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BELGIAN SOCIETY FOR MICROBIOLOGY

ABSTRACT BOOK OF THE SYMPOSIUM

BIOFILMS
MICROBIAL ECOLOGY AND THEIR ROLE IN NATURE AND DISEASE

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BIOFILMS
MICROBIAL ECOLOGY AND THEIR ROLE IN NATURE AND DISEASE

Programme

09.00 h. Welcome address. **Alfons Billiau**, President of BSM

09.15 h. **Sören Molin,** BioCentrum DTU Molecular Microbial Ecology Group Lyngby, Denmark
"Cellular interactions in the course of bacterial biofilm development"

10.00 h. **Jean-Marc Ghigo**, Institut Pasteur, Dep. Microbiologie fondamentale et médicale, Groupe de Génétique des Biofilms, Paris, France.
"Role of natural conjugative plasmids in bacterial biofilm development"

10.45 h. Coffee

11.10 h. **Willy Verstraete,** Ghent University, Agricultural and Applied Biological Sciences, LabMET, Belgium
"Analysis of biofilms performing the oxygen-limited autotrophic nitrification denitrification (OLAND) process"

11.35 h. **Paul Rouxhet,** Unité de Chimie des Interfaces, Univ. Cath. de Louvain, Louvain-la-Neuve, Belgium
"Surface analysis by X-ray photoelectron spectroscopy in the study of bioadhesion and biofilms"

12.00 h. General Assembly BSM

12.15 h. Poster presentations

13.00 h. Lunch + poster presentation discussion groups

14.20 h. Short communications of selected posters

14.20 h. **V. Auquier (ULB).** Identification of new membrane proteins involved in the resistance mechanisms to copper and silver in *Ralstonia metallidurans* CH34

14.35 h. **T. Coeyne (RUG).** Identification of novel *Ralstonia* species from the respiratory tract of persons with cystic fibrosis

14.50 h. **L. Fontaine (U.C.L.).** Characterisation of a bacteriocin-like locus (BLL) from *Streptomyces thermophilus* and analysis of its dedicated two component regulatory system (BLLH/R)

15.05 h. **R. Van Houdt (K.U.Leuven)**. Quorum sensing in surface-adherent gram-negative bacteria isolated from an industrial kitchen

15.20 h. **John William Costerton,** Montana State University Center for Biofilm Engineering, Bozeman Montana, United States
"Biofilms in nature and disease"

16.10 h. **Johan Van Eldere,** K.U.Leuven, Fac. Medicine, Dep. Microbiology & Immunology, Laboratory of Bacteriology, University Hospital Gasthuisberg
"Staphylococcal biofilms and foreign body infections"

16.35 h. **Gerda M. Bruinsma**, University Groningen, Department of Biomedical Engineering, the Netherlands
"Dental biofilms and their removal by different modes of brushing"

17.15 h. General conclusions

ABSTRACTS

ORAL COMMUNICATIONS

CELLULAR INTERACTIONS IN THE COURSE OF BACTERIAL BIOFILM DEVELOPMENT

Soeren Molin
Molecular Microbial Ecology Group, BioCentrum-DTU, Lyngby, Denmark

Bacterial biofilms have become intense study objects in recent years – mainly due to the realization that this mode of growth is dominating in both environmental settings and in connection with many societal activities. In particular, the role of biofilms in many bacterial infections has drawn considerable attention to the field, and among the best studied biofilm developing organisms are opportunistic pathogens such as *Pseudomonas aeruginosa*. Also bacteria related to environmental activities are studied under biofilm growth conditions, and in pace with the increasing number of studies there is now a broad platform of knowledge about biofilm development and properties, which can be used to search for common traits. The current model suggests that after a first phase of cellular adhesion to the surface, the attached bacteria increase their bio-mass forming micro-colonies, which eventually turn into a mature biofilm with a highly structured appearance, consisting of alternating mounds of cells and voids between which water flows through the biofilm. These structures are embedded in a matrix of polymeric substances (polysaccharides, nucleic acids and proteins), which probably stabilize the entire surface community and protect it from various influences from the environment.

Although it is often possible to describe biofilm development in a manner as indicated above, both the actual structural features and the involved processes may vary quite substantially in biofilms with different organisms. Likewise, significant differences are often apparent when the growth conditions are shifted for the same organism. It may therefore be concluded that there is no such thing as a consensus biofilm development scheme – each case is new and requires specific characterization. However, it seems as if common features of significant importance may be identified and characterized from a number of such different biofilm communities, and that we are getting closer to defining some of the differentiation determining events involved in the process.

Through investigations of biofilm development for a number of different organisms we now suggest a general model pointing out some of the key features that seem to be important in determining the overall structure of the mature biofilm. After the first step of adhesion of planctonic cells to the surface micro-colonies will develop with a density and growth rate determined by the nutritional conditions in the near environment. The most common cause of colony formation is clonal growth – we do not normally find any evidence for cell aggregation. The further development is to a large extent determined by cell-cell agglutination: non-cohering cells do not seem to mature structurally, cells sticking to extra-cellular macromolecules such as DNA or polysaccharides form more or less loose structures, and tightly associated cells may form very large ‘mushroom’-like structures. There are indications that even very minor changes of the cell-cell coherent properties have significant impacts on biofilm structure development. Bacterial motility appears to be a very important factor in final shaping of the mature biofilm. Flagella conditioned swimming may be involved in dissolution and re-colonization, and regulated twitching motility has been observed to be an essential factor in formation of mature mushrooms. Some species show an apparent lack of biofilm development, which may allow genetic variants to settle and occupy the available niches.

The lecture will include information obtained from a number of different organisms forming biofilms in laboratory based flow-chamber systems.

NATURAL CONJUGATIVE PLASMIDS INDUCE BACTERIAL BIOFILM DEVELOPMENT.

JEAN-MARC GHIGO.
Genetics of Biofilms Laboratory Institut Pasteur (CNRS URA 2172).25 rue du Dr. Roux, 75724 Paris CEDEX 15, France. ***E-mail address:*** *jmghigo@pasteur.fr*

Horizontal gene transfer is recognized as a major source of evolution leading to change in the ecological character of bacterial species. Bacterial conjugation, which promotes the horizontal transfer of genetic material between donor and recipient cells by physical contact, is a phenomenon of fundamental evolutionary consequence. Whereas conjugation has been primarily studied in liquid, most natural bacterial populations are found associated with environmental surfaces in complex multispecies communities called biofilms. Biofilms are ideally suited to exchange of genetic material of various origins and recent reports demonstrated that bacterial conjugation occurs within biofilms. We studied the direct contribution of conjugative plasmids themselves to the bacterial host capacity to form a biofilm. We showed that natural conjugative plasmids expressed factors that induced planktonic bacteria to form or enter biofilm communities which favor the infectious transfer of the plasmid. This general connection between conjugation and biofilms suggests that medically relevant plasmid-bearing strains are more likely to form a biofilm. We used this property to produce mature biofilms in F plasmid carrying *E. coli* and investigated on a genomic scale their compared expression profile of free floating and biofilms population expressing the F conjugative plasmid. This approach led to the identification of new biofilm functions.

ANALYSIS OF BIOFILMS PERFORMING THE OXYGEN-LIMITED AUTOTROPHIC NITRIFICATION DENITRIFICATION (OLAND) PROCESS

Willy Verstraete

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For the treatment of N-rich wastewaters in the absence of degradable carbon, novel N removal processes are being developed and introduced in wastewater treatment systems. The most important one in that respect is the completely autotrophic N removal via a combination of partial aerobic nitrification to NO_2^- followed by anoxic ammonia oxidation (ANAMMOX) to N_2 with NO_2^- as the electron acceptor. At the Laboratory of Microbial Ecology and Technology (LabMET), this N removal process is examined under the name Oxygen-Limited Autotrophic Nitrification Denitrification (OLAND). The hypothesis is that aerobic ammonia-oxidizing bacteria (AAOB) play a role in the anoxic part of the process. Elements to support this hypothesis are: 1) ANAMMOX bacteria can thus far not be grown in pure cultures, the slow growth rate may indicate the involvement of a second, rate-limiting species, 2) the persistent presence of AAOB in anoxic reactors and re-establishment of NO_2^- production after only 1 day of aeration, 3) homogenised RBC biofilm shows lower anoxic activity and higher H_2 production, perhaps indicative of loss of reducing equivalents between separated groups of bacteria and 4) the observed juxta-position of AAOB and Planctomycetes in biofilms. The activity of ANAMMOX-like bacteria in these autotrophic N removing systems is beyond doubt, however a more pronounced role for the AAOB is proposed.

Further information:

Kuai, L., and W. Verstraete 1998. Ammonium removal by the Oxygen-Limited Autotrophic Nitrification-Denitrification system. *Applied and Environmental Microbiology*. 64:4500-4506.

Pynaert, K., R. Sprengers, J. Laenen, and W. Verstraete 2002. Oxygen-limited nitrification and denitrification in a lab-scale rotating biological contactor. *Environmental Technology*. 23:353-362.

Pynaert, K., S. Wyffels, R. Sprengers, P. Boeckx, O. Van Cleemput, and W. Verstraete 2002. Oxygen-limited nitrogen removal in a lab-scale rotating biological contactor treating an ammonium-rich wastewater. *Water Science and Technology*. 45:357-363.

Wyffels, S., K. Pynaert, P. Boeckx, W. Verstraete, and O. Van Cleemput 2002. Identification and quantification of nitrogen removal in a rotating biological contactor by 15N tracer techniques. *Water Research*: in press.

Pynaert, K., B. F. Smets, S. Wyffels, D. Beheydt, S. D. Siciliano, and W. Verstraete 2002. Characterization of an autotrophic nitrogen removing biofilm from a highly loaded lab-scale rotating biological contactor. Submitted to *Applied and Environmental Microbiology*.

SURFACE ANALYSIS BY X-RAY PHOTOELECTRON SPECTROSCOPY IN THE STUDY OF BIOADHESION AND BIOFILMS

Paul G. Rouxhet and Yves F. Dufrêne

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X-ray photoelectron spectroscopy (XPS) is based on recording the kinetic energy of electrons emitted under irradiation by X-rays in vacuum. Its provides an elemental analysis of the surface layer (about 5 nm thick) of a solid. Due to the influence of the chemical environment of the element, the peak position and shape give further information on the chemical functions present at the surface.
The method can been applied to microbial cells after freeze-drying in controlled conditions. The reliability of the analysis of elements and chemical functions at the surface of microbial cells has been demonstrated by quantitative relationships between different XPS data (C, O, N, P concentrations; C, O, N peak components). Its relevance to the surface composition of cells in an aqueous environment is supported by correlations with data of other kinds (surface, structure, hydrophobicity, isoelectric point …). Atomic force microscopy (AFM) observations under water have recently demonstrated the significance of XPS data obtained previously on bacteria and fungal spores. The surface concentrations of elements and chemical functions can be used to figure out roughly the cell surface composition in terms of molecular compounds (proteins, polysaccharides, …).
Application of this analysis to materials often shows that the chemical composition of their surface is different from the expected composition, due to contamination, migration of constituents or surface reorganization.
The study of different materials after immersion in industrial water circuits shows that, in a very short time, their surface is conditioned by adsorption of macromolecules. The adhesion of *Azospirillum brasilense* in laboratory conditions was found to be strongly influenced by proteins at the cell-substratum interface. It was demonstrated that proteins were released by the cells into the solution and then absorbed at the substratum surface where they favored cell adhesion.

Surface analysis by XPS thus helps to understand the primary adhesion, which initiates biofilm formation. It has shown that the process is more complex that accounted for by basic approaches such as DLVO theory or the balance of interfacial free energies. It also reveals the major features of the molecular composition of a biofilm surface, as obtained after freeze-drying.

BIOFILMS IN NATURE AND DISEASE

John William Costerton
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During the late 1970s direct observations of natural ecosystems showed, clearly, that the majority of bacteria grow in matrix-enclosed biofilms attached to available surfaces. This microbial tendency to form sessile communities is now accepted as being virtually universal, in all natural ecosystems, with the notable exception of nutrient-starved ecosystems like the deep oceans and the deep subsurface. This mode of growth appears to be favored, in natural and industrial ecosystems, by the advantages that biofilms have in matters of resistance to environmental hazards (drying, predatory amoebae) and in the metabolic integration of many species into a functioning community. The question has been asked "Can bacteria sense whether they are in a natural ecosystem or in a mammalian body?", and our negative answer to that question led us to assume that biofilms would be formed on mammalian tissues in both health and disease.

Similar methods of direct observation have shown that the bacteria that colonize exposed human tissues, like the mouth and the vagina, live predominantly in biofilms that closely resemble those seen in nature. In the mouth, supragingival plaque is largely composed of Gram-positive bacteria growing in very well-developed biofilms, and subgingival plaque is composed of a rich mixture of predominantly Gram-Negative bacteria growing in equally prolific sessile communities. Recent direct examinations of human vaginal populations have shown a similar predominance of biofilms, most of which are formed by Lactobacilli, and these sessile communities have been shown to protect of the tissues that they colonize from attack by extraneous pathogens. Human skin has been shown to harbor numerous bacterial cells (mostly *Staphylococcus epidermidis),* that form extensive biofilms in the spaces between the squamous cells, and resist killing by routine surgical preparations.

When bacteria are given access to deeper tissues, often by the surgical installation of medical devices, they assume the biofilm mode of growth on the plastic or metal surfaces of these devices. In this sessile form, the biofilm bacteria are functionally resistant to antibodies and phagocytes, and many studies have shown that they are also resistant to antibiotics at concentrations > 1,000 times those that would kill their planktonic (floating) counterparts. This phenomenal resistance of biofilm bacteria to host defense mechanisms, and to antibiotics, has led clinicians to conclude that colonized medical devices must usually be removed before these device-related infections can be resolved. Many non-device-related bacterial infections follow the same clinical pattern, of chronicity and recalcitrance to therapy, and direct observations have shown that they are also caused by bacteria growing in well-developed matrix-enclosed biofilms. Examples include middle ear infections, osteomyelitis, pneumonia in cystic fibrosis, and prostatitis. These device-related and other chronic infections are now seen to comprise at least 65% of infections treated by physicians in the developed world.

We have now concluded that the resistance of biofilms to antibodies and phagocytes derives from their exclusion by matrix material, but that antibiotic resistance derives from phenotypic changes in gene expression in biofilms. *Pseudomonas aeruginosa* changes the expression of 70% of its genes when it forms biofilms, and many antibiotic targets are simply not produced in these sessile organisms. However biofilm-specific proteins are produced and may provide new biofilm-specific targets. Additionally, biofilm cells have been shown to communicate by means of chemical signals, and blockage of these signals have been shown to influence biofilm formation, and to reduce the pathogenic consequences of colonization. Many new developments in biofilm control will be discussed.

