofilm-grown *P. acnes* towards antimicrobial agents commonly used in the treatment of acne. Our results indicate that *P. acnes* can form biofilms *in vitro*. The results also show that sessile *P. acnes* cells are more resistant to most antimicrobial agents tested (including erythromycin, clindamycin and benzoyl peroxide) than planktonic cells. Benzoyl peroxide in combination with erythromycin or clindamycin has the greatest potential to eradicate *P. acnes* biofilms. Our data also indicate that, of the classical antibiotics, erythromycin has the greatest activity, and that salicylic acid and triclosan may be of value in the treatment of (antibiotic resistant) *P. acnes* in acne.

Negative regulation of T cells by immature dendritic cells is mediated by TGF- β- and IL-10-producing T-helper cells

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Dendritic cells, professional antigen-presenting cells, are currently seen as the key regulators in directing the immune system to a state of tolerance or immunity. In a steady state condition, the main function of immature dendritic cells is the capture of auto-antigens and keeping the immune system quiescent to these self-antigens. After encounter of an inflammatory danger signal, mature dendritic cells migrate to the secondary lymph nodes and present (foreign) antigens to T cells to induce a robust immune response to eradicate the infection. Recent data have indicated that the presentation of antigens by immature dendritic cells results in an inferior immune response. The mechanism of this induction of anergy or tolerance is still poorly understood. The general idea is the lower presentation of costimulatory molecules by immature dendritic cells as compared to mature dendritic cells. However, it is speculated that dendritic cells can also actively induce a state of peripheral tolerance by the induction of negatively regulating T cells.

In a CMV model-system, we used antigen-presenting cells in different activation states, pulsed with the cytomegalovirus-pp65 protein-specific CMV-peptide, to stimulate autologous T cells in a 7 day during co-culture flask. According to their activation state, the antigen-presenting cells, immature or mature dendritic cells respectively, failed to stimulate or efficiently mounted a CMV-peptide specific IFN-γ immune response as detected with IFN-γ ELISA.

However, we demonstrated a significantly higher amount of TGF-β produced in the 7 day co-culture of T cells with autologous immature dendritic cells as compared to the co-cultures with mature dendritic cells or in the absence of dendritic cells.

In order to show if immune-suppressive cytokines were responsible for the non-responsiveness of T cells after stimulation with autologous peptide-pulsed immature dendritic cells, we used neutralizing antibodies for TGF-β and IL-10. After administration of these blocking antibodies, we demonstrated that T cells stimulated once with autologous immature dendritic cells properly gave rise to a CMV-peptide specific IFN-γ response as compared to the response to a control peptide. Moreover, both neutralizing antibodies had a synergistic effect.

Next, we characterized the induced TGF-β-and IL-10-producing cells by multi-parameter flow cytometry. These were CD4+ CD25- T cells.

Ongoing experiments will further address the antigen-specificity of the induced non-responsiveness of T cells after co-cultivation with immature dendritic cells. We will also investigate cellcontact-dependence of the negative regulation by immature dendritic cells.

Demonstrated results are of outmost importance for understanding the regulation of the immune system and for the development of new therapies for several ailments, such as cancer and auto-immune disorders.

Study of the endophytic potential of *Cupriavidus metallidurans*, a bacterium specifically adapted to toxic heavy metals for phytoremediation applications

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Cupriavidus metallidurans (previously *Ralstonia metallidurans*) is a non-pathogenic and anthropogenic soil gram β-proteobacterium exceptionally resistant to heavy metals. Strain *C. metallidurans* CH34 carries two chromosomes and the two plasmids pMOL28 (171kb) and pMOL30 (234kb) which possess genes for multiple heavy metals resistance mechanisms: *czc* (resistance to Cd2+, Zn2+ and Co2+), *cnr* (resistance to Co2+ and Ni2+), *chr* (resistance to CrO42-), *mer* (resistance to Hg+), *pbr* (resistance to Pb2+) and *cop* (resistance to Cu2+). Its closely related genome with *Ralstonia solanacearum* suggests a potential endophytic invasion capability within *Solanaceae*, major host-plants of the phytopathogen. This work focuses on the potential of *C. metallidurans* to colonize plants and improve their capacity in heavy metal uptake and accumulatation.

First, in silico analysis allowed the identification inside the *C. metallidurans* genome of the major genes of the *phc* network regulatory system involved in pathogenesis (*phcARSB*, *vsrADBC*, *pehSR*, *solR*, *rpoS*); adherence, swimming and twitching motility (*pilTUE$_2$VWY$_1$MNOPQX*, *fimUT*) and the EPS I biosynthesis (*xpsR*, *epsFEAPB*, *rkpS*, *wbpM*, *pglC*). The identified genes showed similarity with their equivalent in the *R. solanacearum* genome. Genes involved in surface proteins and exopolysaccharides biosynthesis showed lower similarity between *C. metallidurans* and *R. solanacearum*. Whereas genes involved in *pili* and *fimbriae* biosynthesis are located on the chromosomal and clustered in two major operons inside the *R. solanacearum* genome, they are split into several operons on the *C. metallidurans* chromosome, megaplasmid and pMOL28. We also identified some chromosomally encoded genes normally under *xpsR* control although this regulator and related genes are closely located on the *R. solanacearum* megaplasmid. These results suggest a functional difference in the *phc* system of the two organisms.

Then, different inoculation protocols were followed to investigate the capacity of *C. metallidurans* to invade and establish an endophytic colonization within *Lypersicon esculentum*, *Solanum melongena* and *Nicotiana plumbaginifolia*. To monitor plant colonization, we used a strain of *C. metallidurans* emiting a green fluorescence. This strain was auxotroph for phenylalanine and harboured genes of antibiotic resistance (tetracycline and nalidixic acid). *Arabidopsis thaliana* was used as a putative non-host plant control. Plant auto-fluorescence didn't allow us to clearly locate Gfp-expressed bacteria inside plant tissues. Nevertheless, microbiologic studies using the three others markers confirmed plant endophytic colonization by *C. metallidurans* in the densities ranging between 0,6.101 and 41.104 CFU.g-1 FM; depending on the analysed species and plant organs. For all the plants, roots were the main plant organs colonized and *N. plumbaginifolia* seemed to be the best *Solanaceae* study model.

Antibiotic induced autolysis in *Escherichia coli* is independent of the level of lysozyme inhibitor IvY, but increases with plasmid insertion

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The product of the *E. coli ykfE* gene is an inhibitor of C- type lysozyme (Monchois *et al.*, 2001) and goose egg white lysozyme (Callewaert *et al.*, 2005). This inhibitor, designated as Ivy (Inhibitor of Vertebrate Lysozyme), is the first lysozyme-inhibiting protein to be reported. To date it is still unclear whether besides the role in lysozyme resistance (Deckers *et al.*, 2004) other functions could be ascribed to Ivy. The periplasmic localisation of Ivy, however, suggests a possible role for Ivy in the regulation of the endogenous lysozymes of *E. coli*. Also the structural similarity between Slt70, the most abundant lysozyme of *E. coli*, and goose egg white lysozyme on the one hand, and between the bacterial lysozymes MltC, MltD and the C-type lysozymes on the other, implicate a putative role for Ivy as inhibitor of their hydrolytic activities. Therefore we examined the effect of the presence or absence of Ivy on the sensitivity of *E. coli* MG1655 towards the β-lactam antibiotic ampicillin that disturbs the controlled activity of bacterial lysozymes and consequently leads to autolysis.

Regarding *E. coli* MG1655 (pAA420), which overexpresses Ivy from an arabinose-inducible promoter, and the wild-type *E. coli* MG1655 strain grown in the presence of arabinose, our results seems to suggest that the susceptibility of *E. coli* to ampicillin-induced autolysis is inversely related to the level of Ivy produced. However, inactivation of the *ivy* gene (*E. coli* MG1655 *ivy*::Kan) did not result in a higher tolerance towards ampicillin compared to the wild-type strain. Moreover, when MG1655 wild-type and MG1655 (pAA420) were grown in the absence of arabinose, treatment with ampicillin caused a similar enhanced lysis of the MG1655 (pAA420) strain compared to the wild-type. These results were unexpected and indicate that either the different phenotype is already the result of leak expression of the arabinose promoter and that higher concentrations of Ivy have no additional influence, or that the antibiotic sensitivity of *E. coli* MG1655 (pAA420) is not due to the overproduction of Ivy. To discriminate between these two possibilities, the *ivy* gene was cut out from the pAA420 plasmid, resulting in pAA421, and was electroporated into MG1655 wild-type and MG1655 *ivy*::Kan, and the resulting strains were exposed to ampicillin. The introduction of the pAA421 plasmid did render both wild-type and MG1655 *ivy*::Kan more sensitive to ampicillin induced autolysis, indicating that the production of Ivy does not influence β-lactam sensitivity.

Based on the comparable sensitivity observed for the wild-type *E. coli* MG1655 and *E. coli* MG1655 *ivy*::Kan strains, this study shows that Ivy does not exhibit a regulatory function in ampicillin induced autolysis, although such a function would not have been surprising. Moreover, it appeared that the increased β-lactam sensitivity of MG1655 (pAA420) is unrelated to overproduction of Ivy but, by contrast, was found to rely only on the presence of the plasmid vector carrying this gene. These results point to the profound impact of plasmids on cellular physiology and warn for possible interference with experimental design.

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Sialoadhesin, a macrophage-specific lectin, is an endocytic receptor mediating porcine arterivirus internalization

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Sialoadhesin (Siglec-1) was previously identified in man and rodents as a macrophage restricted lymphocyte adhesion molecule. The protein belongs to the family of sialic acid binding immunoglobulin like lectins (Siglec) and although its exact functions are not known, it mediates interactions with other cells of the immune system, such as neutrophils and T-cells, and these interactions are believed to be crucial in immune regulation. Recently, we have identified porcine sialoadhesin as a receptor on macrophages for the porcine arterivirus. The porcine arterivirus, which belongs to the Arterivirus family (grouped in the order Nidovirales, containing also human viruses such as SARS Coronavirus) exclusively infects sialoadhesin expressing macrophages distributed throughout the body. We found that sialoadhesin, although it was not described as an internalization receptor, is capable of performing clathrin mediated internalization, both in macrophages naturally expressing the protein and in cells expressing recombinant sialoadhesin. The capacity of sialoadhesin to mediate clathrin-dependent internalization, which was shown to occur upon addition of the porcine Arterivirus or upon addition of a sialoadhesin specific monoclonal antibody, is most likely mediated via a potential internalization motif in its cytoplasmic tail, a feature that is now being investigated. Although we observed that sialoadhesin internalizes the virus into endosomes, infection does not occur in any of the tested cell types that express recombinant sialoadhesin. Further studies indicated that the virus is apparently not disassembled in the endosomes and that the viral genome is not released in the cytoplasm. The nature of the other, macrophage-specific, factors that are needed for full infection are now being investigated. Furthermore, we observed that the porcine arterivirus misuses the sialic acid binding capacity of sialoadhesin to interact with this receptor, and to gain entry in the macrophage. In contrast to what is observed with other viruses, such as influenza virus, we found that sialic acid on the surface of PRRSV is essential for the virus to attach with sialoadhesin, and for infection to occur.

The *cyd* operons coding for a bd type of oxidase homologue and a nitrogen-fixation related gene from purple sulfur bacterium *Allochromatium vinosum*

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Microaerobic growth properties of the photosynthetic purple sulfur bacterium *Allochromatium vinosum* has been shown previously. However, there was no information on the gene coding for a terminal oxidase. Here, we present two independent operons, one of which may code for a bd type of oxidase as well as a nitrogen-fixation related gene, and the second one, coding for an ABC type of membrane transporter.

The presence of a *bd* type of quinol oxidase is not surprising in *Allochromatium vinosum* as it has an important role in microaerobic nitrogen fixation in other diazotrophs. *cydAB* are two contiguous open reading frames in the operon, which show high homology to two subunits of *bd* type of terminal oxidase. The deduced amino acid sequence of subunit I shows % 81 homology with *bd* oxidase subunit I of *Azotobacter vinelandii* and % 82 to *Escherichia coli* and deduced amino acid sequence of subunit II shows % 67 homology with *bd* oxidase subunit II of *Azotobacter vinelandii* and % 70 to *Escherichia coli*. Two histidines and a methionine, which are the axial ligands of the three hemes, are conserved in the open reading frames. Upstream of *cydA*, a nitrogen fixation related gene is present. Northern blot analysis is on progress to determine whether these ORFs are co-transcribed.

A non-contiguous *cydDC* operon has also been revealed. *cydD* gene shows up to 79% homology to other bacterial genes, while *cydC* homology is upto 63%. The gene product probably codes for an ABC (ATP binding cassette) type of membrane transporter that plays important role in cysteine transport as well as the membrane assembly of some *bd* oxidases from other species.

