EUROPEAN SCIENCE FOUNDATION
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REPORT FROM A COMPLETED LESC AND EMRC EXPLORATORY WORKSHOP
"Does adaptability to environmental stress favour adaptation to new niches, including the clinical environment"

Aussois, France, 22-25 September 2002-10-28
(Convenors: J. Balandreau and B. Cournoyer)

Workshop Background and Objectives:

The increased occurrence of nosocomial infections, and the appearance of new infections in hospitals, caused by bacteria classically known as environmental taxa have prompted several national health organisation of Europe to launch new research programmes to understand the origin of these new health problems. A somewhat comparable but less critical situation is found in Europe for plant diseases with the occurrence of an outbreak of bacterial wilt in potatoes caused by *Ralstonia* (ex Pseudomonas) *solanacearum*. In several instances, bacteria involved are not only of environmental origin, but also appear to belong to taxa with an exceptional ability to adapt to environmental stress. This is particularly obvious for *Ralstonia* which is able to develop an efficient resistance to many heavy metals or *Burkholderia* which is involved in the degradation of many pollutants as well as *Pseudomonas aeruginosa*. The three taxa are able to acquire multiple antibiotic resistance genes as easily as catabolic genes. This makes these taxa, viz. *Ralstonia, Burkholderia* and *Pseudomonas aeruginosa* an interesting material to understand:

- how an environmental bacterium can adapt to new highly selective environments such as plants, animals, the human body and/or the hospital.
- the possible link between the efficient adaptability of these taxa to environmental stress and their success as new infection agents.

Both questions can benefit from an interdisciplinary approach in the frame of an ESF structure, for which this Exploratory Workshop constituted a preliminary step.

The report of the workshop, as well as the programme and list of participants, are attached.

Final Programme

Sunday 22 nd

17h30: bus ("Trans Savoie") at St Exupery airport, Lyon. Meeting point: in front of the Satobus desk (Satobus is the name of the shuttle going to Lyon.), close to the post office, between terminal 1 and terminal 2. J. Balandreau will wait for participants with an "ESF" poster. On its way to Aussois, the bus will stop at the railway station in Modane, at 19h45 to collect those arriving from Paris or Lille.

20h 30: dinner

21-22h
Welcome address, purpose of the Workshop, ESF presentation: Hui Wang, Benoit Cournoyer and Jacques Balandreau.

Monday 23rd

9-10 : Do environmental and clinical isolates pertain to identical taxa ? Importance of molecular taxonomy (*Burkholderia, Pseudomonas aeruginosa, Ralstonia*) : Tom Coenye.

10-12 : Anthropogenic stresses
Pollutants :

Burkholderia, PCBs and life in biofilms : Ken Timmis
Heavy metals : Max Mergeay
Temperature : temperature induced mutators : Max Mergeay
Metalloids: Benoit Cournoyer

12h30: lunch

Free time

15h30-18h30: Niche changes
Population genetic structure, the case of *Staphylococcus* : Edward Feil
Phase variation and pathogenicity (*Ralstonia*): André Trigallet
Adaptative plasmid-based strategies : Chris Thomas

Importance of pathogenicity markers in *P. aeruginosa* : Jose Luis Martinez
What makes a good opportunist ? : Eshwar Mahentiralingam
Pathogen transition in Enterobacteriaceae: Ariena van Bruggen

19h30: dinner

21-22h: Interactions

Bacterial adhesion: Alain Filloux
Life in biofilm : Søren Molin

Tuesday 24 th

9-11h30: Interactions

Mutators : Ivan Matic
Contingency loci : Christopher Bayliss
Oxidative stress : Niels Høiby
Rhizosphere : Jacques Balandreau
Impact of evolutionary elements, especially IS and the clc element in *Pseudomonas*: Jan Roelof van der Meer

11h30-12h: **Epidemiology** :
The role of the viable but non culturable cells in epidemiology : *Ralstonia* Dirk van Elsas

12h30: lunch

Free time

15h30- 16h30: Epidemiology (continued)
Epidemiology of *Burkholderia*: Gérard Chabanon
Recombinations and epidemiology : Burkhard Tümmler

16h30-17h30: New ways opened by entire genome sequences :
Christian Boucher and Max Mergeay

17h30-19h: Prospect
ESF network, programme, EUROCORE, EU integrated project ?
Selection of a few key issues critical to establish a causal link between adaptability to environmental stress and adaptability to the clinical environment.
Organisation of a transdisciplinary subgroup around these issues.
Preparation of the ESF and maybe EU initiatives as well

19h45: dinner (Swiss cheese fondue)

Wednesday 25th

9-10h45: Concluding session.