STAPHYLOCOCCAL BIOFILMS AND FOREIGN BODY INFECTIONS

Johan Van Eldere
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Laboratory of Bacteriology, University Hospital Gasthuisberg and Rega Institute

Infections that originate at the surface of a foreign material are called foreign body infections. Medical devices constitute the majority of these foreign body infections and due to the increasing use of these foreign devices, infections of these medical devices are a major and still growing problem of modern medicine. The added costs of these infections in terms of prevention and treatment are very high. For example, for central venous catheters, bloodstream infection rates are between 3 and 10 %, for hip and knee arthroplasties, the combined 3 year infection incidence rate is over 1%, resulting in more than 2500 infections annually in the USA alone.

The most common cause of foreign body infections are staphylococci and in particular the coagulase negative staphylococci. Contrary to the virulent *Staphylococcus aureus*, coagulase negative staphylococci are opportunistic pathogens that only rarely cause infections unless specific conditions are present such as the presence of a foreign body.

However, due to the widespread use of intravascular catheters and the ease with which coagulase negative staphylococci cause foreign body infections, coagulase negative staphylococci are now the leading cause of hospital-acquired bloodstream infections, with an attributable mortality from 0.7% overall to 13.6% in Intensive Care Units. Coagulase negative staphylococci are also major pathogens associated with other indwelling or implanted devices such as heart valves, vascular grafts or orthopaedic prostheses etc.

A crucial role in the ability of coagulase negative staphylococci to cause foreign body infections is their capacity for colonising these foreign bodies. Colonisation of foreign bodies by coagulase negative staphylococci is linked to the formation of biofilms. This staphylococcal biofilm contains staphylococci that are embedded in a slimy extra cellular substance that is composed of polysaccharides. Bacteria present in this biofilm also behave differently from their free-living or planktonic counterparts and this is one of the factors responsible for the persistent nature of foreign body infections.

The basic process of biofilm formation by coagulase negative staphylococci consists of the following phases; adhesion, accumulation and finally the establishment of a persistent infection.

Adhesion includes an initial phase of non-specific interaction of the bacteria with the surface of the foreign body followed by a more specific and adhesin-mediated interaction. Initial adhesion starts within a few seconds after exposure and is reversible. Because it is dependent on physical forces, it is strongly influenced by surface irregularities and the nature of the material. Several molecules have already been identified as potential adhesins that mediated specific adhesion. The atlE gene encodes an amidase and a glucosamidase that might function in the adhesion to polystyrene and vitronectin. Other potential adhesins are the fimbria-like proteins SSP-1 and 2 and the capsular polysaccharide adhesin (PS/A) encoded by the *ica* genes. Conditioning of the surface of the foreign body with host-derived factors such as collagen or fibrinogen promotes adhesion.

The adhesion phase is followed by an accumulation phase. In this phase, multi-layered cell clusters are formed that are surrounded by a slimy matrix. The most important component of this slimy matrix is the polysaccharide intercellular adhesin (PIA). Biosynthesis of this molecule is also dependent on the *ica* operon. Although its exact role is as yet unknown, other factors such as the accumulation-associated protein (AAP) are also involved in the accumulation phase.

A persistent and difficult to eradicate infection follows the accumulation phase. The difficulty in eradicating foreign body infections are also due to the local deficiency in host defences. Contact between a foreign body and granulocytes leads to prompt degranulation of these cells and results in exhausted neutrophils with a markedly reduced capacity for phagocytosis, chemotaxis and oxidative burst reactions. Bacterial factors such as the reduced growth however are also hypothesised to contribute to biofilm persistence.

To study the pathogenesis of foreign body infections, we have developed an animal model that allows the establishment of staphylococcal biofilms on implanted catheters. The role of specific genes in adhesion, accumulation and persistence, is studied by monitoring the expression of these genes over time in bacterial cells isolated immediately after initial adhesion, in the accumulation phase and in cells isolated from several days old persistent biofilms. From these studies it has become clear that housekeeping gene expression is indeed reduced in biofilm-associated bacteria and that this might be a factor responsible for the antimicrobial resistance encountered in

these biofilm associated bacteria. Other genes such as atlE remain expressed at high levels in persistent infections, suggesting an as yet undefined function of this gene in established biofilms.

Suggested reading:

Vandecasteele S.J., Van Wijngaerden E., Van Eldere J., Peetermans W.: New Insights in the Pathogenesis of Foreign Body Infections. Acta Clinica Belgica, 55, 3, 148-153, 2000.

von Eiff, C. and Heilmann, C. 1998. *Staphylococcus epidermidis*: why is it so successful ? Clin. Microbiol. Infect. 4:297-299.

Dunne, W.M., Jr. 2002. Bacterial Adhesion: Seen Any Good Biofilms Lately? Clin. Microbiol. Rev. 15:155-166.

Donlan, R.M. and Costerton, J.W. 2002. Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. Clin. Microbiol. Rev. 15:167-193.

Vandecasteele, S.J., Peetermans, W.E., Merckx, R., and Van Eldere, J. 2001. Quantification of the expression of *Staphylococcus epidermidis* housekeeping genes with taqman quantitative PCR during in vitro growth and under different conditions. J. Bacteriol. 183:7094-7101.

REMOVAL BY THREE MODES OF BRUSHING UNDER DIFFERENT WEIGHTS OF CO-ADHERING AND NON-CO-ADHERING BACTERIAL PAIRS FROM PELLICLES AND *DE NOVO* ADHESION.

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Co-adhesion between oral microbial pairs is an established factor in dental plaque formation.

Objectives: This study compares removal of pairs of co-adhering and non-co-adhering oral actinomyces and streptococci from salivary pellicles by hand, mechanic and sonic brushing applying different weights.

Methods: First, actinomyces were allowed to adhere to a pellicle in a flow chamber. Then streptococci suspended in saliva were perfused through the chamber at 33°C and removal was examined by hand, mechanic and sonic brushing applying weights up to 200 gr, after which *de novo* adhesion of streptococci was facilitated.

Results: On average, 34% to 43% of the adhering bacteria were adhering as single organisms. For co-adhering and non-co-adhering pairs, 33% and 10% of the adhering bacteria were involved, respectively in aggregates comprising more than 10 organisms. Brushing by hand removed 79% to 89% of the adhering bacteria and removal increased with the application of weight. Mechanic (92%) and sonic (84% to 92%) brushing removed more bacteria than hand brushing and removal also increased with increasing weight. *De novo* adhesion of streptococci yielded only 2% to 16% of bacteria involved in large aggregates for the non-co-adhering pair. *De novo* streptococcal adhesion to hand brushed pellicles yielded 34% to 57% of bacteria involved in large aggregates for the co-adhering pair, while mechanic and sonic brushing left 22% to 35% of the bacteria involved in large aggregates.

Conclusions: A strong influence of co-adhesion exists in *de novo* streptococcal adhesion after removal of adhering pairs of streptococci and actinomyces. These observations suggest that the three modes of brushing, but particularly manual brushing, leave footprints to which streptococci preferentially adhere.

ABSTRACTS OF

POSTERS AND SHORT COMMUNICATIONS

- List is ordered alphabetically
- Posters are grouped according to topics:

    A. Biodiversity and Evolution, Environmental Microbiology

    B. Detection, identification, genes, genomes and taxonomy

    C. General microbiology and Pathology

    D. Biochemistry and Molecular Biology

- Poster abstracts selected for oral communication

1. **V. Auquier**, D. de Clock, R. Wattiez, S. Monchy, M. Mergeay, J.-M. Ruysschaert and G. Vandenbussche. Identification of new membrane proteins involved in the resistance mechanisms to copper and silver in *Ralstonia metallidurans* CH34
2. **T. Coeyne**, P. Vandamme, J.J. LiPuma. Identification of novel *Ralstonia* species from the respiratory tract of persons with cystic fibrosis
3. **L. Fontaine**, B. Grossiord, P. Hols and J. Delcour. Characterisation of a bacteriocin-like locus (BLL) from *Streptomyces thermophilus* and analysis of its dedicated two component regulatory system (BLLH/R)
4. **R. Van Houdt**, A. Jansen and C. Michiels. Quorum sensing in surface-adherent gram-negative bacteria isolated from an industrial kitchen

ALPHABETIC LIST OF POSTER ABSTRACTS

Poster #	Poster group	Authors and poster title
1	A	V. Auquier, D. de Clock, R. Wattiez, S. Monchy, M. Mergeay, J.-M. Ruysschaert and G. Vandenbussche **Identification of new membrane proteins involved in the resistance mechanisms to copper and silver in *Ralstonia metallidurans* CH34**
2	D	S. Baatout, P. De Boever, F. Marty, L. Hendrickx and M. Mergeay **Flow cytometric study of membrane permeability in six bacterial strains under stress conditions (temperature, X- and UV-irradiations)**
3	B	T. Coeyne, P. Vandamme, J.J. LiPuma **Identification of novel *Ralstonia* species from the respiratory tract of persons with cystic fibrosis**
4	C	E. De Buck, E. Lammertyn, I. Lebeau, E. Meyen and J. Anné **The twin-arginine dependent secretion pathway as a possible determinant of virulence in *Legionella Pneumophila***
5	B	E. De Clerck, T. Vanhoutte, T. Hebb and P. De Vos **Study of bacterial load in gelatine, focussed on *Bacillus* and related genera**
6	C	M. Deghorain, L. Fontaine, J.-L. Mainardi, S. Kotsonis, B. Hallet, P. Hols and J. Delcour **The determinants of the innate vancomycin resistance of *Lactobacillus* plantarum: a comparative study with *Lactococcus Lactis***
7	C	K. Dierick, I. Wybo, M. Jouret, M. Cornelis, Y. Ghafir, L. De Zutter and G. Daube **Antimicrobial resistance in *Salmonella* strains isolated during the zoonosis monitoring in slaughterhouses, meat plants and retail shops in 2001**
8	C	D. Ercken, L. Verelst, P. Declerck, L. Duvivier, A. Van Damme, F. Ollevier **Inactivation of *Naegleria lovaniensis*: the effect of monochloramine and peracetic acid**
9	C	L. Fontaine, B. Grossiord, P. Hols and J. Delcour **Characterisation of a bacteriocin-like locus (BLL) from *Streptomyces thermophilus* and analysis of its dedicated two component regulatory system (BLLH/R)**
10	D	C. Galloy, V. Vanhooff, C. Normand, J. Delcour and B. Hallet **Communication between the core and accessory regions of the TNPI-IRS site-specific resolution system of TN*4430***
11	A	R. Gelsomino, M. Vancanneyt, T.M. Cogan and J. Swings **The effect of raw-milk cheese consumption on the enterococcal faecal flora**
12	D	P. Goffin, F. Lorquet, T. Ferain, M. Kleerebezem, J. Delcour and P. Hols **Lactate dehydrogenase-independent lactic acid racemization in *Lactobacillus plantarium***
13	A	J. Goris, N. Boon, W. Verstraete and P. De Vos **GFP-tagging as a tool to track the dissemination of the 3-chloroaniline-degradative plasmid PC1 in activated sludge**
14	D	I. Hallemeersch and E.J. Vandamme **Characterization of novel fungal cellulases and hemicellulases**
15	A	L. Hendrickx, F. Marty, S. Baatout, R. Wattiez, P. Janssen, A. Wilmotte and M. Mergeay **Genetic stability and axenicity in the biological life support system Melissa**
16	B	M. Heyndrickx, L. Herman, D. Vandekerckhove and L. De Zutter **Molecular epidemiology of *Salmonella* contamination of broilers from hatchery to slaughterhouse**

17	B	G. Huys, P. Kämpfer, M.J. Albert, M. Pearson and J. Swings ***A. hydrophil* subsp. *dhakensis* subsp. nov. and A *hydrophila* subsp. *ranae* subsp. nov., isolated from diarrhoeal children in Bangladesh and from septicaemic farmed frogs in Thailand, respectively**
18	A	P. Janssen, S. Garcia-Vallvé, B. Audit and C. Ouzounis **Genome stability and gene flux in *Helicobacter pylori***
19	D	V. Ladero, M. Kleerebezem, J. Delcour and P. Hols **Polyol production in *Lactobacillus plantarum* by metabolic engineering**
20	C	I. Lebeau, E. Lammertyn, E. De Buck and J. Anné **Promoter activity of LpnR 259/6, a LuxR homologue in *Legionella Pneumophila*, and the effect of gene inactivation as revealed by proteome analysis**
21	D	F. Marty, R. Wattiez, P. Bertin, S. Baatout, A. Wilmotte, L. Hendrickx, M. Mori, P. Janssen and M. Mergeay **Evaluation of two different strategies for the identification of stress related genes in *Arthrospira Platensis* and *Rhodospirillum rubrum***
22	B	L. Masco, G. Huys and J. Swings **REP-PCR fingerprinting for identification of *Bifidobacterium* species**
23	C	F. Meurens, G. Keil, B. Muylkens, F. Schynts, P. Gallego and E. Thiry **Effect of superinfection delay on production of bovine herpesvirus 1 recombinants**
24	A	N. Michelet and J. Mahillon **Distribution, diversity and potential biological role of cereolysin O in the *Bacillus cereus* group**
25	A	S. Monchy, V. Auquier, M. Mergeay, P. Bertin, A. Toussaint and S. Wodak **Metal resistance operons (of the RND efflux family) in *R.metallidurans* CH34**
26	D	A. Onraedt, W. Soetaert and E.J. Vandamme **Accumulation of compatible solutes in *Brevibacterium* sp**.
27	D	E. Palumbo, P.Hols, M. Kleerebezem, R. Leer, C.J.P. Boonaert and J. Delcour **Genetic study of the functional role of D-alanine substitutions of teichoic acids in *Lactobacillus plantarium***
28	D	S. Parmentier, F. Arnaut and E.J. Vandamme **Application of *Leuconostoc mesenteroides* mannitol dehydrogenase for the enzymatic production of D-mannitol**
29	D	J.-Y. Springael and M.J. Penninckx **Expression of the yeast *Saccharomyces cerevisiae* γ-glutamyltranspeptidase requires nitrogen regulatory network including GATA zinc fingers *gln3*, *nil1/gat1* and *gzf3***
30	A	A. Taton, B. Elmandil, S. Grubisic, P. Balthasart and A. Wilmotte **Genotypic diversity of cyanobacteria in microbial mats from coastal lakes in eastern Antarctica**
31	B	R. Temmerman, I. Scheirlinck, G. Huys and J. Swings **Culture-independent analysis of probiotic products using DGGE**
32	C	S.J. Vandecasteele, W.E. Peetermans, R. Merckx and J. Van Eldere **Changes in bacterial metabolism during biofilm-associated infections could explain at least part of the biofilm-resistance**
33	C	S.J. Vandecasteele, W.E. Peetermans, R. Merckx and J. Van Eldere **Early but not late *in vivo* foreign body associated growth provokes a transient peak in ICAC gene expression in *Staphylococcus Epidermidis***
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IDENTIFICATION OF NEW MEMBRANE PROTEINS INVOLVED IN THE RESISTANCE MECHANISMS TO COPPER AND SILVER IN *RALSTONIA METALLIDURANS* CH34

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Ralstonia metallidurans CH34 (formely *Alcaligenes eutrophus* CH34), which was initially found in sediments of a zinc factory in Belgium, is a β-Proteobacterium able to grow in the presence of a variety of heavy metals and is used as a model for bacterial interactions with these compounds. Strain CH34 carries two megaplasmids, pMOL28 (180kb) and pMOL30 (280kb), that contain genes for multiple metal resistance mechanisms: *czc* (resistance to Cd^{2+}, Zn^{2+} and Co^{2+}), *cnr* (resistance to Co^{2+} and Ni^{2+}), *chr* (resistance to CrO_4^{2-}), *mer* (resistance to Hg^{+}), *pbr* (resistance to Pb^{2+}) and *cop* (resistance to Cu^{2+}). These resistance mechanisms are generally based on efflux of heavy metal ions to the extracellular medium via a complex of proteins forming a RND-HME system (Resistance Nodulation and cell Division family/group Heavy Metal Efflux). One of these systems can be exemplified by the genes *czcCBA* products associated in a tricomponent cation/proton antiporter efflux system. These RND-HME systems involve an inner membrane protein (RND family), a periplasmic Membrane Fusion Protein (MFP) and an outer membrane protein (OMF family).