Infection of trigeminal ganglion neurons with the alphaherpesvirus pseudorabies virus induces the formation of synaptic boutons that may serve as axonal exit sites for the virus

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Alphaherpesviruses are a subfamily of the herpesviruses, containing closely related human and animal pathogens, including human herpes simplex virus (HSV), porcine pseudorabies virus (PRV) and bovine herpesvirus 1 (BoHV-1). These viruses mostly cause mild disease, e.g. cold sores for HSV-1, but in rare cases, they are associated with encephalitis. Many of the symptoms observed after infection with alphaherpesviruses are associated with their neurotropic behaviour and their ability to establish a lifelong latent (non-replicating) infection in neurons of the peripheral nervous system of their host. Repeated reactivation from latency may lead to recurrent disease symptoms and viral spread between hosts. Sensory neurons of the trigeminal ganglion (TG) are major target cells for latency/reactivation of different alphaherpesviruses, including HSV-1, PRV, and BoHV-1. Egress of newly produced virions (during primary infection or reactivation) from TG neurons occurs via anterograde spread along the axon and virus release at the axon terminus. However, based on *in vivo* experiments with rat optic nerves, it has been suggested that alphaherpesviruses can also be released from axons along the axon shaft (Tomishima & Enquist, J.Virol., 2002).

We have developed a homologous *in vitro* two-chamber model, in which porcine TG neurons are cultured in an inner chamber where they develop axonal processes that grow through a virus-impermeable silicon barrier into an outer chamber. PRV inoculation in the outer chamber results in infection of the neurons via the axons, similar to the *in vivo* route of infection. Interestingly, using this model, we observed that PRV infection of TG neurons induced the formation of pre-synaptic boutons (varicosities) along the length of the axon (72% of TG neurons with varicosities at 24hpi with PRV compared to only 12% for non-infected TG neurons). Varicosities were positive for the synaptic marker synaptophysin and could be observed from 6 to 48hpi (end of the experiment). Furthermore, using confocal microscopy and live cell imaging, we obtained strong indications that these newly formed synaptic boutons constitute sites for efficient egress of PRV from the axonal shaft to neighbouring cells.

In conclusion, we showed that PRV infection induces the formation of pre-synaptic boutons along the axon shaft of TG neurons *in vitro* and that the virus may use these boutons as axon exit sites to infect neighbouring cells.

Limb defects induced by X-irradiation in mouse foetuses

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In utero irradiation of the foetus during the period of organogenesis can induce an increase in malformation. However, the mechanisms underlying irradiation-induced teratogenesis are far from being elucidated. In the present study, we focused on malformations of the limbs to explore at the molecular level the complex development of limb bud, following irradiation exposure. C57BL mice were exposed to 3 Gy X-rays *in utero* on day 12 of gestation during the period of organogenesis of limb buds.

External examination under a stereomicroscope of 19 day X-irradiated embryos revealed 100 % of forelimb defects. Coexistence of different limb malformation variants in a single limb segment was evidenced during the limb defect characterization and classification steps. All foetuses were hypodactyl (fewer than five digits on a limb) with various extent in the severity: 43% of the examined fetuses had 3 digits, 23 % exhibited 1 digit, 18,5 % 4 digits, 8 % 2 digits and 5,7 % had stump. Syndactyly (having two or more fused digits) was highly present as 71 % of the foetuses showed cutaneous syndactyly upon external examination while 99 % of them revealed the presence of osseous syndactyly after the whole-mount skeletal examination. Ectrodactyly (missing digit and metacarpal) and aphlangy (missing digit but metacarpal present) concerned respectively, 80 and 49 % of the total foetuses observed. Further experiments are currently under progress using microarrays to investigate early and late gene expression modulations in limb buds from 12 day old embryos irradiated with 3 Gy. Future studies will also be directed towards the investigation of telomere length variations in skin fibroblasts issued from control and X-irradiated mouse foetuses. In this context we will use the Flow-FISH technique (flow cytometry combined with Q-FISH analysis) that had been previously optimised on human cancer cell lines in our laboratory.

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Capping of viral cell surface proteins in pseudorabies virus-infected cells is associated with tyrosine phosphorylation and lipid raft association of gE

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Pseudorabies (PRV) is a swine alphaherpesvirus that is closely related to the human herpes simplex virus (HSV). Upon infection of a cell with an alphaherpesvirus, many differeny viral envelope proteins are expressed on the surface of the infected cell and render the cell recognizable for virus-specific antibodies and subsequent antibody-dependent cell lysis. We and others have shown that interaction between antibodies and the viral proteins on the plasma membrane of specific cell types infected with PRV or HSV results in redistribution of the antibody-antigen complexes to one pole of the cell (capping)(Favoreel et al., 1997, J. Virol., Rizvi & Raghavan, 2003, J. Virol.). Both for PRV and HSV, initiation of this process depends on the presence of the viral envelope protein gE. The exact function of this gE-mediated capping process remains unclear, although it has been suggested to lower the efficiency of antibody-dependent lysis of the infected cells and it has also been associated with enhanced spread of the virus from one cell to another.

This viral capping process resembles the physiological process of lymphocyte receptor capping, a process involved in lymphocyte activation. Key events in the latter process are association of the lymphocyte receptor with special cholesterol-enriched microdomains in the plasma membrane (lipid rafts) and subsequent tyrosine phosphorylation of YXXΦ motifs in its cytoplasmic tail by lipid raft-residing Src kinases. Since the viral capping process is mediated by two YXXΦ motifs in the cytoplasmic tail of gE, and is inhibited by disruption of lipid rafts or inhibition of tyrosine phosphorylation (Favoreel et al., 1999, Virology, Favoreel et al., 2004, J. Virol.), we hypothesize that the process of capping of viral cell surface proteins is a viral mimicry of lymphocyte receptor capping. The aim of the current study was to assess whether, in further analogy with lymphocyte receptor capping, PRV gE associates with lipid rafts and/or is tyrosine phosphorylated during gE-mediated capping.

Use of a phosphotyrosine-specific monoclonal antibody on a Western blot provided evidence for tyrosine phosphorylation of a fraction of gE during capping. Immunoprecipitation of gE from cells infected with wild type PRV or mutant PRV strains that either lack the cytoplasmic domain or contain point mutations in the YXXΦ motifs of gE (Tirabassi & Enquist, 1999, J. Virol.) showed that tyrosine phosphorylation of gE occurs in the cytoplasmic domain, at least partly on the YXXΦ motifs. Moreover, the Src kinase inhibitor pp2 reduced tyrosine phosphorylation of gE. Specificity testing of the phosphotyrosine monoclonal antibody with phosphotyrosine-BSA, phosphothreonine-BSA and phosphoserine-BSA allowed us to attribute the entire detected phosphorylation signal to tyrosine phosphorylation. Using detergent lysis and density ultracentrifugation, we also found indications that a fraction of gE associates with lipid rafts.

Thus, we have indications that PRV gE is tyrosine phosphorylated in its YXXΦ motifs by Src kinases and may associate with lipid rafts during gE-mediated capping. These results further suggest that gE-mediated capping of viral cell surface proteins constitutes a mimicry of lymphocyte receptor capping.

Mutational analysis of the operator region of the *Escherichia coli carAB* operon encoding carbamoylphosphate synthetase

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In *E. coli* a single carbamoylphosphate synthetase, encoded by the *carAB* operon, provides carbamoylphosphate for the biosynthesis of both arginine and the pyrimidines. Its production is essentially regulated at the level of transcription initiation at two tandem promoters. P2, downstream, is regulated by arginine and the arginine repressor. Pyrimidine-specific control of the upstream promoter P1 requires the multifunctional proteins, IHF (Integration Host Factor), PepA (Aminopeptidase A) and PyrH (UMP-kinase) which cooperate to form a regulatory nucleoprotein complex (Charlier *et al*., 2000). Recent studies demonstrated that P1 activity is also regulated by excess purines and the purine repressor and that this purine-specific repression is functionally and structurally coupled to pyrimidine-specific repression (Devroede *et al.*, 2004). P1 is also downregulated by UTP-sentive reiterative transcription (Han and Turnbough, 1998) and, upon nutritional stress, by stringent control (Bouvier *et al.*, 1984).

Our knowledge of the predominant protein-dependent pyrimidine-specific repression of P1 is still incomplete, especially the structural and molecular details of how these proteins interact to form the regulatory nucleoproteincomplex and how this complex influences the promoter activity. The binding sites for these regulatory proteins are spread over a control region of about 450 bp with large intervening sequences. Here we focus on these linker sequences, investigating the importance of the phasing and spacing of the regulatory targets by introducing insertion and deletion mutations of various lengths, and combinations thereof, in the four linkers. Their effects on promoter activity and repressibility were assayed with a single-copy *car*P1-*lacZ* reporter gene fusion construct. Finally, the analysis of a P1-*lacZ* reporter construct devoid of its upstream control region has lead to a reappraisal of the effect of excess adenine on P1 activity, and revealed that P1 has no UP element.

The analysis of half and full-turn insertion and deletion mutants in different zones of the operator, indicates that pyrimidine-dependent repression of P1 activity is highly sensitive to perturbations of the distribution of regulatory targets on the surface of the double stranded helix. Moreover, the systematic analysis of the L2 linker length demonstrates that the two PepA binding sites must be properly aligned, also relative to the promoter, and their separation is equally strictly constrained in both, pyrimidine and purine-mediated repression. Similarly, the phasing and the spacing of the IHF and PEPA2 sites is strictly constrained, but for the pyrimidine-specific repression only. A correct alignment of these sites is not required, and the IHF binding site is even dispensable for purine-mediated regulation. Thus, PepA turns out to be the key element and correct localisation of PepA within the higher order regulatory nucleoprotein complex is an absolute prerequisite for the establishment of both, pyrimidine and purine-mediated repression.

The analysis of a *car*P1 derivative (P1-ΔO1) disconnected from its downstream tandem partner (*car*P2) and devoid of its upstream control region has allowed: -1- to demonstrate that the A+T rich region upstream of the -35 element does not function as a UP element, -2- to distinguish the major protein-dependent pyrimidine-specific repression from other direct and indirect regulatory effects, and -3- to reveal the antagonistic effects of excess adenine on P1 activity: an indirect stimulation related to variation in the internal purine and pyrimidine nucleotide pools and a downregulation by purines and the purine repressor, resulting in a net twofold repression.

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Demonstration by flow cytometry that $CD5^+CD8^+$ cells carry alcelaphine herpesvirus 1 in inoculated rabbits developing malignant catarrhal fever

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Alcelaphine herpesvirus 1 (AlHV-1), carried by wildebeest (*Connochaetes taurinus*) asymptomatically, causes malignant catarrhal fever (MCF) when cross-species transmitted to a variety of susceptible species of the *Artiodactyla* order. MCF is a fascinating disease described as a combination of lymphoproliferative and degenerative lesions. The study of MCF pathogenesis has been impeded by an inability to produce recombinant virus, mainly due to the fact that AlHV-1 becomes attenuated during passage in culture. Here, we have overcome these difficulties by (*i*) cloning the entire AlHV-1 genome as a stable, infectious and pathogenic bacterial artificial chromosome (BAC), and (*ii*) by using prokaryotic recombination technology for the production of an AlHV-1 recombinant. Firstly, the AlHV-1 genome was BAC cloned using one insertion site in a region containing no open reading frame. This insertion allowed the production of an AlHV-1 BAC clone stably maintained in bacteria and able to regenerate virions when transfected into permissive cells. BAC-derived AlHV-1 virions induced MCF in rabbits comparably to the AlHV-1 wild-type (WT) strain. Secondly, a two-step mutagenesis procedure in *E. coli* was used to generate a recombinant strain expressing *enhanced-green fluorescent protein* (EGFP) as a reporter gene. After reconstitution of recombinant virions into permissive cells and excision of the BAC cassette, flow cytometry analyses were performed to validate the recombinant strain and to investigate the pathogenesis of MCF. The results of these analyses can be summarized as follows: (*i*) the validity of the EGFP expression cassette as a reporter gene has been demonstrated by *in vitro* infections; (*ii*) inoculation of rabbits revealed that the recombinant strain has retained the pathogenicity of its parental strain and that the cell types carrying AlHV-1 in peripheral blood mononuclear cells, lymph nodes and the spleen are mainly CD5+ CD8+ cells.

Enrichment of PCE/VC reductively dechlorinating microbial consortia from river sediment infiltrated by VC contaminated groundwater originating from a PCE polluted aquifer

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Chlorinated aliphatic hydrocarbons (CAHs) are one of the main groundwater pollutants in Europe and result into surface water contamination through infiltrating groundwater at many sites. However, the contaminants have to pass a zone with a large potential of natural attenuation activity before reaching the main surface body, i.e., the sediment zone. This zone is characterized by steep redox potentials and can provide niches for a wide variety of metabolic diverse microorganisms. Considering the generally high abundance of bacteria in sediments, the bacterial activity is often high and could play a major role in the degradation of CAHs infiltrating via the groundwater. The Belgian river Zenne at Vilvoorde was selected for this study. In this area, groundwater polluted with vinyl chloride (VC) and *cis*-1,2-dichloroethene (cDCE) infiltrates the Zenne.
Microcosms with Zenne sediment and anoxic reduced media were incubated with either tetrachloroethene (PCE; in hexadecane; 40 µM aqueous concentration) or VC (100 µM aqueous concentration) at 25°C. Methanol or lactate was added as electron donor or no electron donor was added (biotic control). Autoclaved sediment was used for abiotic controls.