Initiating the writing of draft projects

11 h: lunch

12h15: bus departure to Modane and St Exupery (arrival around 15h -15h15)

Description of the general objectives of this exploratory workshop

The main workshop objective was not to present new data. It was to provoque exchange of information, ideas, concepts about a set of bacteria adapted both to the hospital and the environment and the way they can react to selective pressures, including the hospital environment. The ultimate objective was to combine our expertises (taxonomy, ecology, medical microbiology) to understand how these organisms can enter hospitals from the environment. This public health problem can best be tackled by a collaborative research.
The output of this meeting is a series of proposals to the ESF (using one of its four tools). A scientific board (J. Balandreau, T. Coenye, B. Cournoyer, E. Feil, K. Timmis, and B. Tümmler) was elected to organize the work around the preparation of these proposals. Two main tasks were assigned to this board: (1) the elaboration of a proposal for the organization of an ESF network on the topic of this exploratory workshop but focusing on "The role of stress and anthropogenic changes in the emergence of human opportunistic pathogens from environmental reservoirs" - this proposal is practically in its final form and should be submitted to the ESF by spring 2003; and (2) the elaboration of a proposal for a network of excellence on the ecology of bacterial infectious agents.

Knowing each other

Participants to this exploratory workshop belong to several cultural and disciplinary groups who do not usually meet. To know each other several means were employed:
- a list of publications of all the participants was sent to each other
- the time schedule had gaps, especially during afternoons to help getting acquainted
- a fondue was pleasantly served with the same objective of getting participants to know each other

Presentations

An overhead projector was available. PowerPoint presentations were possible. The time allowed was around 30 minutes per talk. Particpants used some of their time to present their research groups and organisations. Participants also presented in a few words the way they were seing future coming actions of our group to make the field of the exploratory workshop alive and growing in activities.

A poster session was also organised.
Participants could bring posters describing their know how. The idea was to identify the potential of each group, and, in that respect, "second hand" posters were welcomed.

Feedbacks from the participants

Participants agreed to say that this exploratory workshop was quite original in trying to bring together distinct fields (environmental and medical microbiology), and trying to build up scenario to better understand the reality behind emerging infectious agents and the evolution of pathogen reservoirs. Unanimously, participants also agreed to say that most scientific meetings these days become useless, and that they would appeaciate seeing more of these exploratory workshops. According to the participants, the main strong points of this workshop were:

- the interdisciplinarity
- the attempts, by the various speakers, to cover gaps between knowledge on bacterial population genetic structures, environmental reservoirs of opportunistic pathogens, medical microbiology, and adaptative processes (recombinations, gene transfers and hypermutability).
- the diversity of methodological approaches presented
- the identification of several fields of investigation - like the effect of anthropogenic stresses on the evolution of opportunistic pathogens; the importance of plasmids in the adaptation of *Burkholderia cepacia* to diverse environments (environmental and clinical ones); etc
- the observed and well-documented metabolic versatility of the bacteria investigated; going from gene regulation (sensory systems) to metal/metalloids/antibiotics resistance processes

All participants agreed to say that several original ideas emerged from this workshop, and that we should move a step further by proposing an ESF network to maintain these novel trends alive. Such a network would favor, in the long run, the build-up of collaborative research programmes.