Using a two-dimensional electrophoresis approach, we have analyzed the membrane proteomic response of *Ralstonia metallidurans* CH34 to copper or silver stress. Two-dimensional gels of the membrane proteins expressed in the absence or in the presence of the heavy metal ions were compared and the proteins presenting an increase in their expression level were identified by mass spectrometry. We observed that CopB and at least two other proteins were overexpressed in the presence of both copper and silver. CopB is a protein encoded by the pMOL30 *cop* cluster. This cluster of 14 genes or ORFs contains the genes *copABCD*, which have homologs in *P. syringae* and in *E. coli* (*pcoABCD*), encoding a complex detoxification system. Although the copper resistance mechanism of this complex is only partially known, CopB appears to be an outer membrane protein probably involved in the metal efflux. The two other proteins, named CusB and CusC were so called due to the high identity with *E. coli* genes. The corresponding genes are located in *Ralstonia metallidurans CH34* side by side on the chromosome and can form with the adjacent genes a new metal resistance operon. The proteins CusABC are probably associated in a tricomponent cation/proton antiporter efflux system (like CzcCBA) belonging to the RND-HME subfamily. The CusB and CusC proteins belong to the MFP (Membrane Fusion Protein) and the OMF (Outer Membrane Factor) proteins family, respectively.

A fourth protein has been detected only in response to silver stress. This protein, chromosomally encoded and called AgrR, is a new protein which has never been described before in heavy metal resistance. This protein shares identity with CzcR and similar regulators of a two-component regulation system. The coresponding gene forms with the adjacent genes the putative metal resistance operon: *agrRSABC*.

The membrane proteome of the mutant Agr-2720 of *Ralstonia metallidurans* CH34 has been studied using the same approach. This mutant is characterized by its capacity to grow at a concentration of silver in the medium that fully inhibits the wild type strain CH34. We have demonstrated that this mutant expressed constitutively the proteins CopB and AgrR. This result brings additional arguments for the role of these two proteins and their corresponding genetic loci in the resistance mechanism to silver.

In conclusion, this proteomic approach shows direct evidence, at the protein level, for the involvement of new membrane proteins in the resistance mechanisms to heavy metals.

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FLOW CYTOMETRIC STUDY OF MEMBRANE PERMEABILITY IN SIX BACTERIAL STRAINS UNDER STRESS CONDITIONS (TEMPERATURE, X- AND UV-IRRADIATIONS)

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Whilst flow cytometry is useful for measuring proteins, nucleic acids, size and immunofluorescence in a variety of cells it is not yet widely used in microbiology. In order to study fine changes associated with temperature, oxidative or irradiation stresses, preliminary flow cytometric tests were performed to estimate the maintenance of membrane integrity in six bacterial strains chosen for their potential use in either nuclear bioremediation or life support systems for use in space (*Ralstonia metallidurans, Escherichia coli, Shewanella oneidensis, Deinococcus radiodurans, Rhodospirillum rubrum and Arthrospira platensis*). The maintenance of membrane integrity is commonly measured as an indicator of cell damage (disturbance of ion transport, respiratory activity) or cell death. Propidium iodide (PI) is a fluorescent stain that can be used to estimate membrane integrity. It passively enters stressed or injured cells and intercalates into DNA and RNA. Flow cytometry can be used to measure the fluorescence associated with the damage of several thousand cells within a few minutes, each cell being assessed individually. Since the influx of PI can be correlated with the extent of bacterial wall permeability, the mean fluorescence of the bacterial population submitted to the stress will be inversely proportional to the number of viable cells. In this study, membrane integrity analysis was based on the capacity of the above-mentioned bacterial strains to exclude PI. The various stress conditions applied to these strains were 1) incubation at different temperatures for 1 hour (-20, 4, 15, room temperature, 28, 37, 43, 50, 60 or 70°C), 2) X-irradiation with 4 (except for *A. platensis* and *R. rubrum*) or 8 Gy (0,375 Gy/min) or 3) UV-irradiation for 30 minutes, 2 or 3 hours (dose rate : 491 +/- 36 µJ/s·cm²) (except for *A. platensis* and *R. rubrum)*. Variations in forward scatter (size), side scatter (granularity) and red fluorescence were monitored for each cell on an EPICS XL (Beckman-Coulter) using logarithmic amplification gain. The discriminator was set to trigger on forward scatter. Triplicate samples were analysed for each experiment. For all six strains, significant variations in size, granularity and fluorescence were observed after exposure to 50, 60 or 70°C. For *S. oneidensis* and *D. radiodurans*, variations in size, granularity and fluorescence were also observed after incubation at 43°C for 1 hour. *A. platensis*, *S. oneidensis* and *E. coli* showed a sensitivity to -20°C. Interestingly, variations in size, granularity and fluorescence could be detected immediately after exposure to X- or to UV-irradiation. These variations were proportional to the dose applied.
In conclusion, this study confirms that flow cytometry can be useful for monitoring the physiological status of bacteria under stress conditions and that the maintenance of cell integrity, as indicated by fluorescence intensity, was almost always supported by both size and granularity data. Further studies are underway to measure the variations in membrane potential, enzymatic activity and intracellular pH, as well as survival studies using the above stress conditions.

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IDENTIFICATION OF NOVEL *RALSTONIA* SPECIES FROM THE RESPIRATORY TRACT OF PERSONS WITH CYSTIC FIBROSIS

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Cystic fibrosis (CF) is the most frequent hereditary disease in Caucasians and is caused by a defect in the cystic fibrosis transmembrane conductance regulator gene. Defects in this gene mainly affect the respiratory tract and the pancreas, and exacerbations of pulmonary infections are the cause of significant morbidity and mortality in patients with CF.

The frequency and clinical significance of respiratory tract infections by *Ralstonia* species in persons with CF are not well-documented. This is in part due to a lack of understanding of the total biodiversity in this group of organisms, which hinders accurate identification. We used a polyphasic-taxonomic approach (employing 16S rDNA sequence analysis, DNA-DNA hybridizations, determination of the DNA-base composition, whole-cell protein analysis, biochemical characterization and PCR-based assays) to identify 44 isolates tentatively identified as belonging to the genus *Ralstonia*. These were recovered from 40 CF patients receiving care in 21 CF centers in 20 US cities. Twenty-five isolates were identified as *Ralstonia mannitolilytica*, nine as *Ralstonia pickettii*, two as *Ralstonia gilardii* and one as *Ralstonia taiwanensis*. Six CF isolates could not be identified to the species level and represented novel *Ralstonia* species. Four of these were classified as the novel species *Ralstonia respiraculi*, while two were classified as the novel species *R. insidiosa* (together with isolates from other clinical samples and the environment). The remaining isolate was closely related to *R. respiraculi* but most likely represents another novel *Ralstonia* species. These novel species can be differentiated from other *Ralstonia* species and phenotypically similar species (including *Burkholderia cepacia* complex and *Alcaligenes xylosoxidans*) by a variety of biochemical tests, whole-cell protein analysis and several PCR-based assays.

These data indicate that the CF lung may become infected with a variety of related bacterial species, including heretofore undescribed taxa. Methods to reliably identify these species will allow studies to better assess their potential roles in the pathology of CF lung disease.

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THE TWIN-ARGININE DEPENDENT SECRETION PATHWAY AS A POSSIBLE DETERMINANT OF VIRULENCE IN *LEGIONELLA PNEUMOPHILA*

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Legionella pneumophila is a facultative intracellular pathogen, which is the causative agent of a very severe form of pneumonia, better known as Legionnaires' disease, or a milder, flu-like self-limiting disease named Pontiac fever. In the environment this Gram-negative bacterium multiplies primarily within protozoa. During infection, replication takes place in human alveolar macrophages. Since several identified virulence factors are secreted in the extracellular environment, different protein secretion pathways have been characterised as important determinants of virulence in *L. pneumophila*. Two different types of secretion pathways, responsible for secretion across the outer membrane, were already identified in this parasite.

In this respect we were interested in the possible presence in *L. pneumophila* of the twin-arginine dependent translocation (Tat) pathway, a recently discovered secretion route. Generally this secretion pathway is used to transport folded proteins across the cytoplasmic membrane. The substrates that are transported by the Tat-pathway contain a characteristic twin arginine (RR) motif in their signal peptide. In *Escherichia coli* four different *tat*-genes were identified and three of them are organised in an operon (*tatABC* and *tatE*).

In *L. pneumophila* we showed the presence of a *tatA*-, *tatB*- and *tatC*-homologue on the chromosome. *TatA* and *tatB* were shown to consist an operon. The possible role of the Tat-pathway in the virulence of *L. pneumophila* was investigated by constructing a *tatB*-mutant through insertion of a kanamycine resistance gene. This mutation resulted in a clearly decreased ability of *L. pneumophila* to infect and replicate in the amoeba *Acanthamoeba castellanii*. Similar experiments are now being performed in differentiated U937 cells, used as a model for human macrophages.

These results indicate that the Tat-pathway acts as a determinant of virulence in *L. pneumophila*. Further investigations are focussed on the identification of possible Tat-substrates in *L. pneumophila*. Finally this project will enable us to identify new virulence factors and to come to a better understanding of *L. pneumophila* pathogenesis.

STUDY OF BACTERIAL LOAD IN GELATINE, FOCUSSED ON *BACILLUS* AND RELATED GENERA

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Gelatine is a proteinaceous colloid of animal origin. Because of its gelling and stabilizing properties, it is applied in several industries, mainly food industries (confectionery, dairy products, meat preparations, etc.) and pharmaceutical industries (hard and soft capsules, tablets, etc.). Because of endospore forming capacity, members of the genus *Bacillus* and related genera may survive extreme conditions of pH and temperature as achieved during the production of gelatine. At present, quality control is based on detection methods that are time consuming and of low sensitivity, resulting in problems concerning delivery terms and undetected contamination. The latter may affect the physical characteristics of gelatine and some of the contaminants may be pathogenic for men. An extensive characterisation of *Bacillus* contaminants in order to design a rapid and sensitive detection method for the most relevant species seems indispensable to insure the quality and safety of gelatine products.

In a first phase, the total diversity of bacterial contaminants and the contribution of the genus *Bacillus* and related endospore forming genera was analysed by isolating contaminants from different points along a gelatine production process. Gaschromatographic methyl ester analysis of fatty acids was performed to differentiate isolates at the genus level. Members of the genus *Bacillus* or related endospore forming genera were found. Members of *Salmonella*, *Kluyvera*, *Staphylococcus*, *Burkholderia*, *Lactobacillus, Enterococcus* and *Pseudomonas* were also present. Isolates belonging to *Bacillus* or related genera were further characterised by gelatinase tests, rep-PCR and 16S rDNA sequencing. All showed the ability to liquefy gelatine. Isolates could be assigned to *Bacillus licheniformis*, *B. fumarioli*, members of the *B. cereus* group, *B. badius*, *B. coagulans*, *B. subtilis*, *Brevibacillus agri* and probably yet undescribed *Brevibacillus*, *Paenibacillus* and *Alicyclobacillus* species.

In a second phase, samples were analysed from batches of gelatine that were rejected for consumption because of suspected bacterial contamination as shown by the quality control of the company. All contaminants belong to the genus *Bacillus* or related endospore forming genera, which supports the hypothesis that only spore forming bacteria are able to survive the extreme conditions of the production process. Furthermore, all the isolates were gelatinase positive. The majority of the isolates were identified as *B. licheniformis*, *B. fumarioli* and members of the *B. cereus* group.

In conclusion, endospore forming bacteria are contaminating gelatine production processes and persist in the final product. They destroy the gelling capacity and may exhibit pathogenic properties for humans. Based on the identification results, Taqman probes were developed for *B. licheniformis*, *B. cereus* and *B. fumarioli* strains for testing in a real-time PCR system. Therefore direct DNA extraction from gelatine samples was performed. DGGE patterning is applied to reveal culture-independent characterisation of the contamination.

THE DETERMINANTS OF THE INNATE VANCOMYCIN RESISTANCE OF*LACTOBACILLUS PLANTARUM* : A COMPARATIVE STUDY WITH *LACTOCOCCUS LACTIS*

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Vancomycin inhibits cell wall biosynthesis of Gram-positive bacteria through binding to the dipeptide D-ala-D-ala located at the end of the petidoglycan precursor. Most vancomycin resistant strains contain a modified peptidoglycan precursor in wich the terminal D-alanine residue is replaced by a D-lactate. Specific Ddl ligases are responsible of the formation of the dipeptide D-ala-D-ala or the depsipeptide D-ala-D-lac. In enterococci, vancomycin resistance requires a D-ala-D-ala dipeptidase (VanX) in addition to an exogenous D-ala-D-lac ligase.