Both PCE and VC were completely degraded (reductively) within 40 days. PCE was eventually fully dechlorinated to ethene within 100 days. In VC enrichment cultures, the degradation rate was about 0.6 µmol/day/g sediment (wet weight) after repeatedly adding VC and increasing the concentration in time. There was no significant difference in degradation between the different added electron donors or the microcosms with no added electron donor, indicating that Zenne sediment contains sufficient potential electron donor for reductive dechlorination of CAH's.
A *Dehalococcoides* specific PCR-DGGE was performed to check for the presence of *Dehalococcoides* and to compare the different cultures. In all microcosms, the presence of *Dehalococcoides* was observed. Two clear bands were present in the PCE microcosms and one clear band was present in the VC microcosms and in Zenne sediment. The band in the VC microcosms was the same as in Zenne sediment and was on the same height as *Dehalococcoides* CBDB-1 (control). This band was also present in the PCE microcosms, but here it was not the dominant one. The dominant band in the PCE microcosms was not found in Zenne sediment or in the VC microcosms and did not match with CBDB-1 or *Dehalococcoides ethenogenes*. Most likely, the dominant band represents a *Dehalococcoid*es strain that dechlorinates PCE to cDCE or VC. The second, less dominant, band probably corresponds with a strain that further dechlorinates cDCE and/or VC to ethene. This latter strain is the dominant one in the VC microcosms and in the Zenne sediment, which is in agreement with the pollutants that are infiltrating the Zenne sediment, namely cDCE and VC.
PCR performed on enrichment cultures with primers for the two known VCR reductase genes (*vcrAB* and *bvcA*) gave no PCR products, suggesting that the VC degrading strain uses another VC reductase and is probably a not yet known strain of *Dehalococcoides*.

Adherence of *Acinetobacter baumannii* to Human Bronchial Epithelial Cells

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Acinetobacter baumannii is an important nosocomial pathogen, but the mechanisms contributing to its epidemicity and virulence are unknown. Adherence of microorganisms to host cells is an important virulence factor as it is the initial step of the colonization process. In the present study, adherence of *A. baumannii* to human bronchial epithelial NCI-H_{292} cells was examined by light and scanning electron microscopy. Thirty-seven strains were investigated including 18 from outbreaks, 16 not associated with outbreaks, and three of which an epidemic implication was unknown. Eight and 11 isolates belonged to European clone I and II respectively. Two types of adherence were observed, dispersed adherence of bacteria to the cell, and adherence of clusters of bacteria at localized areas of the cells. Bacteria with dispersed adherence interacted with the epithelial cells through fimbriae, but were also entrapped by protrusions extending from the epithelial cells. Quantitative adherence varied considerably among strains but there was no significant correlation of the outbreak associated strains and the percentage infected cells. There was, however, a correlation between the clonal lineage and the percentage infected cells with clone II being more adherent than clone I ($P < 0.05$). Ten consecutive isolates from one outbreak were investigated to test whether adherence increases during passage among patients, but this appeared not to be the case. This study showed that *A. baumannii* adheres to human bronchial epithelial cells *in vitro* and that *A. baumannii* strains of clone II had a relatively high capacity to adhere to these cells.

High Pressure Mediated Lysogenic Conversion of *Escherichia coli* with Shiga-Toxin Encoding Bacteriophage

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High hydrostatic pressure (HHP) treatment is a nonthermal technique that is finding increased application in food processing. Its use is often advantageous over that of classic thermal processes, because of the better preservation of nutritional and sensorial characteristics of the food product, combined with an efficient inactivation of food borne microorganisms and quality-degrading enzymes. Recently, our group demonstrated the induction of Shiga-toxin (Stx) converting bacteriophage H-19B (Stx1) and 933W (Stx2) in lysogenic *Escherichia coli* MG1655 cultures after HHP treatment. The last two decades, Shiga-toxin producing *E. coli* (STEC) strains have become an important group of food borne pathogens, causing human disease ranging from haemorrhagic colitis to the potentially life-threatening haemolytic uraemic syndrome. In this report we demonstrated a concomitant increase in lysogenic conversion of nonlysogenic *E. coli* when these cells were pressurized together with Stx lysogens. Our results imply that HHP treatment can promote the lateral spread of certain virulence factors.

Induction experiments were done with Stx lysogens of MG1655 and LMM1010 (a pressure resistant mutant of MG1655; Hauben *et al*., 2005, *Appl*. *Environ*. *Microbiol*. 63, 945-50), either in LB broth or whole milk, a complex food matrix that confers an elevated pressure resistance to bacteria. Pressures ranging from 100 to 400 MPa were used, with 50 MPa increments. For MG1655 lysogens in LB broth, obtained results showed a pressure-mediated induction of bacteriophage H-19B and 933W, with clearly visible optimal induction pressures. Moreover, when pressure resistance was elevated through the use of (i) whole milk instead of LB broth, or (ii) a pressure resistant mutant of MG1655 (LMM1010), an upward shift of the optimal inducing pressure was observed, as well as a broadening of the pressure range in which induction occurs (Aertsen *et al*., 2005, *Appl*. *Environ*. *Microbiol*. 71, 1155-62).
To examine lysogenic conversion, *E. coli* strain MG1655 H-19B::Cm was constructed using λNK1324 lysate (Kleckner *et al*., 1991, *Methods Enzymol*. 204, 139-80). To ensure that the inserted transposon sequence did not interfere with normal phage induction pathways, pressure induction of H-19B::Cm was compared with that of H-19B at the previously determined optimal pressure of 100 MPa (Aertsen *et al*., 2005, *Appl*. *Environ*. *Microbiol*. 71, 1155-62). In a following experiment, cultures of MG1655 H-19B::Cm and MG1655 *lacZ*::Tc were mixed and subjected to a pressure treatment at 100 MPa. Afterwards, lysogenic conversion of MG1655 *lacZ*::Tc with prophage H-19B::Cm was examined. Results showed that 3 hours after pressure treatment, there was a ca. 400-fold increase of lysogenic convertants of MG1655 *lacZ*::Tc in the pressurized sample, in comparison to the control sample. This increase could not trivially be caused by enrichment of a few earlier convertants, since total plate counts increased only marginally during the experiment, and was therefore due to independent lysogenic conversion events.

The US3 kinase of an alphaherpesvirus induces cytoskeleton- and virus-filled cell projections that enhance intercellular virus spread

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The *Alphaherpesvirinae* constitute a subfamily of the *Herpesviridae* with closely related human and animal viruses, including human herpes simplex virus and varicella-zoster virus, porcine pseudorabies virus, bovine herpesvirus 1, and equine herpesvirus 1.

All alphaherpesviruses identified thus far encode an orthologue of the viral US3 protein kinase. Using confocal laser scanning microscopy, we have shown that the US3 protein kinase of pseudorabies virus (PRV) induces dramatic rearrangements of a component of the cytoskeleton of the host cell: the actin microfilaments (Van Minnebruggen et al., 2003, Favoreel et al., 2005). Using confocal microscopy and live cell imaging, we found that US3 – both during infection with PRV and during transfection with a US3-encoding expression vector – induces disassembly of the actin stress fibers and the *de novo* synthesis of long, actin-containing cell projections. These cell projections were often found to be branched and the total length of the projections could reach several times the length of an entire, uninfected cell. In infected cells, stress fiber disassembly and projection formation occured simultaneously from 4h post inoculation onwards. No stress fiber disassembly or projection formation could be observed in cells infected with a US3null virus.

Alphaherpesviruses are known to be able to migrate along microtubules during entry and egress of a host cell (Smith & Enquist, 2002). By confocal microscopy, we found that the US3-induced cell projections contain microtubules and that some of the projections contact other cells, which opens the possibility that the virus uses these structures for spread to other cells.

A recombinant PRV strain in which the VP26 capsid protein has been tagged with GFP (Smith et al, 2001) enables visualisation of single virus particles in living cells during analysis with fluorescence live cell imaging using a spinning disk confocal system. Using this setup, we found that virus particles indeed are present in the projections and that they migrate at a fast rate inside the projections towards the tip.

We found additional strong indications that this US3-induced formation of cell projections is associated with enhanced spread of the virus: (i) a US3null PRV strain is impaired in intercellular spread when compared to wild type PRV, (ii) GFP-positive virus particles were present inside the cytoplasm of cells that were contacted by projections originating from cells infected with VP26-GFP PRV, (iii) projection formation could be inhibited by the actin-stabilizing drug jasplakinolide, and addition of this drug resulted in a decrease in intercellular spread of wild type PRV, and (iv) projection formation could be artificially induced using the actin-rearranging Rho-kinase inhibitor Y27632, and addition of this drug resulted in an increase in intercellular spread of US3null PRV.

In conclusion, we report that the US3 protein kinase of an alphaherpesvirus induces the formation of long, cytoskeleton-filled cell projections in which virus migrates towards the tip and that are associated with enhanced intercellular spread of the virus, thereby suggesting a previously undescribed aspect of alphaherpesvirus intercellular spread.

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Phenanthrene directed chemotaxis in soil bacteria

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Biological removal of pollutants in soil environments is highly dependent on the presence of pollutant degrading bacteria. We believe that migration or translocation of bacteria on a micro- and macro- scale towards a pollutant source could be an important feature for biodegradation of organic pollutants in soil. This would especially be the case for hydrophobic poorly water soluble compounds such as Polycyclic Aromatic Hydrocarbons (PAHs) which are non-homogeneously distributed in a contaminated soil. Chemotactic responses based on the detection of and active migration along a gradient towards pollutants like naphthalene, and toluene has already been described for a number of aromatic-degrading bacteria. Less is known about migration towards less soluble aromatics.

We have investigated two PAH contaminated soils for the presence of bacteria able to perform chemotaxis towards PAHs. Phenanthrene was used as a representative chemoattractant and C-source for isolating the pollutant degrading bacteria. A total of 70 phenanthrene degrading soil isolates were obtained. Based on their BOX-PCR pattern, the isolates represented 12 strains belonging to 5 different genera. Each strain was tested for chemotaxis with a swim-agar setup as well as a revised capillary assay. By these tests at least 5 of the 12 strains proved to be chemotactic towards phenanthrene.

MPN-tubes were used for counting total phenanthrene-degrading bacteria in the soils. 16S r-DNA PCR with subsequent DGGE was performed on DNA extracted from all MPN-tubes. An estimate of the original numbers of each strain was made on the basis of the presence or absence of marker sequences of the 12 strains. We were able to conclude that approximately 50% of the culturable phenanthrene-degrading bacteria possess the ability to actively move towards the contaminant source in liquid as well as in semi-solid media and as such, that the capacity of “phenanthrene steered” chemotaxis was well-represented among the phenanthrene degrading community of the two examined soils.

Bovine herpesvirus 4 induces apoptosis of human carcinoma cell lines *in vitro* and *in vivo*

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The idea of using oncolytic viruses for the treatment of cancers was proposed a century ago. During the last two decades, viruses able to replicate specifically in cancer cells and to induce their lysis were identified and were genetically modified to improve their viro-oncolytic properties. More recently, a new approach consisting of inducing selective apoptosis in cancer cells through viral infection has been proposed; this approach has been called viro-onco-apoptosis. In the present study, we report the property of Bovine herpesvirus-4 (BoHV-4) to induce, *in vitro* and *in vivo,* apoptosis of some human carcinomas. This conclusion relies on the following observations: (*i*) *In vitro*, BoHV-4 infection induced apoptosis of A549 and OVCAR carcinoma cell lines in a time and dose dependent manner; (*ii*) apoptosis was seen to be induced by the expression of an immediate-early or an early BoHV-4 gene, but did not require viral replication; (*iii*) cell treatment with caspase inhibitors demonstrated that apoptosis induced by BoHV-4 relied mainly on caspase 10 activation; (*iv*) infection of co-cultures of A549 or OVCAR cells mixed with human 293 cells (in which BoHV-4 does not induce apoptosis) demonstrated that BoHV-4 specifically eradicated A549 or OVCAR cancer cells from the co-cultures; (*v*) finally, *in vivo* experiments performed with nude mice showed that BoHV-4 intra-tumoral injections reduced drastically the growth of pre-established A549 xenografts. Taken together, these results suggest that BoHV-4 may have potential as a viro-onco-apoptotic agent for the treatment of some human carcinomas. Moreover, further identification of BoHV-4 pro-apoptotic gene(s) and the cellular pathways targeted by this or these gene(s) could lead to the design of new cancer therapeutic strategies.