Plant associated *Burkholderia*, stress and variability

Balandreau J, Tran Van V, **Cournoyer B**, Douillet F, Blot M, Nelson L, Miché L
(F)

In the *B. cepacia* complex, 2 recent problems appeared with strains Ral-3 and TVV75. Both strains are very efficient PGPR but their registration was refused for fear that they could be or become pathogenic. To address this question the variability of TVV75, the type strain of B. vietnamiensis has been characterised. In a first phase, mutagenesis was studied using a clone of TVV75 containing the pGBG1 plasmid with an amplifiable target for mutagenesis making mutants selectable on tetracycline. In the presence of the germinating host plant (rice), TetR mutants are increased ten times. Target amplification showed that 75% of these mutants are due to transposition events, predominantly movements of IS407 and IS1416. TVV75 is also remarkable for its ability to produce hypermutators, i. e. clones with a 100 to 10000 fold increase in the frequency of any mutation. Under normal growth conditions in test tubes, they represent 15% of spontaneous rifR mutants. At the end of the log phase, it increases to 45%. In the presence of rice this increase occurs earlier ($p=0.05$), as early as after 15 hours of contact. In the presence of a chemical stress (10 µM HgCl2, a toxic concentration of mercury), among survivors, 21 to 37.5 % are hypermutators. It is hypothesized that in such a bacterium, a stressful situation such as the shortage of nutrients, the presence of a plant or of toxic compounds, the first reaction is a shift to the hypermutator state, eventually producing high numbers of mutants, and exhibiting a higher ability to recombine foreign genes. This evolution of variants could ease adaptation to new niches, including the hospital.

Does the use of selenium by *Pseudomonas aeruginosa* favor its colonization of the respiratory tract and certain environmental sites?

Benoit Cournoyer (F)

Selenium is an essential element of all living forms but at certain levels it can become toxic. The most frequent symptom of chronic selenium toxicity in human is hair and nail brittleness and loss. Selenium was shown to cause reproductive failure and deformities in fish and aquatic birds. Several environmental sites over the world have high content of bioavailable selenium. These environments selected particular microbial flora that can reduce and volatilize selenium. We have chraracterized one of the biochemical process that can lead to the volatilization of selenium by bacteria. This process involves a bacterial thiopurine methyltransferase (bTPMT). This methyltransferase can volatilize inorganic and organic selenium. The gene encoding bTPMT was found in all environments producing methylated selenium i. e. dimethyl selenide (DMSe) and dimethyl diselenide (DMDSe). An homolog of this gene was detected in *Pseudomonas aeruginosa*. It is well-established that seleno-proteins like glutathione peroxidases play a key role in the immune system by preventing excessive damages by reactive oxygen species (ROS). *P. aeruginosa* bTPMT could have been conserved to create a selenium deficiency in the host, and increasing ROS levels. These ROS levels could then drive the generation of *mucA* mutations in *P. aeruginosa*, resulting in alginate overproduction and an efficient colonization of the respiratory tract. Observations supporting a role of *P. aeruginosa* bTPMT for the colonization of immuno-depressed patients, and of environments polluted by high selenium levels were presented.

Life in biofilms.

Søren Molin (DK)

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 as a pathogenicity factor 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 and Escherichia coli. 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.
Soeren

What makes a good opportunist?

Eshwar Mahenthiralingam (UK)

To debate this question examples from the epidemiology and pathogenesis of the genus *Burkholderia* were discussed with particular focus on the *Burkholderia cepacia*. This species has recently been shown to be a complex of closely related species which prior to formal naming are known as genomovars. At present there are nine taxonomic groups in the complex: the new *species B. multivorans, B. vietnamiensis, B. stabilis, B. ambifaria, B. pyrrocinia* and *B. anthina*, and genomovars I, III, and VI. Isolates from all the latter taxonomic groups can be recovered from clinical infections in vulnerable patients such as those with cystic fibrosis. The prevalence of these *B. cepacia* complex taxa in natural environment is understood in less detail, however, it is known that *B. cepacia* genomovars I and III, together with the species *B. pyrrocinia, B. ambifaria* and *B. vietnamiesis* are all prevalent in natural habitats.