The lactic acid bacterium *Lactobacillus plantarum* is vancomycin resistant whereas *Lactococcus lactis* is sensitive. The heterologous expression of the *ddl* gene from *L. lactis* in *L. plantarum* resulted in vancomycin sensitivity while the reciprocal was insufficient to make *L. lactis*. Vancomycin resistant A *L. lactis ddl* mutant strain expressing solely the Ddl ligase of *L. plantarum* was still sensitive, although 60 % of the PG precursors were ending by D-ala-D-lac. This result indicates that the Ddl enzyme from *L. plantarum* is bispecific. A resistant *L. lactis* strain could only obtained by coexpressing the *L. plantarum ddl* gene and the *vanX* gene from *Enterococcus faecalis*. This finding suggests that a *vanX*-like gene recently identified in the *L. plantarum* genome may contribute to its natural vancomycin resistance.

ANTIMICROBIAL RESISTANCE IN *SALMONELLA* STRAINS ISOLATED DURING THE ZOONOSIS MONITORING IN SLAUGHTERHOUSES, MEAT PLANTS AND RETAIL SHOPS IN 2001

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Introduction

The appearance of antimicrobial resistance in *Salmonella enterica* serovars has, over the last decades, become a matter of great concern for public health workers. Therefore the Institute of Veterinary Expertise (IVE) decided in 1998 to monitor the antimicrobial resistance of *Salmonella enterica* serovars isolated during their national zoönosis monitoring plan , according to the directive 92/117/CEE, in slaughterhouses, cutting plants and meat retail shops. This poster presents de results of the antibiotic resistance monitoring in 2001.

Material and methods

245 strains of *Salmonella enterica* were isolated from beef, porc, chicken and spent hens during february-december 2001. Samples included swabs from carcasses, meat cuts and minced meat. After a pre-enrichment in buffered peptone water, Diasalm (Lab M) was used as a semi-solid enrichment medium. The strains were isolated on XLD and confirmed by biochemical tests. (rapid ID 20e, bioMérieux) Serotyping was performed in the National Reference Center for *Salmonella* and *Shigella* and phagetyping was done in the Pasteur Institute in Brussels. Antimicrobial susceptibility testing was performed by the disk diffusion method (Kirky-Bauer) following NCCLS recommendations. The following antibiotics were tested: ampicillin (AMP), ceftriaxone (CTRX), chloramphenicol (CHL), ciprofloxacin (CIP), kanamycin (KAN), nalidixic acid (NAL), streptomycin (STR), sulfonamides(SUL) tetracycline(TET), trimethoprim (TMP) and trimethoprim+sulfonamides(SXT). An inocculum of 0.5 MacFarland was plated on a Müller-Hinton II agar (bio-Mérieux, France) to ensure semiconfluent growth and antibiotic tablets were applied on the agar surface. Inhibition zones were measured with a digital caliper after incubation at 37° for 16-18h. Interpretation of inhibition zones according to NCCLS. Quality control was performed by using an *Escherichia coli* ATCC 25922 strain.

Results

matrix	n	AMP %	CTRX %	CHL %	CIP %	KAN %	NAL %	STR %	SUL %	TET %	TMP %	SXT %
Broiler	64	35.9	0	9.4	0	1.6	29.7	50	32.8	18.8	21.9	20.3
Beef	15	6.7	0	6.7	0	0	6.7	13.3	13.3	26.7	6.7	6.7
Hen	36	2.5	0	2.5	0	0	10	7.5	12.5	2.5	5	5
Porc	126	13.4	0	13.4	0	2.4	0.8	52	24.4	31.5	11.8	10.2
Total	245	17.1	0	10.6	0	1.6	10.2	41.9	24.0	23.2	13.0	11.8

In 2001 there was no resistance to ciprofloxacine and ceftriaxone among the *Salmonellae,* isolated during the Zoonosis monitoring by the Institute of Veterinary Expertise. Resistance to streptomycin was high. Over 20% of the isolated strains were resistant to sulfonamides and tetracyclines. The resistance to ampicillin, chloramphenicol, nalidixic acid, trimethoprim an trimethoprim-sulfonamide was about 10%. In broilers the highest resistance was noticed. However, compared to the results of 2000 a decrease of resistance could be noticed which was mainly due to the lower number of *S*. Hadar isolated in 2001. On the other hand the increase in resistance in *Salmonellae* isolated from porc was caused by more frequent isolation of S. Typhimurium. Strains isolated from porc show more frequent resistance to tetracycline and sulfonamides than strains isolated from other meat species.

In strains originating from beef little resistance was found. Only the resistance to tetracycline exceeds 20%.

Salmonellae isolated from spent hens show very little resistance. This is linked with *S*. Enteriditis as the predominant serovar in this animal species.

The serovars Hadar, Typhimurium, Typhimurium var Copenhagen and Paratyphi B are the most resistant and show the highest number of multiresistant strains.

INACTIVATION OF *NAEGLERIA LOVANIENSIS*: THE EFFECT OF MONOCHLORAMINE AND PERACETIC ACID

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Biocidal activities of monochloramine and peracetic acid were studied on cysts of *Naegleria lovaniensis*. Until recently the most commonly used biocide to disinfect cooling water systems was hypochlorite. Due to its negative impact on the aquatic environment, ecologically less harmful alternatives have been looked for. The biocidal activity of monochloramine and peracetic acid makes them good candidates to inactivate pathogenic *Naegleria* species. These biocides were therefore tested on *Naegleria lovaniensis,* a relative of the pathogen *Naegleria fowleri*, as an alternative treatment to hypochlorite.

Under laboratory conditions the biocidal activity of hypochlorite is 8 to 10 times stronger than that of the two investigated substances. Hypochlorite, at a concentration of 0.5 ppm, kills *Naegleria lovaniensis* 100% after 1 h exposure (25°C, pH 7.3-7.4). To achieve similar results with monochloramine and peracetic acid, 3.94 ppm or 5.33 ppm had to be used respectively (25°C, pH 8).

It is known that ‘in situ’ biota of the biofilm, along with any organic material in the water column, have a negative impact on the efficiency of biocides. There are, however, indications that the relative efficacy of monochloramine and peracetic acid is quite good under such ‘in situ’ conditions compared with hypochlorite.

CHARACTERISATION OF A BACTERIOCIN-LIKE LOCUS (BLL) FROM *STREPTOCOCCUS THERMOPHILUS* AND ANALYSIS OF ITS DEDICATED TWO COMPONENT REGULATORY SYSTEM (BLLH/R)

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Streptococcus thermophilus is one of the main economically important lactic acid bacteria used in the dairy industry. Increasing knowledge of its metabolic properties and physiology is a prerequisite for the improvement of its industrial properties. Determination of the entire genome sequence of *S. thermophilus* LMG18311was used in this study to investigate the presence of two-component systems (TCSs) and their respective functions. TCSs are often used in bacteria to sense and respond to their environment. They play a key role in important physiological mechanisms such as virulence in group A streptococci, natural competence, bacteriocin production, stress responses or viability of the cells. They consist of a sensor or histidine protein kinase (HPK) and an effector or response regulator (RR). Nine TCSs have been identified in the *S. thermophilus* genome and classified by comparison of their deduced amino-acid sequences with previously identified proteins. Among those TCSs, a unique one (named BllH/R) is potentially involved in a quorum-sensing regulated mechanism. Analysis of the Bll locus revealed strong identity levels with the BlpH/K and ComD/E systems from *Streptococcus pneumoniae* respectively involved in bacteriocin production and development of natural competence. The organisation and possible functions of the BllH/R TCS will be presented and discussed.

COMMUNICATION BETWEEN THE CORE AND ACCESSORY REGIONS OF THE TNPI-IRS SITE-SPECIFIC RESOLUTION SYSTEM OF TN*4430*

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Transposon Tn*4430* from *Bacillus thuringiensis* encodes a DNA site-specific recombination system which functions to resolve cointegrate intermediates arising from its replicative mode of transposition. The recombination reaction is catalysed by the TnpI protein, a member of the tyrosine recombinases family. The internal resolution site of Tn*4430* (IRS) contains a recombination core region (IR1-IR2) and two directly repeated motifs, DR1 and DR2. The presence of DR1 and DR2 prevent intermolecular recombination events by restricting recombination to sites that are present on the same DNA molecule. The *in vitro* deletion product exhibits a specific topology (two-noded catenane), demonstrating that DR1 and DR2 contribute to the formation of a complex in which catalytically inert recombinase subunits act as architectural elements to control synapsis between the recombination sites.

In the present study, different approaches were used to further characterise the assembly and organisation of the TnpI-IRS recombination complex.

DNase I footprint analysis demonstrated that TnpI specifically binds to the four IRS motifs. In addition the digestion pattern showed hypersensitive sites, indicating that TnpI binding locally distorts DNA. Band shift experiments showed that TnpI bind the IRS site through a hierarchical and cooperative manner. Binding to both the IR1-IR2 core site and to the DR1 and DR2 accessory motifs is cooperative. TnpI affinity for these two IRS regions is in the same range, indicating that TnpI binding to the core and accessory sequences may occur independently. This is consistent with these two regions having separate role in recombination complex assembly.

To characterise the respective function of the IRS core and accessory motifs, a hybrid recombination site was constructed by replacing the DR1-DR2 region with the accessory sequences of the Xer recombination site *psi. In vivo* experiments revealed that *psi* accessory sequences are able to constrain TnpI-mediated recombination, conferring resolution selectivity to the hybrid site. Topology analysis of the products showed that the hybrid site primarily yields 4-noded catenanes. This result favours a model for the TnpI-IRS synaptic complex, in which 1 negative supercoil is trapped by the accessory motifs DR1 and DR2.

Communication between the core and regulatory sequences of the IRS was investigated by modifying the spacing between the two regions. Recombination demonstrated that the spacing, but not the phasing between IR1-IR2 and DR1-DR2 may be altered without affecting resolution selectivity.

THE EFFECT OF RAW-MILK CHEESE CONSUMPTION ON THE ENTEROCOCCAL FAECAL FLORA

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Enterococci reside in the human gastrointestinal tract. They are also naturally present in artisanal fermented foods such as cheeses. In clinical environments, enterococci have become one of the major causes of nosocomial infections. Considering this safety aspect, the use of enterococci as probiotics or their presence in food preparations is debated.

In the present study the composition and dynamics of the enterococcal faecal flora in healthy humans was analysed before, during and after the daily consumption of ~125g of a raw-milk Cheddar cheese (3.2×10^4 enterococci/g). Enterococcal counts in human faecal samples varied within subjects and from week to week. SDS-PAGE of whole cell proteins and PFGE were used to identify cheese and human enterococcal isolates at the species level and to type them to the strain level, respectively. The cheese mainly contained *Enterococcus casseliflavus* and a small sub-population of *Enterococcus faecalis*. Before and after consumption of the cheese, human samples contained mainly *Enterococcus faecium* with some of the strains being resident. During consumption of the cheese, human faecal samples were largely dominated by one particular transient clone of *E. faecalis,* originating from the cheese.

LACTATE DEHYDROGENASE-INDEPENDENT LACTIC ACID RACEMIZATION IN *LACTOBACILLUS PLANTARUM*

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Lactobacillus plantarum produces a mixture of D- and L-lactate, due to the presence of two NAD-dependent lactate dehydrogenases with opposite stereospecificities (LdhD and LdhL, respectively). This bacterium also possesses two stereospecific NAD-independent lactate dehydrogenases (LoxD and LoxL), enabling the bacterium to oxidise D- and L-lactate, respectively.

In this study we demonstrate that *L. plantarum* also possesses an additional, direct pathway for lactic acid racemization. Studies of lactate production from glucose and lactate racemization by Ldh-deficient strains show that this pathway is Ldh-independent and is strongly induced by L-lactate (about 500-fold increase in specific activity). Using Lox-deficient or over-expressing mutants, we show that this racemization system operates independently of either Ldh or Lox activities. We also provide evidence that the lactic acid racemization system of *L. plantarum* consists of a single lactate racemase, catalysing the direct inter-conversion of D- and L-lactate.

Attempts to purify the enzyme have been unsuccessful due to high instability of the lactate racemase. Therefore, global proteomic and transcriptomic analyses are currently being used in order to clone the lactate racemase gene, based on the fact that lactate racemase activity is strongly induced by L-lactic acid.

The physiological role of a lactate racemase in *L. plantarum* is unclear. This enzyme may play a role in lactic acid production, under conditions where LdhD is less active, ensuring that the bacterium produces D-lactate, which is involved in peptidoglycan biosynthesis. Alternatively, it could be involved in L-lactate utilisation at high pH.

GFP-TAGGING AS A TOOL TO TRACK THE DISSEMINATION OF THE 3-CHLOROANILINE-DEGRADATIVE PLASMID PC1 IN ACTIVATED SLUDGE

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Several reports in the literature indicate that the dissemination of a catabolic plasmid could be a potential bioaugmentation strategy in soil or activated sludge. The use of a suitable marker gene, as the gene encoding the green fluorescent protein (GFP) of *Aequorea victoria*, enables the tracking of a plasmid within such an ecosystem.
In this study, the 3-chloroaniline (3-CA) degrading plasmid pC1 of *Delftia acidovorans* CA28 was tagged with a mini-transposon containing the GFP gene and a kanamycin resistance gene. The labelled plasmid, designated pC1::*gfp*, was transferred to *Pseudomonas putida* UWC3 and the plasmid transfer from this donor to the bacterial community in activated sludge was studied. Conjugation experiments were performed with concentrated activated sludge on LB agar plates or directly in liquid activated sludge. Green fluorescent colonies appearing on mineral medium containing 3-CA as sole nitrogen source and kanamycin were picked up and verified to be true pC1::*gfp*-harbouring transconjugants. These isolates were subsequently identified using REP- and BOX-PCR genomic fingerprinting and partial 16S rDNA sequencing.

Repetitive element-PCR profiling revealed a large diversity in the transconjugant collection, indicating multiple plasmid transfer events. Remarkably, LB agar plate conjugations yielded a different set of transconjugants compared to conjugations in liquid activated sludge. From the plate matings, mainly *Aeromonas* sp. transconjugants were isolated, while *D. acidovorans* strains dominated the transconjugant collection from liquid activated sludge. Several transconjugants were shown to perform a rapid and complete (no aromatic ring structures present) 3-CA degradation. The latter observation suggests a potential for bioaugmentation, although more direct evidence for stimulated 3-CA degradation by dissemination of the pC1::*gfp* plasmid should be shown in experiments with lab-scale activated sludge reactors.