This work, relying mostly on flow cytometry analysis has been accepted for publication in Cancer Research (Cancer Res 65(20), October 15 2005).

Isolation and screening of potentially probiotic lactic acid bacteria from pig intestinal sources for use in fermented liquid feed

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The aim of this study was to select for *Lactobacillus* strains with *in vitro* probiotic properties that can be used as inoculum for fermentation of pig liquid feed. Using this strategy, outgrowth of pathogenic organisms such as *Salmonella* in the pig intestine can be inhibited in two distinct ways: (1) by the effect of elevated lactic acid concentrations in fermented feed and (2) through ingestion of large numbers of probiotic lactic acid bacteria with their feed. Reduction of pathogen excretion via faeces is a prerequisite to prevent cross-contamination of animals in stables and slaughterhouses and is therefore the first step in the production of safer meat products. Potential probiotic lactobacilli were isolated from ileum and caecum samples of pigs raised on different feeds. All isolates were screened for lactic acid production on maltose, taking both acidification rate and maximal achieved lactic acid concentration into account. Repetitive-element PCR using the $(GTG)_5$ primer revealed a large genotypic diversity within the strain collection. From representative strains, a partial 16S rDNA sequence was determined for identification purposes. Furthermore, strains were screened for their ability to inhibit a panel of 20 pig-related *Salmonella* strains. Resistance to low pH and bile salts were determined to asses their ability to survive transit through the animal stomach and intestine. Since the presence of transferable antibiotic resistances is undesirable for a probiotic, resistances against widely used antibiotics in pig husbandry were checked. The best performing strains in this screening will be used in lab-scale fermentations of liquid feed.

Effect of heavy metals on the glutathione status in different ectomycorrhizal *Paxillus involutus* strains

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Four *Paxillus involutus* strains isolated from different - heavy metal polluted and non-polluted - regions of Europe were investigated. We did not find any correlation between the heavy metal [Cr (VI), Cd^{2+}, Hg^{2+}, Pb^{2+}] tolerance and accumulation as well as the origin of the strains. Surprisingly, heavy metal treatment did not decrease or even increased the intracellular glutathione/glutathione disulphide ratios in 11 cases out of 16 (four isolates treated with four different heavy metals). This remarkable feature of *Paxillus involutus* glutathione metabolism could explain the high heavy metal tolerance of this ectomycorrhizal fungus.

Fluorescence activated cell sorting of plasma cells for further cytogenetic analysis

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Background
Multiple Myeloma (MM) is a malignant clonal neoplasia of plasma cells commonly resulting in overproduction of monoclonal immunoglobulins. The plasma cells are phenotypically characterised by a strong expression of CD38 and CD138 but can display an aberrant phenotype compared to normal plasma cells. Besides other markers, asynchronous expression of CD56 is reported in the majority of MM patients.
Cytogenetic abnormalities, mostly evaluated by fluoresence in situ hybridisation (FISH) and karyotyping, are considered to be the most important prognostic markers in MM. However the detection and characterization of genetic aberrations involved in MM can be hampered by the low proliferative index of plasma cells, the limited extent of bone marrow involvement or the limited proportion of cells bearing the abnormality. Preceding purification of malignant plasma cells may offer some important advantages like higher sensitivity and specificity.

Aims
In order to overcome most difficulties associated with the detection and characterization of chromosomal abnormalities in MM patients, we developed a protocol for fluorescence activated cell sorting (FACS) of clonal plasma cells from the bone marrow. The protocol was optimised for subsequent cytogenetic and molecular analyses like FISH and comparative genomic hybridisation (CGH).
Methods
Thirty-five bone marrow samples of MM patients at various stages of treatment and disease were processed. Plasma cells were purified by flow sorting using the FACS Aria (BD). Following red blood cell lysis, immunophenotyping of cells was performed by a five colour staining procedure, using the following mouse anti-human monoclonal antibodies: Igκ-FITC, Igλ-PE, CD138-PerCP, CD56-PeCY7, CD38-APC (BD). Intracellular staining of light chains was performed using the Fix and Perm intracellular staining kit according to the manufacturers' instructions (Kaumberg, Austria). For CGH the protocol described by Franke et al. (Blood 2001) was followed and for interphase FISH studies commercial probes were used (Vysis).
Results
Plasma cells were purified based on the clonal expression of Ig light chains within the CD138+/CD38++ plasma cell gate. The purity of the sorted cell population was determined by reanalysing a small aliquot of the sample by flow cytometry. A purity between 90-95% of the desired population could be obtained. In addition, a May-Grünwald-Giemsa staining of cells sorted directly on microscopy slides showed undoubtedly the typical plasma cell morphology. Subsequent two colour FISH analyses demonstrated clearly the presence of translocations (eg. IgH) and deletions (eg. 13q-) while these abnormalities were often undetectable or at the cut-off level on the corresponding smears. Furthermore, high molecular weight DNA suitable for DOP-PCR could be isolated and successfully used for CGH.
Conclusions
By optimising the FACS protocol we were able to obtain an almost pure clonal population of plasma cells as confirmed by cell morphology and flow cytometry. In these sorted cell populations, chromosomal aberrations could be detected using FISH and CGH. These data clearly illustrate that the quality of sorted cells is suitable for further cytogenetic and molecular analyses in general. This could be particularly important for more sensitive techniques like micro-array analysis in which a pure homogenous sample is highly desirable.

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Development of Selective Growth Media for Denitrifying Bacteria using an Evolutionary Algorithm

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Commonly, microbial communities are investigated through a 16S rRNA gene approach to avoid limitations in culturability. The culture-independent study of denitrification is however problematic because of widespread occurrence in the microbial world. Cultivation is thus necessary for the identification of denitrifying bacteria. It is generally accepted that (i) application of easy-to-use complex isolation media limits the range of feasible denitrifying isolates, and (ii) defined media are known to be more satisfactory for the growth of many denitrifiers. The aim of this study was to determine a set of optimal isolation conditions for denitrifying bacteria.

Eleven growth medium components were varied in their value by a simple evolutionary algorithm for optimisation (seao) to determine different defined growth media, which were tested on their success as isolation medium for denitrifiers using activated sludge as inoculum. This success was defined by the number of denitrifiers isolated per growth medium and their diversity on genus level, attained with fatty acid methyl ester analysis. Newly developed defined growth media with a pH of 7, a nitrogen concentration of 6 mM, ethanol or succinate as carbon source, with addition of 1 ml of vitamin solution but with exclusion of NaCl or riboflavin solution were most successful for isolation of denitrifiers.

Study of *gyrA* and *rpoB* sequence diversity of the '*Bacillus subtilis*-group'

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Aims. The '*Bacillus subtilis*-group' contains the strain-rich species *B. licheniformis*, *B. pumilus*, *B. subtilis* (subsp. *subtilis* and *spizizenii*), as well as the species *B. amyloliquefaciens*, *B. atrophaeus*, *B. axarquiensis*, *B. malacitensis B. mojavensis*, *B. sonorensis*, *B. vallismortis* and *B. velezensis*. Several strains of the '*Bacillus subtilis*-group' are important producers of industrial enzymes. In addition, *B. subtilis* spores are commercially available as probiotic. In contrast, *B. licheniformis*, *B. subtilis* and *B. pumilus* have been associated with a spectrum of clinical records and are often isolated from a variety of food products where they cause food spoilage responsible for food poisoning. *B. licheniformis* has also been associated with abortion in cattle. At this moment, there is no regulation on the presence of members of the '*B. subtilis*-group' in sensitive foods (such regulation exists for *B. cereus*), despite the high numbers that are often found. A correct identification is an absolute prerequisite in the search of possible (dangerous) contaminations. However, the lack of fundamental understanding in the relationships and existence of species and subspecies within this large group of bacteria makes a reliable identification impossible. This study aims to improve the current situation by Multi Locus Sequence Analysis (MLSA) of the '*Bacillus subtilis*-group'.

Methods and Results. The following genes were studied: *gyrA* (DNA gyrase) and *rpoB* (RNA polymerase, beta subunit). *GyrA* and *rpoB* genes of more than 150 isolates were sequenced, aligned (this alignment was translated to amino acids to evaluate its accuracy) and grouped using the neighbour joining method. The dendrograms of both genes were largely in agreement, with clear separation of the different species belonging to the '*Bacillus subtilis*-group'. *GyrA* analysis resulted in more diverse sequences (about 13% of the amino acids were variable, while this was only 3% for *rpoB*), and a clear separation of the subspecies of *B. subtilis* (subsp. *subtilis* and *spizizenii*). In contrast, the 16S rDNA sequences of the type strains of *B. subtilis*, *B. amyloliquefaciens*, *B. atrophaeus*, *B. mojavensis*, *B. vallismortis* and *B. velezensis*, show similarities ranging from 99.2-99.8% (pairwise similarities based on the UPGMA algorithm of 16S rDNA sequences obtained from the GenBank/EMBL/DDBJ database). When the sequences of six different strains designated to *B. subtilis* are compared using the same method, similarities between 99.4-99.9% are obtained.

Conclusions. The above results clearly reflect the limitations of 16S rDNA sequence analysis to discriminate closely related taxa. The use of other housekeeping genes, in this case *gyrA* and *rpoB*, is needed for the separation of some problematic groups of closely related species such as the '*Bacillus subtilis*-group'.

Significance. The analysis of additional housekeeping genes and the comparison with results of other techniques (including DNA-DNA relatedness study) should provide a tool to clearly classify members of the '*Bacillus subtilis*-group'. In addition, the data could lead to fast detection of harmful strains.

Flow cytometric/fluorometric assays for the evaluation of the anti-HIV activity of chemokine receptor inhibitors

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In order to infect a target cell, the HIV envelope glycoprotein gp120 has to interact with both the cellular CD4 receptor and a chemokine receptor, CCR5 or CXCR4, the so-called HIV co-receptors. Several flow cytometric/fluorometric assays are developed in our lab and are very helpful in deciphering the mode of action and interaction site of several classes of HIV entry inhibitors, such as the chemokine receptor antagonists.

Chemokine receptor antagonists can be identified by their ability to inhibit ligand binding to their receptor. Chemokine binding assays are mostly performed with radiolabeled chemokine ligands. Recently, we have developed a flow cytometric technique using an Alexa Fluor 647 conjugate of CXCL12 ($CXCL12^{AF647}$), the specific ligand of CXCR4. CXCR4 inhibitors blocked the binding of this chemokine at concentrations that are comparable with their anti-HIV activity. Also analysis of the chemokine binding in heterogeneous cell populations, such as PBMCs, via multi parameter flow cytometry is feasible. This assay circumvented the poor stability, high backgrounds and the expensive labeling of the radio-active chemokine and the cost/problems of the radioactive waste. In addition, chemokine receptor expression levels and dose-dependent inhibition of compounds can be measured by monitoring the binding of specific fluorescent-labeled anti-CXCR4 and CCR5 mAbs. Mabs recognizing different epitopes on the receptor give already information on the interaction site(s) of the compound in the receptor. Also, chemokine-induced intracellular calcium signaling, with fluorescent probes, will provide further information on the specificity of the compounds and their potency in inhibiting the interaction of the chemokine with the receptor. Thus, flow cytometric/fluorometric assays are very valuable with regard to the intense search for CXCR4 and CCR5 inhibitors to combat HIV transmission and infection.

Cleaning resistant bacteria hitch-hiking to space.

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β-Proteobacteria such as *Cupriavidus, Ralstonia* and *Burkholderia* have been repeatedly isolated from NASA spacecraft assembly rooms (floor and air) (La Duc *et al.*, 2003), spacecraft surfaces (La Duc *et al.*, 2003), spacecraft cooling water (Venkateswaran et al., unpublished)(Pyle et al., unpublished) and spacecraft drinking water Venkateswaran et al., unpublished)(Ott et al., Unpublished). Despite the fact that absolute sterility is not required for robotic missions, such contamination must be prevented and eliminated to protect the forward contamination of the extraterrestrial planets. Moreover, as some of the identified species could be opportunistic pathogens the prevention, early detection and counteraction of this contamination is crucial for a variety of biosafety issues for human space flight including pathogenicity for the crew and the biodetoriation of materials.

Spacecraft intend to land in other celestial body or spacecrafts for human space flight are assembled in clean rooms and undergo strict disinfection protocols to reduce the microbial burden to the maximum. Nevertheless, some *Cupriavidus* and *Ralstonia* strains seem to be able to survive this extremely rough environment. It was shown that some of these isolated strains were capable of growing autotrophically and form biofilms. In addition, they showed a remarkably accumulation of moderate to high resistances to physical and chemical cleaning agents such as UV-irradiation, heavy metals, organic solvents (ethanol, acetone), organic acids, halogens (iodine), oxidizing reagents (hydrogenperoxide), quaternary ammonium compounds, surfactants, antibiotics, etc. Some of these resistances (such as Zinc resistance) seem to be plasmid encoded.