Three examples of overlap between phenotypic and genotypic traits of clinical and environmental *B. cepacia* complex bacteria were illustrated. Firstly, resistance to antimicrobial agents including disinfectants, appears to be shared by both clinical and environmental strains and is an intrinsic feature of *B. cepacia* complex bacteria. Secondly, there is preliminary evidence of low G+C genomic segments in the genomovar III clinical strain, J2315 (the subject of genome sequencing) that have overlap with mobile nitrogen fixation symbiosis islands in environmental Mesorhizobium species.

Finallly, there are now a number of studies which present evidence that strains recovered from clinical infections may possess a genome fingerprint which is identical to strains recovered in the natural environment. These data indicate that many genes encode functions which overlap in both clinical and environmental settings. The genetic identity of clinical and environmental strains suggest that the natural environment also acts as the reservior for human infection in the absence of patient-to-patient spread of *B. cepacia* complex bacteria. Understanding which genes are required for environmental fitness of B. cepacia complex bacteria in both clinical and natural environment settings will be vital to the safe development of these organisms as biotechnological agents.

Genotypic characterization of *Burkholderia cepacia* genomovar III (poster)

Luigi Chiarini (IT)

Burkholderia cepacia genomovar III comprises most virulent and transmissible isolates of the *B. cepacia* complex and represents, therefore, one of the major causes of mortality among cystic fibrosis patients. *B. cepacia* genomovar III is also widely present in natural habitats. As a consequence, one major concern is the evaluation of the pathogenic potential of environmental isolates belonging to this species.

Preliminary work aiming at a genotypic characterization of clinical and environmental isolates has been carried out on a panel of 70 strains isolated from the rhizosphere of maize and from the sputum of patients attending the pediatric Gaslini hospital of Genoa. Isolates were firstly assigned to one of the subgroups (A or B) of genomovar III; subsequently all isolates were RAPD fingerprinted and patterns were subjected to the analysis of molecular variance (AMOVA).

Results are summarized as follows:

1. Isolates belonging to subgroup A were found only among clinical isolates, whereas isolates belonging to subgroup B were found among both clinical and environmental isolates.
2. No common genotypes were found between clinical and environmental isolates
3. A high percentage of genetic variability was attributable to divergences among clinical and environmental isolates
4. Significant differences in the degree of genetic polymorphism were found between clinical and environmental isolates: clinical isolates had significantly lower mean genetic distance values than environmental ones. In particular, clinical isolates belonging to subgroup A showed very low mean genetic distance, whereas higher values were observed in the case of clinical and environmental isolates belonging to subgroup B.

In conclusion, clinical and environmental populations show marked differences in their genetic structures; moreover, our results suggest that even between clinical isolates belonging to subgroups A and B there is a marked difference in the degree of genetic polymorphism.

Transitions of enteropathogens from animal manure into the vegetable production chain

Ariena H.C. van Bruggen[1], Alexandre M Semenov[2], J. Dirk van Elsas[3], and Carolien Zijlstra[3].

To illustrate potential avenues of transition of bacteria from the environment to the human body and the hospital environment, a presentation was given of recently initiated research on niche transitions of human enteric pathogens. First, an overview was given of recent outbreaks of foodborne pathogens, particularly Enterobacteriaceae. Arguments were presented why survival of introduced pathogens may differ in organic and conventional food chains, as microbial composition and diversity have been shown to be more complex in organic systems, probably due to lower nitrogen levels in soil, plants, animal feed, and manure. Thus resistance against introduction of allochthonic bacteria may be higher in organic farming systems and possibly on organic produce. This hypothesis was partially tested and proven to be true for gfp-marked *Pseudomonas fluorescens* introduced into three pairs of organic and conventional soils. An example was given from the literature of transitions of an introduced pathogen (*E. coli* O157:H7) along the food chain from manure to soil, rhizosphere, and plant surface. Tracking a pathogen is a difficult task (expecially in manure and soil), and a proposal was presented to develop micro-arrays that include both pathogens and saprotrophic bacteria, so that risk of pathogen spread could be related to its biological environment.