CHARACTERIZATION OF NOVEL FUNGAL CELLULASES AND HEMICELLULASES

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In recent years, there is an intensified interest in the microbial and enzymatic conversion of renewable raw materials into useful products, such as feed, chemicals and energy. Moreover enzymes, involved in these conversions, can find commercial applications in quite different fields. During a screening programme for grass (*Lolium perenne)* cell wall converting microorganisms, an interesting *Ascomycetes* fungus was isolated, which converted respectively up to 30% and 55% of the grass cell wall material after 7 days and 21 days of growth. This fungus might serve as an interesting source of cellulases and xylanases, since also other cellulosic and hemicellulosic substrates, such as microcrystalline cellulose, cotton, paper and xylan were hydrolyzed.

Depending on the type of substrate used as a carbon source, enzyme synthesis profiles differed considerably; not only did the level of enzyme activity differ, but also the type and mix of enzymes produced. Electrophoresis was performed to elucidate the molecular weight and the number of different enzymes and iso-enzymes involved during growth on different substrates. Further purification of the enzymes by column chromatography is now in progress.

GENETIC STABILITY AND AXENICITY IN THE BIOLOGICAL LIFE SUPPORT SYSTEM MELISSA

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For a lunar base or mission to Mars, it is essential to rely on a safe life support system including food and water supply, gas and waste management. Such life support systems are based on several regenerative techniques and processes that can consist of biological, physico-chemical or hybrid systems.

For this purpose the MELISSA concept was conceived by ESA to be the European model for ecological life support system applications. The system consists of a loop of interconnected bioreactors, which are envisaged to work as a complete unit, enabling the recycling of wastes and CO_2 generated in a close environment, in order to produce an edible material, water and O_2. The compartmentalised structure of MELiSSA makes the use of an engineering approach and hence a deterministic control of the ecological system possible. Proper functioning of the MELiSSA loop will be dependent on several parameters. However, bio-processes are usually subject to evolution and consequently the use of control systems are necessary for monitoring genomic and behavioral stability during long-term operation in the absence of and during accidental or continuous stress. Next to genomic stability of the MELiSSA organisms, the axenic condition is strictly necessary for the proper functioning and maintenance of a closed artificial ecosystem. One of the most important consequences of internal (from one compartment to the next) or external (nutrient harvesting, reactor maintenance) contamination is the possibility of horizontal gene transfer. Therefore the MELiSSA strains should be investigated for their ability to accept, donate and mobilize plasmids.

The general objective of this project is to establish and validate a method and its associated hardware to detect genetic instability, microbial contaminants and horizontal gene transfer in the MELiSSA compartments. This includes genetic description of the MELiSSA strains, microbial behavior under stress conditions, investigation of the ability of the MELiSSA strains to perform horizontal gene transfer, and detection of chemical, detection of genetical and biological contamination and their effect on microbial metabolism.

The methods used were designed to detect the effect of stress or the presence of contaminants at the cellular, proteomic as well as the genomic level. These include flow cytometry (FCM), proteomic analysis (matrix assisted laser desorption ionization – time of flight (MALDI-TOF), electrospray ionization tandem mass spectrometry (ESI-MS-MS), DNA chip technology, amplified (DNA) fragment length polymorphism (AFLP), total genome/plasmid DNA extraction and PCR. To investigate heterologous and homologous conjugation with the MELiSSA strains, classical gene transfer experiments and the method of exogenous isolation of mobilisable plasmids are used.

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MOLECULAR EPIDEMIOLOGY OF *SALMONELLA* CONTAMINATION OF BROILERS FROM HATCHERY TO SLAUGHTERHOUSE

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Salmonella is one of the major foodborne causes of gastroenteritis and frequently associated with contaminated poultry meat. It is important to know the different possible contamination sources and their relative contribution to the contamination of the end product. This can be accomplished by a combination of an extensive sampling plan and a polyphasic molecular typing approach.

From 1998 to 2000, *Salmonella* isolates were collected from 18 Belgian commercial broiler flocks, sampled both in the animals and in the environment, starting from the hatchery up to the slaughterhouse. The study included 7 different hatcheries, 17 different poultry farms and 9 different slaughterhouses. The isolates were serotyped and typed by pulsed field gel electrophoresis (PFGE) with the enzyme combination *XbaI, SpeI*, *NotI* and *BlnI*. *Salmonella* Enteritidis was additionally typed by RAPD with 4 primers and by plasmid profiling. Antibiotic resistance was determined with the E-test (Oxoid).

From the 18 broiler flocks during the rearing phase, 14 *Salmonella* serotypes were isolated. *Salmonella* Enteritidis was isolated from broken egg shells and from paper tray liners after transport of the one-day-old chicks from two different hatcheries. These isolates could be discriminated from each other by PFGE with *NotI*. In one of these flocks, the same *S*. Enteritidis genomic type I could be traced from the hatchery up to the carcasses in the slaughterhouse. In the other flock, the broilers were first contaminated with 2 other serotypes (*S*. Braenderup and *S.* Mbandaka) after 2 and 4 weeks, respectively, and then with *S*. Enteritidis after 6 weeks rearing. These *S*. Enteritidis isolates were from the same genomic type II as in the hatchery, but nevertheless showed a genetic variation by the acquisition of a megaplasmid (ca. 100 kb) carrying ampicillin resistance. In the slaughterhouse, carcasses from this flock were contaminated with this variant *S*. Enteritidis genomic type II as well as with yet another *S*. Enteritidis genomic type.

In 2 successive flocks reared in the same broiler house, the same *S*. Hadar genomic type I was dominantly found in the broilers during the whole rearing period. This *S*. Hadar genomic type I was already present in the environment of the farm before arrival of the one-day-old chick. In the environment and also in the poultry house before arrival of the one-day-old chicks of the second followed flock, another *S*. Hadar genomic type III was also present; hower this type was never found in the broilers and can thus be regarded as less virulent for chickens. It is evident that a circulation of contamination with a highly virulent *S*. Hadar type occurs on this farm.

On the slaughterhouse level, it was found that only for 5 flocks the same *Salmonella* strain was present on the carcasses as in the broilers during rearing. In the different slaughterhouses, more different serotypes (e.g. *S.* Indiana, *S*. Bredeney, *S*. Typhimurium, *S.* Paratyphi B) were found in the faeces of the transport crates and/or on the chilled carcasses than found during rearing. Some of the serotypes of the transport crates were also found on the carcasses, but some serotypes were only found on the carcasses. It is evident that the transport of broilers and the slaughterhouse environment are the major contamination sources for broiler carcasses, while the delivery of positive animals seems only to be of minor importance.

About 30% of the isolates were resistant to streptomycin, ampicillin, amoxillin or tetracycline, about 12% to nalidixic acid or trimethroprim/sulfamethoxazole. All *S*. Hadar isolates were resistant to at least 2 antibiotics and most of them to 3 to 5 antibiotics.

A. HYDROPHILA SUBSP. *DHAKENSIS* SUBSP. NOV. AND *A. HYDROPHILA* SUBSP. *RANAE* SUBSP. NOV., ISOLATED FROM DIARRHOEAL CHILDREN IN BANGLADESH AND FROM SEPTICAEMIC FARMED FROGS IN THAILAND, RESPECTIVELY.

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Recently, two groups of *Aeromonas* strains were received that clustered most closely to *A. hydrophila* DNA Hybridization Group (HG) 1 with AFLP fingerprinting although both groups seemed to constitute a separate genotypic subgroup in the latter species. To clarify their taxonomic position, representatives of both groups were subjected to a polyphasic taxonomic study. A first group of 10 strains referred to as group BD-2 mainly comprised haemolytic and cytotoxic isolates from diarrhoeal children in Bangladesh. Determination of DNA-DNA hybridization values with type and reference strains of all known *Aeromonas* taxa revealed that the BD-2 group represented a homogeneous taxon exhibiting 78-92% DNA relatedness with the type strain of *A. hydrophila* HG1. These results indicated that the BD-2 group belongs to *A. hydrophila* HG1, but examination of 152 phenotypic characteristics revealed that the group BD-2 isolates differed from HG1 in eight biochemical properties including utilization of urocanic acid and L-arabinose. Collectively, the reported genotypic and phenotypic data suggest that the BD-2 group represents a subspecies of *A. hydrophila*, for which the name *Aeromonas hydrophila* subsp. *dhakensis* subsp. nov. is proposed (Huys *et al*., 2002).

A second group of 7 sucrose-negative *Aeromonas* strains (referred to as group Au) were isolated from the internal organs of septicaemic farmed frogs (*Rana rugulosa*) in Thailand. Determination of DNA-DNA hybridization values with type and reference strains of all known *Aeromonas* taxa revealed that the Au group represented a homogeneous taxon (internal DNA homology, 93 to 103%) that exhibited the highest genomic relatedness with members of the two *A. hydrophila* subspecies ranging from 75-96%. Phenotypic characterization on the basis of 152 features further revealed that the Au group isolates differed from *A. hydrophila* subsp. *hydrophila* and/or subsp. *dhakensis* subsp. nov. in 13 biochemical properties including acid production from salicin and D-sucrose. From the results of this study, it can be concluded that the *Aeromonas* frog isolates of the Au group represent a new subspecies of *A. hydrophila*, for which the name *Aeromonas hydrophila* subsp. *ranae* subsp. nov. is proposed (Huys *et al*., in press).

GENOME STABILITY AND GENE FLUX IN *HELICOBACTER PYLORI*

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Whole genome clustering. We applied the GeneRage (1) algorithm to identify strain-specific genes of *Helicobacter pylori*. The algorithm constructs a binary matrix containing all similarity relationships from an all-against-all protein sequence comparison by BLAST. The matrix is then processed for transitivity relationships, using successive rounds of the Smith-Waterman dynamic programming algorithm. False relationships within the matrix as well as multi-domain protein families are detected. In all, 110 and 52 strain-specific genes from strains 26695 and J99, respectively, were obtained. This set of genes was compared to the sets obtained with other computational approaches of direct genome comparison as well as experimental data from microarray analysis. In a similar approach, 645 genes of strain 26695 and 558 genes of strain J99 were found to be species-specific in respect to *Campylobacter jejuni*. Interestingly, the majority of strain-specific genes (108 out of 110 in strain 26695 and 49 out of 52 in strain J99) do not have detectable homologs in *C. jejuni*.
Function prediction. All 162 strain-specific genes were analysed with GeneQuiz (2), confirming the 16 previous annotations and listing an additional 14 clear assigments.
Gene position analysis and directional statistics. We introduced a novel approach to gene position analysis by employing measures from directional statistics. Although the apparent differences with respect to strain-specific gene distributions, this is due to the extensive genome rearrangements. If these are taken into account, a common pattern for the genome dynamics of the two *Helicobacter* strains emerges, suggestive of certain spatial constraints that may act as control mechanisms of gene flux.
Compositional analysis (3). A reliable reference set of 78 strain specific genes was used, excluding very small genes (<300 bp) and pseudogenes. Correspondence analysis based on (G+C)-content and codon usage shows that the large majority, or 74 genes (95%), were likely acquired by gene transfer while only four genes (5%) were lost.
Conclusions. Genetic diversity in *H. pylori* seems to be largely determined by a small number of genes that are common to a particular population, but which are variously found in different subsets of strains within that population. This arrangement of a fixed core set and a flexible genepool is very similar to the 'species genome' concept for bacterial species and, keeping in mind its highly isolated lifestyle and natural competence for DNA uptake, likely enables the organism to adapt rapidly to environmental changes (i.e. dietary variation).

(1) http://www.ebi.ac.uk/research/cgg/services/rage/
(2) http://jura.ebi.ac.uk:8765/ext-genequiz/
(3) http://www.fut.es/~debb/HGT/

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S. Garcia-Vallvé, P. Janssen and C. Ouzounis. Genetic variation between *Helicobacter pylori* strains is mainly due to gene acquisition, not loss. *Trends in Microbiol.* (In Press).

POLYOL PRODUCTION IN *LACTOBACILLUS PLANTARUM* BY METABOLIC ENGINEERING

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Body-weight control is a major concern in developed nations, so new food products containing low-calorie sugars are requested by consumers. Polyols are non metabolisable sugar alcohols that could replace sucrose or glucose in food products with equivalent sweetness and taste.

In this context, *Lactobacillus plantarum* possess some interesting characteristics: it is largely found as the dominant species in the last step of natural food fermentation, and, the development of a mutant defective in both lactate dehydrogenases, *L. plantarum* VL103, facilitates the metabolic engineering of this strain.

The goal of the project will be to re-route the sugar flux from an intermediate of glycolysis (fructose-6P), in order to produce sorbitol (stl) and mannitol (mtl), by activating or enhancing its metabolism.

The sequence of the operons involved in stl and mtl catabolism in *L. plantarum* NCIBM8826 were analysed. The operons are compose of several genes with dehydrogenase, regulatory and transport activities. The *stl1D, stl2D* and *mtlD* genes, coding for the different polyol dehydrogenases were cloned under the control of a strong constitutive or inducible promoters (P_{ldh} and P_{nis}), and were introduce into *L. plantarum* VL103. The resulting strains were evaluated, under different growth conditions, for their capacity to produce stl and/or mtl. The results shown that this approach could be useful for the production of other products by re-routing the sugar fluxes from different intermediates of glycolysis.

PROMOTER ACTIVITY OF *LPNR 259/6*, A *LUXR* HOMOLOGUE IN *LEGIONELLA PNEUMOPHILA*, AND THE EFFECT OF GENE INACTIVATION AS REVEALED PROTEOME ANALYSIS

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Legionella pneumophila is a Gram-negative, facultative intracellular parasite of several protozoa and human macrophages. It is found ubiquitously in freshwater environments, free-living or associated in biofilms or in protozoa, which are the primary site for replication. *L. pneumophila* is the causative agent of Legionnaire's disease, a very severe form of pneumonia or of a milder, flu-like self-limiting disease named Pontiac fever.

The understanding of *L. pneumophila* pathogenesis is still rather limited although the number of genes important for infection and intracellular multiplication is constantly increasing. However, little is known about the regulation of these genes. The *L. pneumophila* genome contains four putative LuxR homologues which are known to be a superfamily of transcriptional regulators. These homologues are designated LpnR and are putative DNA-binding proteins since they contain a HTH-motif. A better understanding of the regulation of virulence factors is aimed through the characterisation of these LpnR proteins.

This communication is focussed on the characterisation of LpnR 259/6, one of the four LuxR homologues detected so far in *L. pneumophila*.