The isolated 'space' *Cupriavidus* and *Ralstionia* strains show a remarkably similarity in resistance patterns to the *Cupriavidus metallidurans* (formerly *Ralstonia metallidurans*) species type strain CH34^T. Strain CH34^T, however, was originally isolated from a heavy metal contaminated sludge at a non-ferro metallurgical plant in Belgium (reviewed in Mergeay *et al.*, 2003). The CH34 isolate was shown to be resistant to high concentrations of a wide number of heavy metal ions including Ag(I), Bi(III), Cd(II), Co(II), Cr(VI), Cu(II), Hg(II),Mn(II), Ni(II), Pb(II), Tl(I), and Zn(II). Most of the genetic determinants controlling these resistances are plasmid-bourne. *C. metallidurans* CH34 is a versatile and robust bacterium that is well equipped to deal with both acute and chronically environmental stresses and is thus a good representative of a genus able to survive in harsh oligothrophic environments including heavily polluted anthropogenic sites, and now it seems highly cleaned spacecraft assembling rooms, space robot surfaces, spacecraft cooling water and spacecraft drinking water.

The further study and characterization of such cleaning resistant β-Proteobacteria is of importance to qualify and improve contamination prevention, monitoring and disinfection tool for the future. Remaining questions to be answered are: What is the source of contamination and how could this contamination be prevented?, What are the genetic derminants of resistances and can they be shared among bacteria by mobile genetic elements such as plasmids?, Could certain server cleaning methods select and enrich these resistant strains? How could contaminated area be successfully disinfected?

Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards *Salmonella enterica* serovar Typhimurium reveals a role for lactic acid and other inhibitory compounds

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Introduction: Probiotics can function as a microbial barrier against gastrointestinal pathogens through the competitive exclusion of pathogen binding, modulation of the host's immune system, and production of inhibitory compounds. The aim of this study was to examine the kinetics of the production of compounds with anti-*Salmonella* activity by probiotic lactobacilli.

Methods: The kinetic analysis of growth and production of antibacterial compounds active towards *Salmonella enterica* serovar Typhimurium SL1344 by six *Lactobacillus* strains (*L. acidophilus* IBB 801, *L. amylovorus* DCE 471, *L. casei* Shirota, *L. johnsonii* La1, *L. plantarum* ACA-DC 287, and *L. rhamnosus* GG) was carried out during laboratory fermentations in 10 l of MRS broth. The antibacterial activity of lactobacilli was measured using a killing assay.

Results: All lactobacilli displayed a strong antibacterial activity against *S.* Typhimurium SL1344. Studying the production kinetics of antibacterial activity and applying the appropriate acid and pH control samples, distinction was made between the effect of lactic acid and other inhibitory compounds that were produced. The antibacterial activity of *L. acidophilus* IBB 801, *L. amylovorus* DCE 471, *L. casei* Shirota, and *L. rhamnosus* GG was solely due to the production of lactic acid. The antibacterial activity of *L. johnsonii* La1 and *L. plantarum* ACA-DC 287 was due to the production of lactic acid and an unknown inhibitory substance. The latter was only active at low pH and in the presence of lactic acid.

Conclusion: The production of lactic acid was the main underlying mechanism of the inhibition of *Salmonella* by lactobacilli. Nevertheless, certain *Lactobacillus* strains produced a hitherto unknown anti-*Salmonella* compound too.

Development of an oligonucleotide microarray for *Sinorhizobium* species identification

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Sinorhizobium, a genus of nitrogen-fixing bacteria living in symbiosis with leguminous plants, consists of 11 different species[13]. As in other bacterial groups, its classification is strongly influenced by 16S rDNA sequence analysis. However, the application of this gene as a taxonomic marker is limited by the low resolution of closely related species and possible genetic crossing-over. Multiple housekeeping gene sequence analysis is becoming a powerful tool to improve the reliability of the phylogenetic conclusions[2].

In order to provide a more reliable tool for the identification and classification of the different *Sinorhizobium* species, we are currently experimenting with a microarray that targets many housekeeping genes at the same time. For this purpose we amplified and sequenced internal segments of 14 housekeeping genes from 36 reference strains representing the different *Sinorhizobium* species. The generated sequences were used for the design of diagnostic oligonucleotides for the identification microarray. The sequences also provide data on the divergence and phylogeny of the genus *Sinorhizobium*.

We developed a microarray that contains 2308 oligonucleotides, representing the different housekeeping genes. Experimental conditions are now being optimised and the oligonucleotides are validated by hybridising the microarray with DNA from the *Sinorhizobium* reference strains.

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Zn tolerance of the nitrifying community in long-term Zn contaminated grassland

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Adding metal salts to soil severely disturbs the nitrification process. However, these toxic effects are not observed in long-term metal contaminated soils (Smolders et al., 2003). This paper attempts to show Zn tolerance of the soil nitrifying community in a long-term Zn contaminated grassland.

Long-term Zn contaminated soil was sampled in grassland at different locations near a galvanized electricity transmission tower. Underlying soil was enriched by Zn due to corrosion with concentrations ranging from 76 mg Zn kg^{-1} in a control soil 30 m away from the tower to 1643 mg Zn kg^{-1} under the tower. An $(NH_4)_2SO_4$ solution was added to all soil samples and the Potential Nitrification Rate (PNR) in soil was measured as the linear increase of the NO_3-N concentration in time. PNR in all field soil samples was unaffected by the elevated Zn concentrations whereas spiking a relevant uncontaminated soil with $ZnCl_2$ reduced PNR significantly at 131 mg Zn kg^{-1}. The toxic effect after spiking could be party explained by an elevated ionic strength in soil solution. But comparison of PNR values at similar Zn concentrations and ionic strength gave evidence for Zn tolerance in the field contaminated soil samples.

To test for tolerance, both uncontaminated control soil or field contaminated soil (1643 mg Zn kg^{-1}) were inoculated in sterilized, $ZnCl_2$ contaminated soil and PNR was measured. The PNR after inoculation with uncontaminated control soil decreased by 50% (EC50 value) at 270 mg added Zn kg^{-1} whereas the EC50 value for the field contaminated soil was at 920 mg added Zn kg^{-1}. In a second tolerance test, both control soil and field contaminated soil were suspended in 0.01 M $CaCl_2$ at pH 5.5 and extra Zn was added to the suspensions in different concentrations (0 to 250 mg Zn l^{-1}). The PNR of the control soil decreased by 70% when 250 mg Zn l^{-1} was added while the PNR of the contaminated soil was unaffected at this dose. Both tests showed that the nitrifying community of the field contaminated soil was less sensitive to Zn than the nitrifying community of the control soil.

Moreover, amplification of 16SrDNA of ammonia oxidizers with CTO primers (Kowalchuk et al., 1997) and DGGE analysis showed a gradual change of the nitrifying community structure with increasing Zn concentrations indicating that the original nitrifying community changed during to the long-term Zn contamination. The 16SrDNA sequences of dominant bands in the DGGE fingerprints of control soil and field contaminated soil are now being determined. Results of the sequencing will be available in October 2005.

We conclude that the nitrifying community in the long-term Zn contaminated soil changed structurally and became more tolerant to elevated Zn concentrations.

Quorum sensing dependent production of antimicrobial component influences establishment of *E.coli* in dual species biofilms with *Serratia plymuthica*

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Mixed species biofilms can act as a reservoir of undesirable microflora compromising the safety of medical as well as food processing treatments. We previously isolated a biofilm-forming strain from a food-processing environment (Van Houdt et al., 2003, J. Appl. Microbiol. 96: 177-84), which was identified as *Serratia plymuthica* RVH1 (Van Houdt et al., 2005, FEMS Microbiol. Lett. 15: 265-72). We showed that the strain contains a quorum sensing system consisting of an N-acyl-L-homoserine lacton synthase SplI, producing signal molecules, and a response regulator SplR. Recently it was shown that these proteins regulate the production of an extracellular protease, chitinase, nuclease and the production of an antimicrobial component (AC) (Van Houdt et al., In preparation).

Knockouts were made in the quorum sensing genes *splI* and *splR* from *S. plymuthica* RVH1 and in an unknown gene involved in AC production. These strains were spotted on LB soft agar inoculated with the AC sensitive *E. coli* MG1655 to investigate toxic component production levels by the size of the inhibition zone. As expected, no inhibition zone was seen for the knockout in AC production. The *splI* knockout strain, deficient in the production of signal molecules, produced considerably lower levels of AC, but production could be restored by the exogenous addition of signal molecules. By contrast, the *splR* knockout produced slightly more AC than its parental strain. These results demonstrate that production of AC is regulated by quorum sensing.

We used a continuous flowcell system (adapted from Christensen *et al*., 1998, Appl. Environ. Microbiol. 64: 2247-55) to establish biofilms. *S. plymuthica* RVH1 strains and *E. coli* MG1655 were inoculated in the flowcell in a 100/1 ratio and biofilms were grown for 3 days. Then cells were rinsed from the flowcell and plated on selective media. The CFU/cm² of *E. coli* MG1655 differed significantly. Whereas dual species biofilms containing wild type *S. plymuthica* contained 2-log CFU/cm² *E. coli* MG1655, biofilms with the knockout in AC production contained 5-log more. Biofilms containing the strain that no longer produced signal molecules and thus produced less AC than the wild type strain showed intermediate values of *E. coli* MG1655 counts. Addition of excess signal molecules to these biofilms, however, resulted in almost total absence of *E. coli* MG1655, which is a similar result as in biofilms with the *splR* knockout strain, where no *E. coli* MG1655 could be recovered. In a second approach the different *S. plymuthica* strains were labeled with green and *E. coli* MG1655 with red fluorescent protein. Flowcells were inoculated with *E. coli* MG1655 and either wild type *S. plymuthica* RVH1 or its isogenic knockout in AC production. After 3 days of continuous growth the established biofilms were investigated using a confocal scanning laser microscope. In accordance with our results above, almost no *E. coli* could be seen in biofilms containing wild type *S. plymuthica* RVH1, but *E. coli* was abundant when no AC was produced (Moons et al., In preparation). Taken together, these results show that quorum sensing fine tuning of AC production plays an important role in the possible incorporation of *E. coli* MG1655 in dual species biofilms containing *S. plymuthica* RVH1.

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Organisation of heavy metal resistance genes in the four replicons of *Cupriavidus metallidurans*

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Cupriavidus metallidurans (formerly *Ralstonia*) belongs to the phylum β-proteobacteria and includes various isolates of soil bacteria adapted to harsh industrial biotopes. The genome of strain CH34 is sequenced and contains four replicons: two large plasmids pMOL28 (171 Kb) and pMOL30 (234kb), and two megareplicons, a chromosome (3.9Mb) that is especially rich in biosynthetic genes, and a megaplasmid (2.6 Mb). Analysis of *C. metallidurans* on the genomic level (against existing databases and through phylogenetic approaches), the transcriptomic level (using quantitative PCR and microarrays analysis), and the proteomic level (using 2-D gel electrophoresis and mass spectrometry) indicate a high number of heavy metal resistance or -detoxification genes in comparison to other sequenced bacteria. These genes seem to be mainly associated with the two plasmids and the smaller megareplicon of *C. metallidurans*. Using microarrays we analyse the expression of *C. metallidurans* plasmid-bourne genes after induction with several heavy metal. We found 80 ORFs on a total of 161 located on pMOL28 and 134 ORFs on a total of 242 located on pMOL30 over-expressed in at least one metal condition. Plasmid pMOL28 contains three clusters conferring resistance to nickel and cobalt (*cnr*), to chromate (*chr*) and to mercury (*mer* in Tn*4378*). The whole group constitutes a 35 kb block flanked by IS*1071* on the *mer* side and a deleted form of IS*1071* on the *cnr* side (1). On plasmid pMOL30, heavy metal resistance are clustered opposite to the replication origin of the plasmid. Among other minor determinants, this region contains a cluster of nine genes conferring resistance to Cd^{++}, Zn^{++} and Co^{++} (*czc*), a mercury transposon (*mer* in Tn*4380*), a cluster of six genes conferring resistance to Pb^{++} (*pbr*), a cluster of three genes that respond to Cu^{++} and Ag^{+} (*sil*), and a large cluster of 19 genes conferring resistance to Cu^{++} (*cop*) (1). Expression analysis reveals specific metal response for *czc* cluster induced only by Zn^{++} and Cd^{++}, but mainly cross-response for the other cluster of genes. The pMOL28 *cnr* cluster of genes responds to Ni^{++} but also to Cu^{++} and Cd^{++}, the pMOL30 *cop* cluster of genes responds to Cu^{++} but also to Zn^{++}, Cd^{++} and Ni^{++}, the *mer* cluster responds to Hg^{++} but also to Zn^{++}, Cd^{++} and Pb^{++}, the *pbr* cluster responds to Pb^{++} but also to Zn^{++}. Moreover, such microarray analysis allows use to identify small or unknown ORFs (with not equivalent in data bases) highly express in metal condition that could be involved in a general metal response of *C. metallidurans* against heavy metal. The other genes found to be over-expressed in metal condition are probably involved in membrane repair, and mobility. But, it also appears that in extreme environmental condition with high level of heavy metals, the major resistance response observed is mainly plasmid-bourne.