Alexandre M. Semenov (RU)

My contribution was a co-participation in the presentation of Ariena Van Bruggen: Transition of enteropathogens from animal manure into the vegetable chain production.
The idea of our presentation was to demonstrate that bacteria associated with food, for example Enterobacteriaceae but possibly also Pseudomonadaceae, could be a hazard for human health. Via the food production and marketing chain, representatives of genera *Pseudomonas*, *Burkholderia* and *Ralstonia* could also transit from the farm environment to the hospital environment. Our presentation was mainly from the ecological and epidemiological point of view. In particular for enterobacteria, the natural reservoirs are quite clear, because a lot of research has been done in the fields of evolution of pathogenicity, survival and even transitions from one niche into the next.
In our presentation, we also included recent work on survival of *Pseudomonas fluorescens* in organic versus conventional soil, demonstrating that introduced populations declined significantly faster in organic soil. We are interested in niche transitions of various introduced and indigenous bacteria, in particularly we are tracing now transition of *P. fluorescens* from manure to soil and further on the plants and survival in organically versus conventionally managed soil, in and on organically versus conventionally produced plants.

Changes in the structure of microbial communities of a refinery site in the Czech Republic

Vladimir Brenner (CZ)

(Poster)

Subsurface environments are often severely restricted in the availability of nutrients, carbon substrates and electron acceptors. Hence, as aquifer oligotrophic communities are often adapted to low substrate concentrations, a sudden input of supplied substrates or electron acceptors might have stress effects on the activity and growth of oligotrophic communities. The major goal of our project is to find out how significantly and in which direction the microbial community structure of an oligotrophic aquifer changes when that community is challenged with organic pollutants. Thus, we make a comparison of the functional and genetic composition of the oligotrophic microbial communities before and after exposure to a pollutant plume passing through an aquifer. We have selected refinery as model site contaminated with aromatic pollutants, prevalently with BTEX, to study shifts in microbial community structure in response to changing concentrations of pollutants. The movement of the contaminant plume on this site may be predicted and therefore samples of pristine, recently contaminated, and long-term contaminated aquifer material with a known pattern of pollution, were available for investigations. In preliminary selection we have isolated typical soil strains (including opportunistic pathogens strains Burkholderia cepacia, Pseudomonas aeruginosa, Burkholderia malei) and compared the DGGE (denatured gel gradient electrophoresis) patterns of different soil samples from pristine, contaminated, and freshly contaminated soil. The prevalent bands will be sequenced to find out which bacterial strains are best adapted to environmental stress, in this case to the high concentration of BTEX pollutants.

Abstracts of the talks or posters

Are clinical and environmental isolates the same? The relevance of molecular taxonomy

Tom Coenye (BE)

Members of the genera *Burkholderia* and *Ralstonia* are versatile organisms that easily adapt to changing conditions. Members of these genera are exploited for biocontrol, bioremediation and plant growth promoting purposes, but safety issues regarding human infections (especially in particular patient groups, like cystic fibrosis [CF] patients) have not been solved and an important question that remains largely unanswered is whether 'good' and 'bad' strains are actually the same.

The results of polyphasic-taxonomic studies on *Burkholderia cepacia* indicate (i) that many other bacteria are misidentified as *B. cepacia* (including a variety of known and novel species) and (ii) that *B. cepacia* actually represents at least nine genomic species referred to as the *B. cepacia* complex. It has been shown that *B. cepacia* genomovars II and III dominate in specimens recovered from CF patients, while *B. cepacia* genomovars I, III, VII and IX dominate in the soil samples examined so far. This shows that the same species that occur in the environment can also colonise and infect CF patients. There is now also growing evidence that, at the strain level, human and environmental isolates are not necessarily distinct.

It can be concluded that the genus *Burkholderia* is an extremely versatile genus comprising over 29 species, including primary human pathogens (e.g. *B. pseudomallei*), primary animal pathogens (e.g. *B. mallei*), primary plant pathogens (e.g. *B. glumae*), soil and plant-associated organisms (e.g. *B. graminis*), nitrogen-fixing symbionts (e.g. *B. phymatum*), obligate endosymbionts (e.g. *Candidatus* B. kirkii) and several opportunistic pathogens (e.g. *B. cenocepacia*, *B. vietnamiensis*).