In a first instance, promoter activity was analysed with the aid of GFP. Therefore, the promoter located immediately upstream of *lpnR 259/6* was cloned in pBC-GFP. This construct was introduced both into *E. coli* TG1 and *L. pneumophila* serotype 1 using chemo-competent and electro-competent cells, respectively. Fluorescence was detected in each species by fluorometry as well as fluorescence microscopy. As such, it could be concluded that this promoter is active both in *E. coli* and in *L. pneumophila*. Fluorescence was measured at different time intervals to have an idea about the variation of activity of the promoter during growth.

Besides promoter analysis, the importance of LpnR 259/6 on overall protein expression was investigated by 2D-gel electrophoresis. Therefore, a deletion mutant of *lpnR 259/6* was made by replacing the gene by a kanamycine resistance gene. A comparison of the protein pattern between wild-type *L. pneumophila* serotype 1 and the deletion mutant of *lpnR 259/6* was carried out by means of 2D-gelelectrophoresis. This analysis revealed some differences in the protein pattern of the wild type and mutant strain. Identification of the relevant proteins will be carried out in the near future.

EVALUATION OF TWO DIFFERENT STRATEGIES FOR THE IDENTIFICATION OF STRESS RELATED GENES IN *ARTHROSPIRA PLATENSIS* AND *RHODOSPIRILLUM RUBRUM*

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The MELiSSA (Micro-Ecological Life Support System Alternative) loop, divided in four main interconnected bacterial compartments, has been designed to provide life support by food and water supply as well as, gas management and waste management in any extraterrestrial manned activities. Bacteria from the MELiSSA loop will be exposed to different kind of stresses linked to the space conditions (cosmic radiations, absence of gravity...) and to the fact that the loop itself is closed (accumulation of metabolites in the supernatants of each compartment...). Therefore, it is necessary to study the effect of space-related environmental and accidental stress on the gene expression of the MELiSSA strains in the perspective of the maintenance of the reactor stability of the loop.

Here, we present two strategies designed to identify stress-regulated genes in *Arthrospira platensis* and *Rhodospirillum rubrum*, the two MELiSSA loop photosynthetic (and potential food sources) bacteria of compartments IV and II, respectively.

In a first step, axenic cultures were grown and maintained in order to extract DNA and proteins from each strain. The absence of contaminants was controlled by flow cytometry.

In a second step, two main strategies were carried out on the basis of stress proteins identification and their corresponding genes. 1) As a targeted strategy, degenerative PCR experiments were performed after selection of degenerate primers from sequence alignment of homologous genes present in genomic databases. 2) As a global approach, a proteomic approach was used to identify proteins specifically synthesized under thermic and oxidative stress.

The genomic approach did not give the expected results for both strains mainly because of the too high degeneracy of the primers. On the contrary, the proteomic approach based on mass spectrometric analysis of 2D-PAGE gel-separated proteins gave promising results for *R.rubrum*. Seven proteins were over expressed during thermic or oxidative stress. During thermic stress conditions (43°C, 2h15), proteins as DnaK, GroEL and GroES were clearly recovered. During oxidative stress conditions (50µM H_2O_2, 1h25) the expression of Thioredoxine, Alkylhydroperoxide reductase subunit c, HtpG and RecA was induced. The proteins were identified using ESI-MS-MS mass spectrometry and could subsequently lead to the corresponding gene sequences using appropriate primers. For *A.platensis*, the overexpressed proteins still have to be identified.

In this study, the limit of the degenerated approach was shown in the case of non sequenced micro-organisms. With the proteomic approach, it was possible to assess which gene was important in the stress response. Thereafter, the genomic sequence could be deduced and the stress induced genes identified.

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REP-PCR FINGERPRINTING FOR IDENTIFICATION OF *BIFIDOBACTERIUM* SPECIES

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Bifidobacteria inhabit the gastro-intestinal tract of humans. They are the dominant genus within the microbiota of infants and remain the third most common genus as humans age. Their role in public health has long been recognized, resulting in the use of some members of the genus as probiotics. Currently over 30 species of *Bifidobacterium* have been validly described and the taxonomic position for some of these taxa has been heavily debated. Although various methods have reported for the identification of bifidobacteria, many of them turn out to be time-consuming, laborious and do not permit the differentiation of closely related species. In the present study, the applicability of rep-PCR fingerprinting was assessed for identification of a wide range of *Bifidobacterium* species. For this purpose, several primersets (GTG_5, BOX, ERIC and REP) targeting repetitive DNA elements were evaluated on a subset of representative strains. Based on discriminatory power, yield of complex patterns and the distribution of bands within a pattern the BOX primer was found to be the optimal choice for the establishment of a reference framework comprising a broad taxonomic range of bifidobacteria. We were able to differentiate 26 of the 31 included species. The remaining taxonomic inconsistencies within the framework were subsequently analysed with 16S rDNA sequence analysis. We are currently testing the method on a collection of bifidobacterial isolates from probiotic dairy products. In conclusion, rep-PCR fingerprinting using BOX primers is a promising tool for differentiation of a wide range of bifidobacteria at the (sub)species level.

Keywords: *Bifidobacterium*, Identification, Rep-PCR

EFFECT OF SUPERINFECTION DELAY ON PRODUCTION OF BOVINE HERPESVIRUS 1 RECOMBINANTS

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Recombination between different strains of a same alphaherpesvirus after co-infection has been already described both *in vitro* and *in vivo*. In order to evaluate the effect of a temporal delay between infections, epithelial bovine kidney cells (Madin Darby Bovine Kidney, MDBK) were infected with different viruses. In the first experiment, cells were infected with the ST strain of bovine herpesvirus 1 and co-infected or superinfected with a recombinant ST strain which expressed β-galactosidase gene in place of glycoprotein E gene ORF (STBG). In the second experiment, cells were infected with the Lam strain deleted for glycoprotein C ORF and co-infected or superinfected with the Lam strain deleted for glycoprotein E ORF. After incubation, cells of the first experiment were analysed by pulsed field electrophoresis to detect the presence of mixed replication intermediates specific of intramolecular recombination events. In the second experiment, progeny viruses were analysed by double immunofluorescence staining (gC, gE) to determine the percentages of each parental and recombinant strains (gC+gE-, gC-gE+, gC+gE+, gC-gE-). Mixed recombination intermediates ST/STBG were not detected when superinfection occurred 4 hours after infection. Analysis of progeny viruses by immunofluorescence shows an important decrease and an absence of production of recombinant and gE- viruses when cells are superinfected respectively 6 or 8 hours after infection by the gE- strain. These results demonstrate that the delay of superinfection has an important effect on the recombination process.

DISTRIBUTION, DIVERSITY AND POTENTIAL BIOLOGICAL ROLE OF CEREOLYSIN O IN THE *BACILLUS CEREUS* GROUP

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Bacillus cereus sensu lato comprises six closely related bacteria displaying a broad range of virulence spectrum, from the food contaminant and opportunist *B. cereus* (Granum & Lund, 1997) to the entomopathogenic *B. thuringiensis* (Ash *et al*., 1991). Beside the specific virulence factor (e.g. the crystal endotoxins of *B. thuringiensis*), these bacteria possess a large arsenal of others virulence factors (hemolysins, phospholipases or enterotoxins), whose role may be determinant in setting up and/or maintaining non-acute pathogenesis. This research focused on the contribution of one of these factors, the sulfhydryl-activated cytolysin (Alouf, 1999) Cereolysin O (CLO), to the *B. cereus* pathogenic arsenal. More than 80 *B. cereus s.l.* strains originating both from reference collections and natural sources have been analysed. Using PCR, the *clo* gene was shown to be present in all strains, regardless of the bacterial species. The diversity of the *clo* genes, analysed by RFLP, indicated a good conservation among the different strains. However, the most striking observation was certainly the presence, in at least 15 % of the strains, of more than one gene copy. Sequencing of a total of 13 *clo*-derived PCR products from 7 strains revealed a conservation varying between 90 to 99 % among themselves, as compared to the 75 % identity shared with the alveolysin gene of *Paenibacillus alvei. Clo* knockout experiments, as well as cloning and expression of *clo* in *Bacillus subtilis* have been performed to determine the relative contribution of this toxin to the cytolytic activity of these bacteria towards various target cells. Analysis of the *clo* genetic context in relevant strains also revealed striking variations in the immediate vicinity of the genes.

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METAL RESISTANCE OPERONS (OF THE RND EFFLUX FAMILY) IN *R. METALLIDURANS CH34*

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Ralstonia metallidurans strain CH34 is a natural isolate found in the sediments of a Belgian zinc metallurgical plant. This strain possesses a large diversity of heavy metal resistance genes located on its large plasmids pMOL28 (180kb) and pMOL30 (280kb) but also on the chromosome. The main resistance mechanism (*czc* on pMOL30 and *cnr* on pMOL28) is mediated by the RND system (Resistance Nodulation and cell Division) subfamily RND-HME for Heavy Metal Efflux. This system, a tricomponent efflux cation/proton antiporter system, is composed of an outer membrane protein (OMF family), an inner membrane protein (RND family), and a transperiplasmic one (MFP family). The sequencing of the genome carried out by the Joint Genome Institute in California and released on their internet web site: http://www.jgi.doe.gov/JGI_microbial/html/ralstonia/ralston_homepage.html, allows us to complete our information about heavy metal resistance on the plasmids but also on the chromosome.

To our surprise, "in silico" analysis allowed at least to identify 12 *czcA/cnrA* paralog/ortholog proteins: 1 in pMOL28, 3 on pMOL30 and 8 on the chromosome. This high number of *czc*-like cluster regarding to only 0 to 4 found in 64 procaryotic genomes, and particularly of *czcA* paralogs genes, supports the specialisation of *R.metallidurans* in the adaptation to metal-rich biotopes. It is not yet possible to assign functions or metal specificities for most of them. But, using phylogenic analysis and multi-alignment motif search of the *czcA* paralogs, the first representative of this family, we found the 5 different classes of Heavy Metal Resistance genes (RND-HME) with various metal specificity that were described by D. H. Nies. Two operons of *R. metallidurans* are classified in HME1 group (basic *czc* gene) conferring cobalt, zinc, and cadmium resistance one on pMOL30 and one on the chromosome. Two genes and the corresponding operons are classified in the HME2 group conferring nickel resistance. Six operons of *R. metallidurans* are classified in HME3 group, subdivided in HME3a (3 operons) mostly involved in divalent heavy metal cations efflux and HME3b (3 operons) involved in monovalent heavy metal cations efflux. The last class of heavy metal resistance HME4 in *R.metallidurans* comprises two additional operons mostly involved in copper and/or silver efflux. Focusing on the RND regulatory mechanism, at least 7 two-component regulator/sensor (*czcRS)* are associated with genes of the RND family, including 5 RND-HME (1 on pMOL30 and 4 on the chromosome). Moreover, a large diversity in *czc*-like *RS* organisation is observed with respect to their orientation regarding the other genes of the cluster: divergently expressed or organized in operon where both genes are sometimes overlapping.

Recently, the crystallography structure of AcrB, an *E.coli* multidrug resistance pump (paralog to *czcA*) was released. Further studies on the 12 RND-HME of R. metallidurans will be based on the comparison with this structure as well as on genetic (mutant) and proteomic studies (Noël-Georis and al., Global analysis of the *Ralstonia metallidurans* proteome: prelude for the large scale study of heavy metal response, in preparation).

ACCUMULATION OF COMPATIBLE SOLUTES IN *BREVIBACTERIUM* SP.

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Compatible solutes are small organic molecules that can be accumulated in the cytoplasm of microorganisms, where they are responsible for the regulation of the osmotic balance. These compounds are also able to protect and stabilise enzymes, nucleic acids and membranes against a variety of stress factors, and osmotic stress in particular (Galinski, 1995).
In the genus *Brevibacterium* the ectoines (tetrahydropyrimidines) are the main compatible solutes (Bernard *et al.*, 1993; Frings *et al.*, 1993). Intracellular ectoine concentrations are influenced by the osmotic pressure of the cell environment (Nagata & Wang, 2001).
In present work, the effect of an increased osmolarity on growth and accumulation of ectoine in *Brevibacterium* sp. was evaluated. Both electrolytes, NaCl and KCl, and non-electrolytes, sucrose and D-sorbitol, were tested; these compounds all have a similar effect on medium osmolarity.
Growth of *Brevibacterium* sp. on MSGYE medium supplemented with 0 – 3 M NaCl was followed. The osmotic pressure developed by these media ranged from 0.6 osM (0 M NaCl) to approximately 6.7 osM (3 M NaCl). The growth rate was the highest in media containing between 0 en 1M NaCl (generation time 10-11 h); above 1M NaCl growth rate and extent were drastically reduced. Replacement of NaCl by other electrolytes such as KCl did not induce significant changes in the growth pattern. Addition of the non-electrolytes sucrose and D-sorbitol had a slightly stronger inhibitory effect on growth, but also here growth rate and extent were still relatively normal up to 1 M of these compounds.
The influence of the electrolytes NaCl and KCl on the intracellular accumulation of ectoine was evaluated. Two different responses to increasing salt concentrations are observed. For NaCl and KCl concentrations allowing good growth, intracellular ectoine accumulation increases linearly with the osmolarity. At higher osmolarities, ectoine concentrations do not reach the level we expected according to this linearity. This probably explains the growth inhibition at these osmolarities.
An increase in osmolarity induced by the addition of non-electrolytes such as sucrose and D-sorbitol, does not lead to the intracellular accumulation of ectoine in *Brevibacterium* cells. This can be explained in two ways: The synthesis of ectoine can be induced by the presence of salts themselves rather than indirectly by the medium osmolarity. It is also possible that the cells prefer to accumulate sucrose or D-sorbitol from the medium to balance the external osmotic pressure rather than spending energy on the synthesis of ectoine. However, this last hypothesis has to compete with literature findings claiming that these compounds have only very limited potential as osmoadaptors (Galinski, 1995). Probably, a combination of the effects of these compounds with the natural level of the more potent compatible solute ectoine is sufficient to support normal growth up till 3,5 osM.