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Flow cytometric analysis of heat shock protein 32 in human peripheral blood mononuclear cells

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Heat shock proteins (Hsp) are highly conserved throughout evolution and evoke great interest both in basic biology and in medicine. Under normal physiological conditions Hsp are expressed at low levels, but when cells endure stress their synthesis can be greatly accelerated. Several studies have shown the expression of Hsp, especially Hsp32, under a variety of conditions associated with oxidative stress, and evidence is mounting that these proteins might have potential significance in down-regulating reactive oxygen species and offering protection against oxidative damage, most notably, under pathophysiological conditions such as inflammation and shock/hypoperfusion. Because elderly are more susceptible to oxidative stress and inflammation, and because Hsp32 mediated protection could be of special interest in aged individuals during exposure to supplementary acute stress, we used flow cytometry to investigate the production pattern of Hsp32 with aging and during acute infection in peripheral blood mononuclear cells (PBMC) which are central to the inflammatory response. Our results show that Hsp32 is up-regulated in PBMC in the elderly as well as in individuals with inflammation. Several relationships were found between the inflammatory parameters and the expression pattern of Hsp32. We concluded that differences in relationship with the cytokines according to the health status could indicate different triggering mechanisms for the production of Hsp32. In elderly control subjects, increased oxidative load could be responsible for the up-regulation of Hsp32 production. On the other hand, acute inflammation in patients, resulting in a pronounced presence of cytokines, could up-regulate the production of Hsp32.

Atomic Force Microscopy analysis of nucleoprotein complexes formed by Ss-LrpB, a transcription regulator from *Sulfolobus solfataricus*

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The archaeal basal transcription apparatus is a simplified version of its eukaryotic counterpart. It seems to be regulated mostly by bacteria-like transcription regulators, an observation which reveals a hybrid situation (1). Most of the to date best characterized archaeal transcription regulators belong to the Leucine-responsive Regulatory Protein (Lrp)-family, a family with members in both Archaea and Bacteria (2). Ss-LrpB from *S. solfataricus* belongs to this family (3).

Atomic Force Microscopy (AFM) is becoming a widespread and very valuable technique in the study of protein-DNA interactions. The experimental procedures are straightforward and allow direct visualization of nucleoprotein complexes with high resolution (nm resolution). Here, we present an AFM study of DNA binding by Ss-LrpB. This binding occurs at the own promoter/operator region, an interaction which is suggestive of autoregulation. Ss-LrpB binds cooperatively three regularly spaced, palindromic, 15 bp-long binding sites (3).

AFM allowed us to visualize the Ss-LrpB-DNA complexes, using a DNA fragment with the operator approximately in the centre. Complexes were formed at high Ss-LrpB concentration, in order to favorise full occupation of the operator binding sites. The vast majority of the nucleoprotein complexes shows two DNA arms extending from the complexed region, which has a globular shape. The contour length of 350 unbound DNA molecules and 350 complexes was measured and compared: this revealed a condensation of almost 140 bp, which indicates the existence of very strong DNA deformations and even DNA wrapping. Bending angles were analysed using a method based on end-to-end-distances of the complexes. Altogether, these images strongly suggest wrapping of the operator around the regulator with an average bending angle of 260°. AFM images were also made using a Box2-mutant operator fragment, in which Box2 binding was weakened, allowing the observation of complexes bound at one or two Boxes.

Ss-LrpB exists primarily as a dimer in solution, as shown by crosslinking studies. Therefore, we hypothesize that DNA wrapping occurs upon binding of three Ss-LrpB-dimers to three targets aligned on the same face of the DNA helix. These deformations are supposed to play a major role in the establishment of protein-protein contacts that account for the apparent cooperativity in the binding to two high affinity (at the borders) and one low affinity binding site (in the center).

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Comparison of different methodologies to measure ZAP-70 in B-CLL and correlations with mutational status

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Introduction: Although generally regarded as an indolent disease, CLL has a considerable variability in clinical course. The prognostic significance of IgVH gene rearrangements is well established, those patients with unmutated IgVH genes having a worse prognosis than those with somatic mutations. Zap-70, a tyrosine kinase involved in T-cell receptor signalling, was found by gene expression profiling, to be expressed in the unmutated subgroup. The analysis by flow cytometry (FC) of the latter marker is much easier than the elaborate and costly sequencing technique. In this study we evaluated a RT-PCR analysis of Zap-70 on selected B-cells and compared the results with Zap-70 expression measured with flow cytometry and with the mutational analysis (MA).

Materials and Methods: 24 B-CLL patients were included. In samples used for RT-PCR, B-cells were sorted with MACS technology (Miltenyi Biotec) or with EasySep technology (Stem Cell Technologies). Primers and probe were used as described by Wiestner et al. (Blood 2003) and analyses were performed on a ABI PRISM 7000 Sequence Detector. PBGD was used as a housekeeping gene. A ROC-analysis was performed versus IgVH analysis to find an "optimal" cut-off value. For FC, 1.5 µg of anti-Zap-70 antibody (clone 2F3-2, Upstate Biotechnology) was used and further stained with GAM-PE. Then cells were washed and incubated with normal mouse serum for 5 minutes and finally stained with CD19-FITC, CD3PerCP and CD56-APC/CD5-APC. FC was considered positive if >20% of B-cells scored positive when compared with the T-cells present in the sample (cut-off value defined by 95% of T-cells being positive).

Results: 22 results were concordant between RT-PCR and MA (6 positive and 16 negative results). One result was false positive and one was false negative. 19 results were concordant between FC and MA. One sample was false negative and 4 were false positive. Between RT-PCR and FC 15 samples were concordant, one was positive for RT-PCR and negative for FC, and in 8 samples it was vice versa.

Discussion: These data show that ZAP-70 analysis with RT-PCR on selected B-cells is a good alternative to FC. These results need to be further confirmed on a broader group of patients.

Multi-colour labelling of quarter milk neutrophils from heifers in early lactation for the flow cytometric assessment of functional quality changes.

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Mastitis or intramammary infection still is the most costly disease in dairy cattle. Research regarding the development of mastitis prevention and control programs has traditionally focused on lactating or dry cows, while heifers in early lactation have hardly been studied. Nevertheless, there are indications that also in this population which represents the future of the herd, udder quarters are already infected prior to calving. It is known that minor pathogens, mainly coagulase-negative staphylococci, rather than major pathogens are responsible for these intramammary infections. The latter are reflected in the marked increases in milk somatic cell count in early lactating primiparous heifers. It is generally accepted that the first line of innate immune defence against invading pathogens is mediated by neutrophils. In addition to the neutrophil counts, their quality is also important in the udder defence. We postulate that neutrophil quality may be a risk factor at quarter level, associated with heifer mastitis in early lactation.

Therefore, our aim is to investigate whether the bacteriological status of foremilk samples and the milk neutrophil function are related in quarters of early lactating heifers. The prerequisite for an adequate bactericidal function of neutrophils is their residual viability following migration towards the infection site. To measure this critical parameter, two complicating factors had to be overcome: the limited available volume of quarter foremilk and the fact that differentiation between activated milk neutrophils and other milk leukocytes is not possible based on the simple FSC/SSC characteristics used for blood neutrophils. We developed a three-color flow cytometric protocol combining the traditional Annexine-V-FITC/propidium iodide staining with the primary monoclonal antibody CH138A, a specific marker for bovine granulocytes, coupled to secondary antibody labelled with a sensitive red fluorescent dye. The proposed method allows the discrimination between viable, apoptotic and necrotic subpopulations of milk neutrophils at the quarter level and holds promise for the routine application in a clinical veterinary context.

Evaluation of a compact flow cytometer as a microbiological quality-, process- and hygiene control device in the food industry

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The availability of compact flow cytometers with diode lasers of low price, high efficiency, and long lifetime is expected to promote broader applications of flow cytometry (FCM) in food microbiology.

While FCM has been frequently used to analyze bacteria in pure cultures, it has seen more limited use with heterogeneous natural samples such as those encountered in food microbiology. Bacteria are small compared to mammalian cells, and in FCM analysis the signal-to-noise ratio is of greater concern for bacterial cell samples than for mammalian cell samples, especially in food and environmental samples. Due to this the application of FCM is complicated by the difficulties of distinguishing the organisms of interest from noise and debris, releasing bacteria from flocs or aggregates, and by the low signal often obtained from bacteria in food samples. It is possible to circumvented these problems by a good sample treatment and using a multi-parameter approach, e.g. to gate the cytometer to ignore the light scattering and fluorescence properties of background events and to only display those properties exclusive to microbial cells.

The purpose of this study is to evaluate a **compact flow cytometer**, and at the same time to investigate its application possibilities as a microbiological quality-, process- and hygiene control device in the food industry.
Within the research project four domains can be distinguished:

1. Evaluation of the performances of the compact flow cytometer and the matching fluorochromes in function of microbial cell activity and physiology.
2. Creating protocols for sample treatment of different food products for flow cytometry analysis.
3. Tracing cellular damage caused by processing factors through flow cytometry.
4. Developing reliable protocols in function of the available flow cytometer for applications in different food sectors (e.g. dairy, brewery, and vegetables).

FCM analyses were performed with a "BACTIFLOW GL100" (Partec-Chemunex), equipped with an 100 mW YAG green laserdiode, operating at 532 nm, and with 4 parameters (2 scatters FSC & SCC and 2 fluorescence, orange FL1 & red FL2).
Data acquisition, analysis, and real time display were performed with the Partec Flomax software.
The **viability assay PI-Syto81** enables distinction of live and dead bacteria and yeast. Cells with intact membranes allows only Syto81 to enter the cell and fluoresce orange, whereas those with damaged membranes allows the PI dye to enter and fluoresce red. Viability studies after microbial treatment with desinfectans, heat, antibiotics and other antimicrobial compounds were done on the base of the individual staining pattern.
A flow cytometry-based **somatic cell** assay was developed with a sensitivity of 10^4 cow cells ml^{-1}, which is much below the standard level for good quality raw milk.
A total yeast count assay for **fermented milk products** was developed, based on a clearing step and fluorescent staining with PI-Syto81 and flow cytometry analysis. The detection limit of the assay was 5 x 10^4 cells ml^{-1}.

FCM offers a rapid determination of **microbial contaminants of beer**. The minimal detection limit of yeast in beer is log 2 - log 4 cfu ml^{-1} and for lactic acid bacteria in beer is log 3 - log 4 cfu ml^{-1} depending on the beer and sample treatment i.e. centrifuging and washing.
The FCM PI-Syto81 assay was applied to **dairy fermentation starters** (freeze dried and concentrated frozen) and **probiotic products** (freeze dried and commercial probiotic drinks). The FCM assay is very accurate and highly sensitive and provides tools to assess the functionality of different populations in fermentation starters and probiotic products.

Efficient transfection of primary acute myeloid leukemic cells by messenger RNA electroporation

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Leukemic cells are considered as suitable targets for gene therapy. Gene-modified tumor vaccines (GMTV) engineered to express immunomodulating molecules would be an interesting adjuvant strategy for the elimination of minimal residual disease in acute myeloid leukemia (AML). In order to circumvent the safety issues related to viral vectors, we applied a novel gene transfer method for transfection of primary human AML blasts, based on mRNA electroporation (Van Tendeloo et al, Blood 2001). Messenger RNA encoding the enhanced green fluorescent protein (EGFP) was used to evaluate the gene transfer efficiency.

Electroporation efficiency was significantly improved when fresh AML blasts from the bone marrow of newly diagnosed patients were cultured for three to five days in culture medium. The average gene transfer rate in cultured primary AML blasts 24 hours after electroporation was 53% (range 24-66%) and EGFP was still detectable four days after electroporation. Electroporation with mRNA encoding CD80 or CD137 ligand (CD137L) led to a marked expression of these costimulatory molecules at the cell surface of AML blasts. Currently we are investigating the antigen-presenting capacity of these CD137L and/or CD80 mRNA-modified AML blasts.

In conclusion, mRNA electroporation is a promising and safe strategy for gene transfer in primary AML blasts, with clinical applicabilities for cellular gene therapy of AML.

Study of Bovine Herpesvirus 4 LANA protein

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The 73 open reading frame product (pORF73) encoded by gammaherpesviruses plays key roles in the establishment and the persistence of latency. Bovine herpesvirus 4 (BoHV-4) is a gammaherpesvirus. Its pORF73 is by far the smallest protein among gammaherpesvirus orthologues. The present study was devoted to the characterization of BoHV-4 pORF73. First, bioinformatic tools were used to identify several functional domains in BoHV-4 pORF73 sequence, including those previously identified in the human herpesvirus 8 (HHV-8) pORF73 orthologue. Second, the functionality of some of these domains was investigated. Here, we show that the BoHV-4 pORF73 has at least two functional nuclear importation signals that allow its entry into the nucleus. We also show that this nuclear protein is able to interact with mitotic chromosomes. The present study suggests that, despite its relatively small size, BoHV-4 pORF73 could be a functional homologue of the larger orthologues encoded by other gammaherpesviruses. Further studies will determine the functions of this unique protein.