The genus *Ralstonia* also contains species occupying multiple ecological niches and includes plant pathogens (e.g. *R. solanacearum*), opportunistic human pathogens (e.g. *R. pickettii*), nodulating species (e.g. *R. taiwanensis*) and soil organisms with biotechnologically interesting properties (e.g. *R. metallidurans*).

The molecular/physiological background of this diversity and adaptability are largely unknown and further collaborative molecular taxonomic studies are required to further gain insights into the biodiversity of the genera *Burkholderia* and *Ralstonia*.

Pathogenicity markers in *Pseudomonas aeruginosa*.

J. L. Martínez, F. Rojo (ES)

The rate of nosocomial infections range from 6 to 10%, with a mortality of 7-8%. Infections multiply by three the number of deaths at hospitals. A replacement of classical opportunistic pathogens (mainly from the commensal flora) by environmental bacteria such as *P. aeruginosa* is occurring in the last years. This replacement is probably due to the fact that hospitalized patients are under strong antibiotic therapy and environmental bacteria are frequently intrinsically resistant to antibiotics.

A key issue is to know whether the strains causing infections are exactly the same that are present in the environment or are just a specialized subset that have evolved to a pathogenic lifestyle. To that goal, environmental and clinical isolates of *P. aeruginosa* have been compared. No differences have been found among isolates from different ecosystems, neither at the genomic level, nor when the physiological characteristics of the strains were compared: environmental isolates presented a full repertoire of virulence determinants and clinical isolates have biodegradativo properties.

During therapy, *P. aeruginosa* isolates evolve to become antibiotic resistant. A relevant topic should be to know whether those antibiotic resistant isolates are impaired for surviving in the environment. We have found that multidrug resistant mutants are less virulent and are also impaired for surviving in natural ecosystems.

More work is needed to understand the effect of the clinical/natural environments in the evolution of *P. aeruginosa*. Some topics (and tools) that are relevant for this are:

a) Molecular epidemiology of environmental and clinical isolates including physiology (Genomics and functional genomics)

b) Does ‘evolved’ strains survive in the environment? (Models and functional genomics)

c) Do environmental changes produce an enrichment of opportunistic pathogens in the environment? (Models and analysis of natural populations)

d) Does pollution select for antibiotic resistant isolates? (Models and analysis of natural populations).

The simple sequence contingency loci of *Haemophilus influenzae* and *Neisseria meningitidis*

Christopher D. Bayliss, Dawn Field and E. Richard Moxon

Many pathogens have evolved the ability to alter surface-exposed molecules, most often in response to selective pressures associated with the host immune system. Pathogenic bacteria exhibit numerous examples of this adaptive strategy, and a range of molecular mechanisms has evolved in these bacteria for generating genetic variation at individual loci termed "contingency loci". Many contingency genes are controlled by simple sequence DNA repeats that accumulate reversible, rec-independent mutations at high frequency. A striking feature of the complete genome sequences of the human pathogens *Haemophilus influenzae* and *Neisseria meningitidis* is the abundance of loci containing simple sequences. Intriguingly, both of these bacteria are obligate commensals of the upper respiratory tract of humans but can, in some hosts, cause life-threatening invasive disease. In this talk we provide a synthesis of our current understanding of how polymorphisms in simple sequence contingency loci produce phenotypic variation. We also evaluate the role of this variation in pathogenesis and the evolution of virulent bacterial strains.