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GENETIC STUDY OF THE FUNCTIONAL ROLE OF D-ALANINE SUBSTITUTIONS OF TEICHOIC ACIDS IN *LACTOBACILLUS PLANTARUM*

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Lipoteichoic acids (LTA) and cell wall teichoic acids (WTA) represent a major constituent of the Gram-positive cell wall. They consist of polyglycerolphosphate or polyribitolphosphate substituted with D-alanine residues whose amino groups partially compensate for the negative charges of the phosphates. As a first step towards elucidating the function of D-alanine substitutions, the *dlt* genes responsible for teichoic acid D-alanylation in *L. plantarum* were cloned. The 4 *dlt* genes (*dltA*, *dltB*, *dltC* and *dltD*) were found to be clustered, as already observed in other Gram-positive species. In addition, a *pbpX* gene encoding a protein homologous to low molecular weight penicillin-binding proteins (PBP) was found to adjoin the *L. plantarum dlt* cluster. Northern blotting analyses using *dltA* and *pbpX* probes demonstrated that the 5-gene cluster was transcribed as a single polycistronic mRNA.
A *dltB* minus strain unable to incorporate D-alanine in teichoic acids was constructed. In this strain, *pbpX* and *dltA* probes hybridized with a smaller transcript, while no transcript was detected when using *dltD* probe suggesting polar effects on expression of *dltC* and *dltD*. The growth of *dltB* mutant is affected, the curve reaches a lower maximal value in OD and displayed a decrease in stationary phase which might be the result of autolytic activity. However the relation between autolysis and D-alanine absence in teichoic acids is not well understood. It might be a consequence of the more negative character of teichoic acids due to the absence of D-alanine, as the cationic autolysins might bind more tightly to D-alanine deficient teichoic acids. It might also be an indirect effect of modifying the ionic environment of autolysins in the cell wall. Demonstration of an increased anionicity of the mutant cell wall was gained from the observation of an enhanced binding of the cationic protein cytochrome c. Further evidence for charge modification on TA was sought by assaying the sensitivity of the *dlt*$^-$ mutant to the cationic antimicrobial compound nisin. The *dltB* $^-$ strain turned out to be 16 times more sensitive to nisin as compared to the control strain. We characterized the cell surface by physicochemical approaches. X-ray photoelectron spectroscopy (XPS) analysis showed a decrease of protonated nitrogen at the cell surface, in agreement with the loss of D-alanyl ester substituents. The effect of *dltB* mutation on cell morphology was also observed by microscopy. In our preliminary observations, the mutant showed longer cells and filaments compared to the WT which suggest a septation problem. In order to obtain a genetic demonstration of the implication of an autolysin in the OD decrease occuring in stationary phase, we inactivated an homologous to AcmA autolysin of *Lactococcus lactis*, (that we called *lpa),* in the *dlt* mutant. The single *lpa* mutant of *Lb. plantarum* was constructed. It is forming chains suggesting a cell separation problem but the growth curve is similar to the WT strain. Interestingly, the *dlt lpa* double mutant doesn't show any OD decrease in stationary phase anymore but the growth defect is not completely repaired. In addition, morphological phenotype of *dlt lpa* mutant seemed to be a combination of both single phenotypes (*dlt* $^-$ and *lpa*$^-$) : Cells are forming chains containing filaments and longer cells.

APPLICATION OF *LEUCONOSTOC MESENTEROIDES* MANNITOL DEHYDROGENASE FOR THE ENZYMATIC PRODUCTION OF D-MANNITOL

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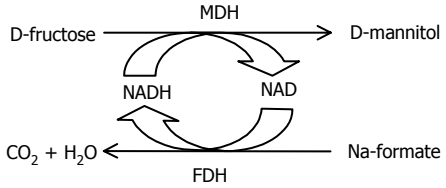
D-Mannitol, a naturally occurring six-carbon sugar alcohol, is usually found in small quantities in most fruits and vegetables and it is used as a sweet builder in food and various pharmaceutical products. Nowadays D-mannitol is mainly produced by metal-catalysed hydrogenation of D-fructose. A very efficient fermentative method for D-mannitol production with great selectivity was developed by Soetaert et al. (1995). However with both methods byproducts are formed, D-sorbitol in the chemical process and acetic acid and lactic acid during the fermentation (Vandamme & Soetaert, 1995). In this respect, a purely enzymatic method for D-mannitol production with *Leuconostoc mesenteroides* ATCC 12291 mannitol dehydrogenase (MDH) extract has been considered. The pH-optimum for D-fructose reduction was situated around pH 5.5 and the enzyme was stable within the pH-range of 6.5 to 8.5. D-Fructose reduction was optimal at 50 °C and loss of enzymatic activity was observed after heating for 10 minutes at 60°C. The crude *Leuconostoc* enzyme extract had a rather high degree of substrate specificity.
Since the enzyme is NADH-dependent, an efficient coenzyme regeneration system is needed for the production of D-mannitol. In this context, formate dehydrogenase (FDH) is an interesting enzyme to be used in a coupled enzyme reaction, since no byproducts are formed in the reaction mixture (Slatner et al., 1998).



The reaction was performed in a 0.1 M Bis-Tris buffer pH 6.5 at 25°C. D-Fructose was converted to D-mannitol and Na-formate was converted to CO_2 and water at the same rate. The optimal substrate concentration was 100 mM for D-fructose and Na-formate. At a coenzyme concentration of 0.5 mM (>>Km (Km_{MDH}(NADH) = 0.024 mM, Km_{FDH}(NAD) = 0.029mM)) the enzymes were saturated with NAD(H), such that a further increase in coenzyme concentration is, from an economic point of view, not needed.

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EXPRESSION OF THE YEAST *SACCHAROMYCES CEREVISIAE* γ-GLUTAMYLTRANSPEPTIDASE REQUIRES NITROGEN REGULATORY NETWORK INCLUDING GATA ZINC FINGERS GLN3, NIL1/GAT1 AND GZF3

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CIS2 gene in the yeast *Saccharomyces cerevisiae* codes for γ-glutamyltranspeptidase (□-GT) the principal glutathione - degrading enzyme. The promoting region of *CIS2* gene contains eight GAT(T/A)A sequences likely involved in the nitrogen regulated transcription and one copy of the STRE sequence (5'-CCCCT-3' stress response sequence. Expression of *CIS2* was shown to be regulated according to the nature of the nitrogen source provided to the yeast cells. The expression was high for cells growing on a medium containing a poor nitrogen source such as urea. Under this condition, both GATA zinc finger Nil1 and Gln3 were required for *CIS2* expression but Nil1 appeared as the most important factor. Gzf3, another GATA-zinc finger protein, inhibited the expression of *CIS2* on urea but not on L-glutamate, indicating that its action depended on the nitrogen source. When cells were grown on a preferred nitrogen source like an ammonium salt, *CIS2* expression was repressed through a mechanism involving the Gln3-binding protein Ure2/GdhCR. We have also shown that the TOR signalling pathway was involved in activation of *CIS2* expression via Gln3 and Nil1factors that occurred when the yeast was deprived from nitrogen. Finally, *CIS2* expression data show that γ-GT biosynthesis in *S. cerevisiae* is only induced by nitrogen deprivation but not by different other stress situations.

GENOTYPIC DIVERSITY OF CYANOBACTERIA IN MICROBIAL MATS FROM COASTAL LAKES IN EASTERN ANTARCTICA

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In the Antarctic biota, cyanobacteria are the dominant phototrophs. The richest biomass accumulation of cyanobacteria occurs in the benthic habitats of lakes and ponds where they form microbial mats. Until now, their genotypic diversity has not been determined.

In the frame of the OSTC project LAQUAN (EV01), molecular techniques based on rDNA sequences were used to study the cyanobacterial diversity of benthic microbial mats samples collected in 20 lakes of the Eastern Antarctic coast. The lakes were selected to cover a wide range of chemical environments and to obtain a picture as complete as possible of the microbial diversity.

A genotypic fingerprint of the samples was obtained by the DGGE technique (Denaturing Gradient Gel Electrophoresis) and 30 DGGE bands were sequenced. Each lake is characterized by a particular DGGE pattern, with only a few bands in common.

In addition, 5 clone libraries were constructed for selected samples collected from the lakes Reid (Larsemann Hills), Ace (Vestfold Hills), Rauer 8 (Rauer Islands), and Heart (Larsemann Hills). For the 3 first lakes, the clones were grouped by the ARDRA technique (Amplified Ribosomal DNA Restriction Analysis) and 130 partial 16S rDNA sequences were obtained for representatives of all the ARDRA clusters. For Heart lake, all the 56 cyanobacterial clones isolated were sequenced.

The 246 clones containing a cyanobacterial insert belonged to 27 phylotypes (16S rDNA similarity higher than 97.5%, E. coli positions: 405-780). The cyanobacterial communities of the studied lakes appeared quite different, 20 phylotypes being unique to the lake where they were found. However, the lakes Heart and Reid shared 6 phylotypes out of the 17 present in these 2 lakes. Only 1 phylotype out of 10 was shared by lakes Ace and Rauer 8. These variations in the genotypic cyanobacterial diversity are probably reflecting the heterogeneity of the ecological characteristics of the lakes (salinity, pH, depth, ice cover,…) in this coastal region of Eastern Antarctica.

The phylogenetic analysis also revealed the existence of 'Antarctic clusters', sequences from putatively novel or yet unsequenced organisms, and a high genotypic diversity of cyanobacteria belonging to the Oscillatoriales order, that have a simple filamentous morphology.

CULTURE-INDEPENDENT ANALYSIS OF PROBIOTIC PRODUCTS USING DGGE

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In order to obtain functional and safe probiotic products for human consumption, a fast and reliable quality control of these products is crucial. Currently, analysis of most probiotics is still based on culture-dependent methods involving the use of specific isolation media and the identification of a limited number of isolates, which renders this approach relatively insensitive, laborious and time-consuming. In the current study, a collection of ten probiotic products including four dairy products, one fruit drink and five freeze-dried products were subjected to microbial analysis using a culture-independent approach in comparison with conventional culture-dependent analysis. The culture-independent approach involved extraction of total bacterial DNA directly from the product, PCR amplification of the V3 region of the 16S rDNA, and separation of the amplicons on a Denaturing Gradient Gel Electrophoresis (DGGE) gel. Digital capturing and processing of DGGE band patterns, allowed direct identification of the amplicons on the species level. This whole culture-independent approach can be performed in less than 30 hours. In comparison with culture-dependent analysis, the DGGE approach was found to have a much higher sensitivity for the detection of microbial strains in probiotic products in a fast, reliable and reproducible manner. Unfortunately, as reported in earlier studies using the culture-dependent approach, a rather high percentage of probiotic products suffered from an incorrect label and yielded low bacterial counts, impairing beneficial probiotic effects.

Keywords: Probiotic Products, DGGE, Identification

CHANGES IN BACTERIAL METABOLISM DURING BIOFILM-ASSOCIATED INFECTIONS COULD EXPLAIN AT LEAST PART OF THE BIOFILM-RESISTANCE

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Objectives: Foreign body associated infections (FBI) are a major cause of morbidity and mortality. During FBI, bacteria embed themselves in a biofilm. Although antibiotics usually suppress the symptoms of the FBI, definite eradication generally requires removal of the device. The exact cause of the persistent nature of FBI remains unclear. A state of reduced bacterial metabolism is hypothesised as an important contributing factor. This hypothesis that the sessile growth mode induces a metabolically slowed state during chronic *in vivo* FBI was examined in *Staphylococcus epidermidis* infections.

Methods: The expression of the *16S* rRNA gene (as a measure of total protein synthesis and growth rate) and of three other housekeeping genes (guanylate kinase, triosephosphate isomerase and *hsp-60*) was quantified with RT quantitative PCR as the cDNA/gDNA quotient as previously described (BBRC, 2002). Gene expression was examined *in vitro* in planktonic (101 samples) and sessile (106 samples) bacteria and *in vivo* in sessile bacteria (253 samples) during the first two weeks of foreign body infection in a rat model.

Results: Housekeeping gene expression differed markedly between sessile versus planktonic bacteria harboured under the same conditions, with the expression of the *16S* and *gmk* being significantly higher and of the *hsp-60* being significantly lower in the sessile bacteria. Initial foreign body colonization is an active process (5-fold increase in *16S* expression, $p<0.0001$) that induced major metabolic changes in bacteria both *in vitro* and *in vivo*. The bacteria recovered from chronic foreign body infection *in vivo* have entered a state of reduced metabolic activity (129-fold decrease in 16S expression, $p=2.1\times10^{-28}$).

Discussion: Housekeeping gene expression differs between planktonic and sessile bacteria harboured under the same conditions from the early foreign body colonization on. During early FBI bacteria have an enhanced metabolic activity, which may render them more susceptible to the effects of antibiotics that act on targets highly expressed during this phase. In clinical practice, this is illustrated by the efficacy of minocycline-rifampin coated catheters in the prevention of catheter-related infections and the efficacy of prophylactic antibiotics in surgery. The state of a markedly reduced metabolism reached during chronic foreign body infections *in se* may protect the biofilm embedded bacteria against the effects of most antibiotics.

EARLY BUT NOT LATE *IN VIVO* FOREIGN BODY ASSOCIATED GROWTH PROVOKES A TRANSIENT PEAK IN *ICAC* GENE EXPRESSION IN S*TAPHYLOCOCCUS EPIDERMIDIS*

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Background: Biofilm forming coagulase negative-staphylococci (CNS) are the main cause of foreign body (FB) associated infections. The *ica*-operon is involved in the pathogenesis of biofilm-associated CNS infections. The aim of this study was to investigate the expression of the *icaC* gene during the course of *in vivo* foreign body infections.

Methods: 205 catheter segments were inoculated with *S. epidermidis* and implanted subcutaneously in rats as previously described. Catheters were explanted after 0 (N=10), 15 minutes (N=18), 60 (N=18), 120 (N=17), 240 (N=18), 360 (N=18), 720 (N=18) minutes, 1 (N=18) and 2 days (N=18) and 1 (N=24) and 2 weeks (N=28). Gene expression on the catheters was determined using a previously described method of instantaneous RNA and DNA isolation from the adherent bacteria, combined with Taqman quantitative PCR and given by the cDNA/gDNA ratio (J.Bact, 2001 and BBRC, 2002).
Results: Initial *in vivo* foreign body colonization induced a sharp 6-fold increase in *icaC* expression peaking after 60 minutes. Thereafter, a progressive decline in expression was noticed to reach very low levels after 1 and 2 weeks ($p<0.0001$ for evolution; 1-way ANOVA). Maximum mean expression (-0.95 log10cDNA/gDNA at t=60 min) was 1.95 log10 or 82 times higher than minimum mean expression (-2.90 log10 cDNA/gDNA at t=2 weeks; $p<0.0001$, Bonferroni test).