A new model for the quantification of *Staphylococcus aureus* biofilms

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Biofilms represent possibly the oldest form of life on our planet, but even now there is no such thing as "the" definition of a biofilm. They are often defined as a structured bacterial population, embedded in a self produced polymeric matrix and irreversibly attached to a surface. (3)

Adherent bacterial communities can form on virtual every surface. They are found as well in natural environments, in industrial settings as in a variety of biomedical situations: in catheters, in wounds, on teeth and so on. (2) The extracellular polymeric substance (EPS) forms the main component of the biofilm matrix, also called slime layer. It is composed of a collection of polymers, such as polysaccharides and glycoproteins, produced by the microorganisms themselves. The most important function of this slime layer is the protection of the germs in a biofilm from negative external factors, including host antibodies and biocides. This unusual high resistance is the major advantage conferred to microorganisms living in a biofilm. All kinds of biocides, often used against planktonic bacteria, are not or far less effective against their sessile counterparts. (4)

In order to gain more understanding in biofilms and their way of life and with a view to develop new anti-biofilm strategies, appropriate *in vitro* models are indispensable. The aim of the current study is to develop a simple and reproducible test system for the quantification of adherent *Staphylococcus aureus* (Sa) populations. This germ is known as an important opportunistic pathogen for humans. The model in question should prove the presence or absence of a biofilm. In anti-biofilm strategies, the desired endpoint is to remove the biofilm. If only the germs are killed, the biofilm will soon be re-established. (5) Moreover, the test method should be efficient and require as little sample as possible, so the use of 96-well plates is favourable.

Here, a simple model for the detection and quantification of Sa biofilms, using the dye dimethylmethylene blue (DMMB) is presented. This assay is based on the ability of sulphated glycosaminoglycans (GAG's) to bind to the cationic DMMB. Afterwards, the excess of the dye is discarded and the DMMB-GAG complex is dissociated. The optical density, which is proportional to the amount of GAG's present, is subsequently measured. Initially, the used method was developed for quantification of GAG's in biological solutions. (1) However, in biofilms sulphated polysaccharides are bound to the surface of a recipient. Our experiments showed that this method could be used to quantify Sa biofilms, grown in a 24-well plate. The use of reference products, such as chondroitin sulphate and N-acetyl-D-galactosamine, showed that this test method is specific for the quantification of sulphated GAG's. Moreover, several Sa strains were tested and biofilm producing strains were distinguished from non-biofilm producing strains. Furthermore, the optimal growth time of biofilms was set to 72h when the growth medium was changed once every day. The DMMB solution was stable for at least 3 months at room temperature. Finally, the test conditions were adapted to use 96-well plates in stead of 24-well plates.

In conclusion, a new model for the quantification of Sa biofilms has been optimized and can be useful to examine the anti-biofilm activity of several biocides.

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Enrichment and characterization of a bacterial culture utilizing pyrene at pH 2 and dominated by a slow-growing *Mycobacterium* sp. from acidic polycyclic aromatic hydrocarbon contaminated soil

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Three polycyclic aromatic hydrocarbon (PAH) contaminated soils with different pH were sampled from different locations at a former manufactured gas plant site and analyzed regarding microbial ecology and the presence of PAH-utilizing bacteria. Soil TM had a pH of 8.0, while soils B7-1 and B7-2 had an extremely low pH of 2.6 and 2.4, respectively. Eubacterial 16S rRNA gene DGGE fingerprinting showed that the soils were inhabited by different communities. From soils B7-1 and B7-2, enrichment cultures were obtained utilizing phenanthrene or pyrene as sole source of carbon and energy at pHs 3, 5 and 7 while from soil TM, PAH-utilizing cultures were only obtained at pHs 5 and 7. Pyrene-utilizing enrichment cultures from soil TM at pH 7, from soil B7-1 at pH 7 and from soil B7-2 at pH 3 and pH 5 were further characterized. All cultures showed rapid ^{14}C-pyrene mineralization at pH 3 and pH 5. On the other hand, the TM culture obtained at pH 7 did only grow on pyrene at pH 5 to pH 7. The B7-1 culture obtained at pH 7 showed growth on pyrene in a pH range from pH 4 to pH 6, while the two B7-2 cultures obtained at pH 3 and pH 5 did grow on pyrene at a pH range from 2 to 6. Eubacterial 16S rRNA gene DGGE fingerprinting and sequencing showed that the 3 pyrene-utilizing cultures from the acidic soils were dominated by one and the same bacterium that was most related to *Mycobacterium montefiorense*, a slow-growing pathogenic *Mycobacterium* sp. isolated from moray eels. In contrast, the culture obtained from soil TM at pH 7 was dominated by strains related to *Pseudomonas putida* and *Mycobacterium aurum*. The occurrence of different *Mycobacterium* spp. in the TM and B7 cultures was confirmed by *Mycobacterium*-specific 16S rRNA gene PCR-DGGE and sequencing of DGGE bands. Moreover, the band in the *Mycobacterium* fingerprints of the pyrene-utilizing cultures obtained from the acidic soils matched the only band in the *Mycobacterium* communities present in the corresponding soils, indicating that the isolated slow-growing *Mycobacterium* sp. also out-competed other mycobacteria in the soil and that it plays an important role in PAH-biodegradation in the examined acidic PAH-contaminated soils.

Physiologicals and transcriptomics studies of the copper resistance in *Cupriavidus metallidurans*

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Cupriavidus metallidurans CH34 is a metal resistant bacterium isolated from the sludge of a zinc decantation tank in Belgium that was polluted with high concentrations of several heavy metals. This bacterium carries two large plasmids pMOL28 (171kb) and pMOL30 (234kb) containing a variety of heavy metal resistance genes[1]. In this study we will focus on the copper resistance carried by plasmid pMOL30 and encoded on a 19 genes cluster (*copVTMKNSRABCDIJGFLQHE)*.

The pMOL30 copper resistance cluster of genes was cloned on a pLAFR3 vector and sequenced. The copper resistance mechanism is not perfectly known, but we can distinghish four group of genes: i) *copSRABCDI* encoding for proteins involved in the periplasm detoxication (as already observed in *E.coli* or *P.syringae*), ii) *copJGFL* encoding for proteins involved in the cytoplasm detoxication via CopF (a P-ATPase), iii) *copK* and *copT* encoding for proteins involved in copper binding that may play the role of a buffer during the detoxification process and iiii) the other genes encoding for small proteins with no or few equivalent have been found in the databases[2]. The copper form in the cytoplasm would be exclusively the monovalent one. Copper II would be reduced in copper I inside the periplasm or when it enters on it (S. Silver, personal communication).

In order to investigate the expression of these 19 genes, transcriptomic analysis using Quantitative PCR and microarrays was performed. *C. metallidurans* was grown on minimal medium supplemented with different concentration of copper for a 30 minutes induction before the RNA extraction. Transcriptomic data show that all the 19 genes are induce by copper and may therefore participate to the transport and resistance mechanism. Flanking genes were not induced by copper allowing a delimitation of the copper resistance cluster of genes. Other plasmid bourne genes were inducted by copper like: *silABC* (involved in silver resistance and located next to the *cop* cluster), additional ORFs located on pMOL30 and the *cnr* operon located on pMOL28 plasmid.

Viable counts using the strain AE1744 that carries only the plasmidic *cop* cluster of genes were made to determine the MIC (Minimal Inhibitory concentration). For copper II, we observe a biphasic pattern suggesting the *cop* cluster of genes alone does not restore the full copper resistance observe for the wild type strain (CH34). More precisely, it phenotype is intermediary between strains carrying the pMOL30 plasmid and those without it suggesting that some additional genes located on pMOL30 are necessary for the full copper resistance phenotype (microarray data give us clue for the possible other ORFs involved in this copper resistance). In the case of copper I, there were no clear differences in resistance between those strain. We observed a MIC value 40 fold more sensitivity to copper I (30 μM) comparing to copper II (1.2 mM). The MIC value can also be increased by adding copper to the initial culture to induce the copper resistance mechanism.

Further transcriptomics experiments will be performed on the strain AE1744, to compare its behavior with the wild type strain. Induction with copper I will also be done to determine the expression of each gene belonging to the cop cluster. The physiological effect of copper resistance will also be studied by flow cytometry. We will than determine the complementarily of both method in the framework of bacterial's heavy metals resistances.

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[2] S. MONCHY, M. A. BENOTMANE, R. WATTIEZ, S. VAN AELST, V. AUQUIER, B. BORREMANS, M. MERGEAY, S. TAGHAVI, D VAN DER LELIE and T. VALLAEYS. (2005) *Microbiology*, (Submitted)

Screening of *in vitro* probiotic activities of lactobacilli isolated from laying hens and *in vivo* evaluation of colonization and inhibition of *Salmonella* Enteritidis

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In Belgium, the majority of salmonellosis cases is caused by *Salmonella* Enteritidis, with contaminated eggs as the major infection source. To control the contamination of industrial poultry with *S.* Enteritidis, specific actions are necessary. The presence of lactobacilli in the vagina and cloaca of laying hens seems important to maintain a microbial ecosystem that prevents the growth and invasion of pathogenic bacteria such as *Salmonella* spp. The use of lactobacilli as probiotics for laying hens is an interesting option to reduce *S.* Enteritidis infection.

From a larger collection of lactobacilli, isolated from the vagina and cloaca of laying hens, a rational selection of about 50 strains was tested for *in vitro* probiotic properties. These included the inhibitory activity towards the growth of *Salmonella*, and the tolerance to low pH and bile salts, both important properties for survival within the gastrointestinal tract. Furthermore, the susceptibility of the strains to the antimicrobial agents enrofloxacin and oxytetracycline, both generally used for breeding of laying hens, was evaluated. Finally, a selection of 15 strains was tested for hydrophobicity, a property which is possibly related to epithelial cell adhesion capacity. From these *in vitro* results, a selection of three potentially probiotic strains (2 *L. reuteri* (R-17485 and R-17753) and 1 *L. johnsonii* (R-17504)) and one strain with low probiotic potency (*L. vaginalis* (R-17362)) was tested in a *S.* Enteritidis challenge experiment. One day post hatch, different groups of 20 chickens were orally inoculated with 2×10^8 lactobacilli, while the control group received medium alone. The next day, all lactobacilli-treated groups showed a clear colonization of the gastro-intestinal tract, as verified by plating of cloacal swabs and identification of individual colonies by Rep-PCR. Also at day two post hatch, all groups were challenged with 10^4 cfu *S.* Enteritidis. At day 6 post hatch, samples of caeca, liver and spleen were taken and analysed for the number of *Salmonella* and/or lactobacilli. Three lactobacilli-treated groups (R-17485, R-17753 and R-17504) had 2 to 4 times more lactobacilli in caeca than the control group. Furthermore, two of the tested groups (one treated with R-17485 (*L. reuteri*) and one with R-17504 (*L. johnsonii*)) showed a statistically significant decrease in colonization by *S.* Enteritidis in caeca, liver and spleen.

Mobilisation of endothelial progenitor cells after acute exercise in young healthy volunteers

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Objectives: To investigate the effect of acute exercise on the number of circulating endothelial progenitor cells (EPC's) in healthy volunteers. Additionally the relation between EPC's, peripheral endothelial function, lipid levels and physical capacity was studied.

Background: Circulating EPC's are characterised by the co-expression of hematopoietic and endothelial markers. In response to tissue injury, their number increases and they contribute to neovascularization and vascular repair. A single episode of exercise mobilizes EPC's in patients at cardiovascular (CV) risk and in patients with coronary artery disease, after exceeding the ischemic threshold. In this study we hypothesized that a short exercise mobilizes EPC's in healthy individuals.

Methods: Eleven healthy medical students without CV risk factors were enrolled (22-26yrs, 6 males). Venous blood was sampled before and immediately after symptom-limited bicycle exercise test with gas-analysis. Flow mediated dilation (FMD) of the brachial artery was assessed at baseline and after exertion using ultrasound. Circulating EPC's (CD34+/ VEGFR2+), were analyzed by FACS after immunomagnetic CD34+ separation. Pre-enrichment and rigorous gating was necessary to obtain robust results.

Results: Circulating EPC's increased acutely in healthy subjects following exercise (median 16.5/ml [4-77] vs. 30.5/ml [7-102], $p = 0.025$). There was a trend for FMD to increase (median 6.7% [5.7-12.0] vs. [7-102], $p = 0.09$). There was no relation between relative or absolute changes in EPC counts and FMD or physical activity. Even though lipid concentrations were within normal levels (median total cholesterol (TC) 173 mg/dl [108-280], ratio TC/HDL cholesterol 3,0 [4-1.6]), the relative EPC increase was strongly related to TC ($r=0.65$, $p=0.043$) and TC/HDL ratio ($r=0.891$, $p=0.001$).