Conclusion: *In vivo* gene expression experiments confirm the important role of the *ica*-operon in foreign body colonization previously established by mutagenesis and epidemiological surveillance. The *ica*-operon is mainly expressed during initial colonization and far less during late *in vivo* foreign body associated growth. These results suggest that the *ica*-operon is more important in the establishment than in the maintenance of a biofilm. This is consistent with the presumed function of the *ica*-operon in the establishment of intercellular connections.

CONTROL OF DIRECTIONALITY IN THE RECOMBINATION REACTION CATALYSED BY THE TYROSINE RECOMBINASE TNPI OF TN4430

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The transposon Tn*4430* from Bacillus thuringiensis is a member of the Tn3 family that encodes a tyrosine recombinase (TnpI) to resolve cointegrate transposition intermediates. The internal resolution site of Tn4430 (IRS, 116 bp) contains two inverted repeats (IR1-IR2) that form the core recombination site, and two additional TnpI DNA binding motifs (DR1 and DR2) that are directly repeated downstream to the core. Both in vivo and in vitro studies have shown that the presence of DR1 and DR2 provides resolution selectivity to the recombination reaction by stimulating recombination between directly oriented sites on the same DNA molecule. The In vitro deletion product exhibits a specific topology (two-noded catenane), indicating that the accessory motifs DR1 and DR2 contribute to the formation of a specific synaptic complex in which catalytically inert recombinase subunits act as architectural elements to control pairing of the recombination core sites. In this study, we investigated the relative contribution of the IRS core and accessory regions in controlling the directionality of the recombination reaction.
TnpI cleavage at the core site was first examined using linear suicide substrates carrying a central nick in the 6-bp spacer between IR1 and IR2. Cleavage reactions were also performed with a perfectly symmetrical core-derived substrate. Kinetics analysis showed that both DNA strands were cleaved with similar efficiencies, albeit with a slight preference for the bottom strand (cleavage at IR2) in the case of the wild type core site sequence.

The order of strand exchange was also investigated on supercoiled recombination substrates carrying the wild type or the symmetrical core site in different orientations with respect to the accessory motifs DR1 and DR2. These plasmids were reacted in vitro, and the Holliday junction intermediates were isolated, labelled and analysed by restriction digest. For all substrates analysed, only one specific pair of DNA strands was found to be exchanged. In all cases, strand exchange was catalysed by the TnpI subunits bound to the distal core motif relative to the accessory sequences (i.e.; IR1 for the wild type IRS).

These results demonstrate that the IRS accessory motifs control the order of strand exchange by imposing that the distal core site recombinases are first activated for the DNA cleavage and rejoining reactions. This supports a model for the organisation of the TnpI/IRS complex in which the C-terminal domains of the core sites-bound TnpI tetramer point toward the accessory motifs-bound subunits in the synpatic complex.

QUORUM SENSING IN SURFACE-ADHERENT GRAM-NEGATIVE BACTERIA ISOLATED FROM AN INDUSTRIAL KITCHEN

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Many species of bacteria are capable of regulating gene expression in response to their population density by a mechanism known as quorum sensing. This cell-to-cell communication is based on the production of low molecular-mass extracellular molecules (autoinducers), which can function as a signal triggering expression or repression of specific genes, when their concentration reaches a certain threshold. To reach this critical concentration, the bacterial population must grow to a certain cell density. We have examined 68 isolates of gram-negative bacteria from food-contact surfaces in a raw vegetable washing and cutting process line for the ability to form biofilms and for the production of different classes of known signalling molecules. A series of reporter strains was used to demonstrate the production of (*i*) N-acyl-homoserine lactones (AHLs), signalling molecules in LuxR-LuxI type quorum sensing; (*ii*) autoinducer 2 (AI-2), first identified as one of the two autoinducers in *Vibrio harveyi* and later in many other gram-negative and also gram–positive bacteria and (*iii*) 2-heptyl-3-hydroxy-4-quinolone (PQS), produced by *Pseudomonas aeruginosa*. AHLs of different specificity (different acyl chain) as well as AI-2 are produced by several of the isolated strains, indicating that these types of cell-to-cell signalling are widespread among surface-adherent gram-negative bacteria in food process lines.

GENOMIC CHARACTERISATION OF FIVE NOVEL SPECIES IN *BACILLUS*, ISOLATED FROM THE DRENTSE A GRASSLANDS (THE NETHERLANDS)

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In a preliminary study, analysis of soil samples from the Dutch Drentse A grassland area via Temperature Gradiënt Gel Electrophoresis of the directly extracted 16S rDNA revealed a prominent group of low % G + C, Gram-positive organisms clustering around *Bacillus benzoevorans*. This group was called the BACREX-cluster, and a culture campaign resulted in the isolation of more then 100 bacterial isolates belonging to it. As part of the EU-project QLK3-2000-01678 (http://mik.gbf.be/bacrex.index.html), 94 of them were studied in a polyphasic taxonomic characterisation. In a first approach the isolates were grouped by rep-PCR fingerprinting (using REP- and GTG5-primers). The resulting patterns were yet too heterogeneous to clearly delineate groups. Therefore, all isolates were subjected to a partial 16S rDNA analysis of the first 400-500 bp. Comparison of the grouping obtained by both techniques, allowed the delineation of 42 isolates into six groups of genomically similar strains. A more complete 16S rDNA sequence of representatives of these groups were determined and a FASTA search with the EMBL database showed that the isolates from all six of these groups are closest related to *Bacillus niacini* (with similarity % varying from 97-99). Further DNA-DNA relatedness studies between representative isolates of the six groups with each other and with *B. niacini* resulted in the delineation of five genospecies. Preliminary results of phenotypic analysis indicate that these five genospecies probably represent five new species within *Bacillus*. Formal description is awaiting completion of phenotypic characterisation.

FLAVOBACTERIUM GELIDILACUS SP. NOV., ISOLATED FROM MICROBIAL MATS IN ANTARCTIC LAKES

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During the MICROMAT-project (November 1998-February 2001) the diversity of heterotrophic bacteria in microbial mats from 10 Antarctic lakes in 3 different regions (Vestfold Hills, McMurdo Dry Valleys and Larsemann Hills) was investigated. Almost 750 heterotrophic bacterial strains were isolated and characterized using fatty acid analysis and 16S rDNA sequence analysis of representative strains (Van Trappen *et al.*, in press).

A group of twenty-two isolates, related to the *Cytophaga-Flavobacterium-Bacteroides* branch and isolated from microbial mats in the east-Antarctic lakes Ace, Watts, Reid and Pendant showed similar fatty acid compositions and were further investigated in a polyphasic taxonomic approach. 16S rDNA sequence analysis placed two representative strains within the genus *Flavobacterium*, with 95 % sequence similarity to *Flavobacterium flevense* and *Flavobacterium tegetincola*, less to other *Flavobacterium* species and less than 90 % to representatives of other genera. Repetitive extragenic palindromic DNA-PCR fingerprinting (using the GTG_5-primer) was performed on the twenty-two strains and three profile types could be detected. DNA-DNA hybridisations between 5 representatives showed more than 87% reassociation to each other, confirming that the 22 strains they represent are belonging to a single species.

The molar G+C content of the DNA of the strains is 30 %. The strains show the typical morphological characteristics of *Flavobacterium* and can be differentiated from related *Flavobacterium* species by several phenotypic characteristics.

The results of the polyphasic analysis support the creation of a new species within the genus *Flavobacterium*, with the name *Flavobacterium gelidilacus* sp. nov.

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pGIL01, A LINEAR TECTIVIRIDAE PROPHAGE ORIGINATING FROM *BACILLUS THURINGIENSIS* SEROVAR *ISRAELENSIS*

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Bacillus thuringiensis, the most widely used entomopathogenic bacterium, belongs to the *B. cereus sensu lato* group, which also includes *B. cereus sensu stricto*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides* and the pathogen *B. anthracis*. *B. thuringiensis* serovar *israelensis* harbours at least 8 extrachromosomal molecules: three small circular plasmids pTX14-1, pTX14-2 and pTX14-3 of 5.4, 6.7 and 7.6 kb, respectively, two large circular plasmids of 113 and 174 kb, the megaplasmid pBtoxis bearing the delta-endotoxin genes, the conjugative plasmid pXO16 and one linear molecule (Madsen *et al*., 1993, Berry *et al*., 2002). Sequence analysis of this linear molecule, named pGIL01, showed the presence of at least 31 ORFs, four of which displayed similarity with proteins involved in phage systems (i. e. a B-family DNA polymerase, a LexA-like repressor and two putative muramidases). Experimental evidences could confirm that pGIL01 indeed corresponded to the linear prophage of a temperate phage. This bacteriophage, named GIL01, produces small turbid plaques and is sensitive to organic solvents suggesting the presence of lipids inside its capsid. Experiments using proteases and exonucleases also revealed that the pGIL01 prophage and the GIL01 phage genomes are protected by proteins at their 5' extremities. These phenotypic and genotypic features are reminiscent of those of PDR1, a Tectiviridae phage found in several Gram-negative bacteria (Bamford *et al*., 1995). Dot blotting and PCR analyses were used to investigate the distribution of pGIL01-related molecules in the *B. cereus* group. Interestingly, pGIL01 variants were observed in 2 *B. cereus* and 3 *B. thuringiensis* strains.

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ACTIVATION OF THE ANAEROBIC ARGININE CATABOLISM BY ARCR IN *BACILLUS LICHENIFORMIS* IS CONTROLLED BY A THIOL-DEPENDENT REDOX MECHANISM

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The facultative anaerobic *Bacillus, B. licheniformis* is able to utilize arginine as an energy source in the absence of respiration. Anaerobic growth on arginine in the absence of glucose fermentation is totally dependent on the induction of the arginine deiminase pathway (Broman *et al*., 1978). When arginine breakdown occurs through this pathway, one ATP is formed for every arginine molecule consumed.
Four genes, organized in an operon, *arcABDC*, encode the pathway. Arginine availability and anaerobiosis are the two conditions needed for induction. Simultaneous binding of two activators to their target site in the regulatory region is required to promote transcription of the *arc* operon. The arginine repressor senses the presence of arginine and acts as an activator after binding an arginine box located 109bp upstream from the transcription origin (Maghnouj *et al*., 1998). The second activator, ArcR, binds at -60.5bp (Maghnouj *et al*., 2000).
The question underlying this work was whether ArcR could be the target of a physiological signal, or be a sensor of anaerobiosis.
The ArcR protein is a member of the Crp-Fnr family (Maghnouj *et al*., 2000). No element in its sequence suggests that it can bind a Fe-S cluster or a heme, which act as oxygen sensors in many proteins. Yet two cysteine residues (C178 and C205), the only ones in the sequence, are found in the small C-terminal domain, each on one side of the helix-turn-helix DNA-binding motif.
The protein was purified from an overproducing recombinant *Escherichia coli* strain. The purification procedure included a chromatographic step on Heparin Sepharose, which appeared dependent on the presence of dithiothreitol (DTT), a thiol-reducing agent. Gel mobility shift assays performed with pure protein showed that the DNA-protein complex could only form in reducing conditions. Further in vitro studies of the ArcR protein with thiol-reacting agents suggest that the ability of ArcR to recognize its DNA target is submitted to a thiol-dependent redox control.
Our results support the hypothesis that transcription activation of the *arc* operon by ArcR is dependent of the cell redox status. In anaerobiosis, the cysteine residues would be reduced and ArcR would be able to bind to the *arc* regulatory region. During aerobic growth, ArcR would undergo a conformational change upon oxidation and would be present under an inactive conformation.

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CLAVAN DEPOLYMERIZING ENZYMES : INDUCTION AND OPTIMIZATION

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Clavan is a L-fucose containing exopolysaccharide (37% w/w), produced by *Clavibacter michiganensis* LMG 5604. The unusual constituent sugar, L-fucose, can be used in biocosmetics, in the medical field and in the food industry (1).
Among many microorganisms screened as to their ability to grow on and hydrolyze the clavan *exopolymer*, a soil isolate identified as *Streptomyces* sp. YSDL-20 was selected for further optimization studies (2).
To check whether this "clavanase" activity was induced by other plant and microbial homo- and hetero-polysaccharides, *Streptomyces* sp. YSDL-20 was cultivated on solid and liquid Czapek Dox medium supplemented with alginate, cellulose, cellulose acetate, chitin, chitosan, dextran, pectin, xanthan, xylan from birchwood or xylan from oat spelts.
On solid Czapek Dox medium, the strain could grow on all the tested polymers except pectin. Large clearing zones (3-4 mm) could be seen when xylan (birchwood and oat spelts) and xanthan were used as sole carbon source. These results indicate that *Streptomyces* sp. YSDL-20 displays a large spectrum of polysaccharases.
After 4 days of cultivation in liquid Czapek medium with different carbon sources, the supernatant was recovered by centrifugation, concentrated 10 times via lyophilization and used as crude enzyme source for clavan hydrolysis : 200 µl of clavan solution (1% w/v) was incubated with 1 ml of the lyophilized supernatant for 5h. Liberated reducing sugars were quantified by the DNS method. For all concentrations of xylan used as carbon source (up to 20 g/l), reducing sugars were liberated from clavan : up to 189 $mg.g^{-1}$ of reducing sugars were found, when 18 g/l of xylan was used as carbon source. 14 $mg.g^{-1}$ and 57.5 $mg.g^{-1}$ of reducing sugars were liberated from clavan respectively, when10 g and 20 g/l of alginate were used as carbon source. These results suggest that the enzymes, induced by xylan and alginate, also have a depolymerizing effect on clavan.
Attempts were also made to optimize the clavanase activity. In this respect, *Streptomyces* sp. YSDL-20 was cultivated on different media : Czapek Dox, minimal medium, yeast malt extract, Czapek peptone and modified Morosoli medium. All the media were supplemented with 2 g/l of purified clavan, prepared from a *Clavibacter* fermentation run. After 16 h of growth, 2.54 $mg.l^{-1}$ of free L-fucose was found in Czapek Dox medium; it was subsequently used by the strain. In yeast malt extract medium, 1.32 $mg.l^{-1}$ and 24.91 $mg.l^{-1}$, respectively of L-fucose and D-glucose could be detected after 16 h of fermentation. In this medium, the highest concentration of liberated free L-fucose was 4.39 $mg.l^{-1}$ after 40 h of cultivation. In Czapek peptone and minimal medium, the monosaccharides could not be detected. In Morosoli medium, 4.12 $mg.l^{-1}$ and 12 $mg.l^{-1}$ respectively of L-fucose and D-glucose were detected after 96 h of fermentation with clavan as carbon source. Growth kinetics of *Streptomyces* sp. YSDL-20 on L-fucose -all or not in combination with other sugars- are now under study.

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