Conclusions: In young, healthy subjects, the number of EPC's increases after a single episode of maximal exertion. The relative increase in EPC's was related to baseline lipid parameters. There was no relation between EPC counts and peripheral endothelial function/physical capacity.

Expression and pathogenic role of MMPs in Experimental Cerebral Malaria (CM)

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Cerebral malaria (CM) is a life-threatening disorder and a major medical problem in developing countries (3000 deaths/day). It is caused by the sequestration of malaria-infected erythrocytes onto endothelia of the brain. Subsequent blood-brain-barrier (BBB) damage and neurological deficit is caused by a combination of metabolic and immune reactions, of which the exact molecular mechanisms are not well understood. Expression levels of 9 matrix metalloproteinases (MMPs), 5 membrane-type (MT)-MMPs, TNF-α converting enzyme (TACE), the 4 tissue inhibitors of metalloproteinases (TIMPs) and α_2-macroglobulin were studied in different organs of mice infected with *Plasmodium berghei ANKA*. For several of these molecules, significant alterations in expression were observed. In addition, activated forms of gelatinase B (MMP-9) were observed on gelatin zymograms of CM brain extracts. Net gelatinolytic activity was detected *in situ* in the endothelia of small capillaries and larger blood vessels in CM brains. However, selective genetic knock-out of gelatinase B did not alter the clinical evolution of experimental CM, but inhibition of MMPs and/or TACE with a broad-spectrum MMP-inhibitor (BB-94) caused a moderate but significant delay of the cerebral symptoms. These results demonstrate the involvement of metalloproteinases as pathogenic molecules in CM.

Oligofructose-consuming species of *Bifidobacterium*, *Lactobacillus* and *Bacteroides* differ in their degradation kinetics

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Introduction: The human colon harbours a complex microbial ecosystem that is indispensable for the well being of its host. Nowadays, probiotics and prebiotics are used to enrich or stimulate health-promoting bacteria in the colon. Among all prebiotics, fructo-oligosaccharides (FOS) are the most frequently studied and used. Several human trials confirmed that FOS specifically stimulate bifidobacterial growth, the so-called bifidogenic effect. It is assumed that other important members of the human colonic microbiota may also metabolise FOS but clear evidence has not been given up to now.

Methods: Several colon bacteria (strains of *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Anaerostipes*, *Roseburia*, *Clostridium*, and *Enterococcus*) were tested for their growth on oligofructose (Raftilose®P95, consisting of glucose, fructose, sucrose, and oligofructose with an average DP of 4). A detailed kinetic analysis was performed for five oligofructose-degrading strains (two *Bacteroides* and *Bifidobacterium* strains and one *Lactobacillus* strain) by means of *in vitro* batch fermentations. Gas chromatography analysis was performed to monitor the oligofructose degradation in more detail.

Results: Besides strains of bifidobacteria, also certain strains of *Lactobacillus* and *Bacteroides* were able to breakdown and metabolise oligofructose. However, detailed kinetic analysis of oligofructose breakdown patterns revealed that different mechanisms were involved. Whereas *Lactobacillus paracasei* subsp. *paracasei* strain 8700:2 and both *Bacteroides* strains tested did not show a preferential metabolism of certain oligofructose fractions, all *Bifidobacterium* strains gradually degraded oligofructose. For the latter strains, it was observed that after glucose and fructose depletion (present as 'contaminant' sugars in the commercial oligofructose used), the shorter oligofructose fractions (such as inulobiose and inulotriose) were preferentially metabolised during the early stage of fermentation. Later on, the longer fractions were also metabolised. The *Bifidobacterium animalis* subsp. *lactis* strain DN-173 010 was not capable of metabolising the monosaccharides and started immediately to metabolise the shorter fractions.

Conclusions: This study proved that not only bifidobacteria but also other important members of the human colon can metabolise FOS. Furthermore, it was shown that different mechanisms are possibly involved in oligofructose degradation, which might explain the bifidogenic nature of FOS.

Conventional versus organic dairy farms: influence of operational management on the harmfull aerobic spore-forming bacterial flora in raw milk

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Aims

In this project, the aerobic spore-forming bacterial flora in raw milk of two types of dairy farming, conventional and organic, are examined. Comparison of these two managements may reveal operational aspects that affect the introduction of aerobic spore-forming bacteria in raw milk.

Methods and Results

Raw milk samples were collected at 4 organic and 4 conventional dairy farms during a summer and a winter sampling campaign. The samples were heated at 80°C for 10 minutes, 500 µl plated on BHI before and after chemical extraction of the milk components and incubated for 72 h at 20, 37, and 55°C. Different isolates were selected based on colony morphology. Potential harmful enzymatic activities (lipolysis, proteolysis) of the isolates were demonstrated on selective media.
Isolates were preliminary identified through analysis of their cellular fatty acid composition (FAME).
A striking difference in the frequency of occurrence of thermotrophic isolates was observed between organic (24%) and conventional dairy farms (48%). Furthermore, some clusters according to the fatty acid grouping were specific for the conventional dairy farms, while others were composed of isolates from both operational management types.

Conclusions

The difference of operational management is translated in a different aerobic spore-forming bacterial flora in the produced raw milk.

Significance

One of the explanations for the observed differences between the spore-forming communities in organic versus conventional dairy farms may be the composition of the feed. Indeed, while conventional dairy farmers use cattle feeds e.g. concentrate, prepared from imported agricultural (waste) products, organic farmers feed locally cultivated crops.

The nitrite oxidoreductase operon in *Nitrobacter*: primer development for *norA*, *norB* and *norX*

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Nitrobacter oxidizes nitrite to nitrate by the enzyme nitrite oxidoreductase (NOR), which is coded by an operon containing the genes *norA*, *norX* and *norB*. Only a limited number of *nor* gene sequences are currently available, and specific primers are lacking till now. In contrast to the 16S rRNA gene, the *norA* and *norB* genes are currently considered as unique for *Nitrobacter*, they do not have high similarities with other known genes and are less conserved. Primers targeting the *nor* genes are hence considered to be more useful for qualitative and quantitative analysis of nitrite oxidizing communities. In this study, primer pairs were therefore developed targeting either the *norA* or *norB* gene, specific and sensitive for all *Nitrobacter* species. Until now, only the *norX* gene of *N. hamburgensis* $X14^T$ has been described. In this study, the inverted complements of the *norA* or *norB* primers were able to amplify the complete *norX* sequence of strains of all other *Nitrobacter* species.

Activating Natural Killer cell Immunoglobulin-like Receptor and HLA-C genes in immunity against human CMV infection

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Natural Killer (NK) cells play a key role in the early phase of defense against viral pathogens, preceding the development of specific adaptive T-cell reactions and antibodies. In view of the remarkable genetic diversity demonstrated by the human killer cell Immunoglobulin-like receptors (KIR) and Human Leukocyte Antigen (HLA) genes which control the function of natural killer (NK) cells, we propose that these genes could mediate specificity in the innate immune response to HCMV infection.

This case-control study consisted of 298 apparently healthy individuals, distinguished on the basis of their IgG seroconversion status for HCMV, 168 individuals were HCMV IgG positive while 130 individuals were IgG negative. Genomic DNA was determined for presence or absence of five groups of inhibitory (KIR2DL1-3 and KIR3DL1-2) and six groups of activating KIR genes (KIR2DS1-5 and KIR3DS1) by the polymerase chain reaction-sequence specific primer (PCR-SSP) technique. HLA-C typing was performed by PCR - sequence specific oligonucleotides (SSO) method.

We detected a small group of individuals (11%) characterized by *KIR2DS1*(+)*2DS4*(+)*2DL2*(-) and *HLA-C* alleles of *group 1 and 2* (*HLA-C1C2*), that were relatively protected against human CMV (HCMV) infection. All individuals in this group, except for one patient, possessed at least one copy of the full length form of KIR2DS4 gene in contrast to 25% of the total study population who were homozygous for the *KIR2DS4 deletion* variant. Our results suggest that innate NK cell protection against HCMV infection might be afforded by a joint effect of two activating KIRs in the presence of their ligands belonging to *HLA-C2*.

Evaluation of *pspA* co-expression for improved protein secretion in *Streptomyces lividans*

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Extracytoplasmic stress caused by, for instance, osmotic shock, ethanol shock, extreme heat shock or bacteriophage infection, may reduce the energy status of the cell which in turn induces the bacterial phage-shock-protein (Psp) system. The consensus so far is that the Psp response, with PpsA being the main effector protein, plays a role in maintaining the proton-motive force (PMF). In turn, the PMF is thought to help the cell to cope with membrane-related stresses, such as overloading or blocking of the export machinery. The most important effects noticed in *Escherichia coli psp* mutants were defects in the maintenance of the PMF, protein export by both the Sec and Tat pathway, and survival in stationary phase at alkaline pH.

Currently, we are investigating the role of the Psp system in the Gram-positive bacterium *Streptomyces lividans*, in which a *pspA* homologue was identified. So far, Psp reponses in Gram-positive bacteria have not (yet) been described in the literature and their function remains elusive. Hence, we have evaluated the influence of *pspA* overexpression on both native and heterologous protein secretion in this host organism.

In a first step, the protein secretion via the "twin-arginine translocation" (Tat) pathway was evaluated in both the wild-type and the *pspA*-overexpressing strain. Tat-dependent secretion of native proteins, assessed by xylanaseC (XlnC) protein yield, showed a fourfold increase in the *pspA*-overexpressing strain as compared to wild-type conditions. On the other hand, heterologous protein secretion using the Tat pathway was investigated using the enhanced fluorescent protein (EGFP). Previous research in our lab showed that, in order to secrete EGFP under the active, fluorescent form, the Tat pathway has to be used while Sec-dependent EGFP secretion yielded inactive EGFP. Notably, it is also the first protein that is secreted in such high levels (upward of 20 mg/l) via the Tat pathway. *PspA* overexpression further increased the Tat-dependent EGFP yield threefold.

The influence of PspA overproduction on Sec translocation was assessed using the native subtilisin inhibitor (Sti) protein, whole secretome analysis and ultimately the heterologous murine tumor necrosis factor (mTNF) α protein. Preliminary results showed that Sec translocation is also positively influenced by *pspA* overexpression, but to a lesser extent than the Tat translocation. This dissimilar effect is likely caused by the differential importance of PMF in both secretion pathways.

Biological safety of wastewater inoculants commercialized in Belgium

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The use of wastewater inoculants for the re-activation of domestic septic tanks became more and more common over the last years. These powders composed by a lyophilized microbial population are sold in almost every Belgian supermarket. In the absence of a safety control of these products, their impact on the public health has been assessed since they can be manipulated for domestic use. An examination of the whole bacterial population was performed by culture dependent and independent approaches. All bacteria growing on the different non-selective and selective media were identified. Construction of 16S rDNA clone libraries with subsequent sequencing, DGGE and selective PCR for pathogenic bacteria were also performed.

For all wastewater inoculants, the CFU were living cell number was between 10^4-10^7 /g powder on non-selective media. The bacterial compositions of the different wastewater inoculants studied displayed similarities. In all of them, *Bacilli* are the predominant cultivable species. This could be attributed to their capacity to survive as spores in desiccated materials such as wastewater inoculants. The sequencing of 16S rDNA clone libraries demonstrated a high prevalence of an uncultured bacteria (EMBL: AY171306) together with the presence of *Bacilli* species. *Enterococci* were also present to a lesser extend in almost all the wastewater inoculants tested. Selective enrichment and plating showed the presence of pathogenic or opportunistic pathogenic bacteria in 6 different products, namely; *Klebsiella pneumoniae* subsp. *pneumoniae* and *Enterobacter cloacae*. These pathogens were not detected by culture independent techniques. Immunocompromised, elderly people or children manipulating these powders can therefore be at risk.

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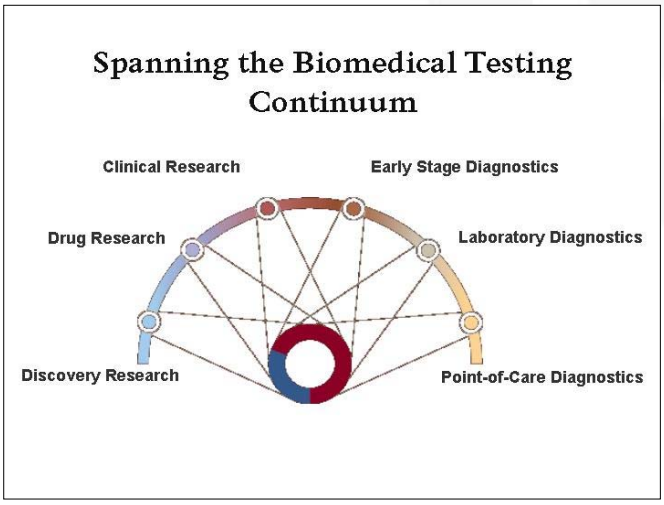

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