Multicellular behaviour in *Salmonella typhimurium*

Ute Römling (SE)

Salmonella typhimurium and *Escherichia coli* display a stationary phase induced multicellular behaviour. This multicellular behaviour is characterized by bacterial cell-cell interactions, which lead to a spreading cell network on plates, to cell clumping in liquid culture, to pellicle formation and to biofilm formation on abiotic surfaces. Thin aggregative fimbriae and cellulose are the two extracellular matrix components, which have distinct roles in cell-cell interaction and biofilm formation. Thin aggregative fimbriae and cellulose are positively regulated by the transcriptional regulator *agfD*. *AgfD* itself is regulated by a variety of environmental conditions. Expression of *agfD* by entry in the stationary phase is triggered by nutrient starvation, while quorum sensing signals do not play a role. Temperature, oxygen tension, the nutrient content of the medium, osmolarity and other environmental conditions influence *agfD* expression. On the other hand, individual point mutations in the *agfD* promoter region lead to a phase variable expression of multicellular behaviour. Thereby, the regulated multicellular morphotype switches to a semi-constitutive multicellular morphotype. In conclusion, the equilibrium between the single cell status and the multicellular status is mainly determined by mutations and secondarily regulated by various environmental conditions.

The importance of VBNC cells in bacterial adaptation – case study with *Ralstonia solanacearum*

Jan Dirk van Elsas and Leo van Overbeek **(NL)**

Ralstonia solanacearum biovar 2, the causative agent of brownrot in potato – and wilting of tomato - , is an emerging pathogen that has established in Dutch open environments (soils and waters), and economic losses incurred by farmers have been enormous in recent years. The organism was shown to be able to survive a winter period in the Netherlands in soil in the field. In water as well as in soil systems, it responded to low temperature by an enhanced population decline when measured as culturable forms (cfu), yet a remarkable stability of total immuno-detectable as well as viable forms measured by CTC membrane activity stains and direct viable counts. These observations provided strong evidence for the enhanced induction of viable-but-nonculturable (VBNC) forms in this species by low temperature. Attempts to recover culturable forms by temperature raises failed. There is ongoing speculation about a possible relationship between the conversion to VBNC forms and the capacity of *R. solanacearum* to change its phenotype to a non-aggressive form, in a process called phenotypic conversion (PC). This PC can have a genetic basis, as shown by A. Trigalet (this meeting).

Serial tenfold dilutions of water cultures treated by low temperature, with a strong dominance of VBNC forms, were used to inject tomato plants. The data showed that as long as there was a statistical chance of any fully culturable cell being present, wilting of tomato plants was observed. However, below the level at which culturable cells were present, plants didn't show any symptoms of wilting, whereas, surprisingly, culturable forms of the pathogen could be re-isolated. These forms invariably had the PC appearance. Even upon re-infection of plants and re-isolation from them, we have, so far, been unable to show conversion back to the aggressive form. A selection of colonies re-isolated from plant tissue were typed using BOX-PCR; the data indicated that several forms, which also showed a changed colony morphology, had a changed BOX pattern. We are currently looking into the possibility that these strains have a relationship to the PC forms with genetic changes in the *phcA* gene such as observed by A. Trigalet.

Epidemiology of *Burkholderia*

Gérard Chabanon and Christine Segonds (F)

➢ The *Burkholderia cepacia* complex strains are mainly involved in the two following groups of infections :

- "nosocomial infections mainly respiratory-tract infection, bacteremias and UTI, occurring in debilitated patients ;
- infections of the respiratory-tract in Cystic Fibrosis (CF) patients. The clinical outcome in such infected patients is extremely variable, however some of them showed a rapid and fatal necrotizing pneumonia with septicemia and fever, the "cepacia syndrome" (CS). Moreover, cross-infections between CF patients has been well documented both in and out of hospital. Finally *B. cepacia* strains demonstrate an innate resistance to a wide range of antimicrobial agents, such as aminoglycosides and polymyxins widely used in CF patients against *P. aeruginosa*. These data led to the introduction of various hygiene preventive guidelines in and out hospitals.
- finally, infections have been reported in patients with chronic granulomatous disease or sickle cell hemoglobinopaties

➢ The French CF Association "Vaincre la Mucoviscidose" decided to found the "Observatoire National B. cepacia" in 1993. (G. Chabanon, E. Bingen, P. Plesiat, Scientific Group ; C. Segonds, responsible) this network now includes 110 Centers spread over France (about 3200 CF patients concerned) ; both bacteriological and clinical informations about *B. cepacia* complex strains are collected ; an annual report is published.

➢ In France, the annual prevalence of *B. cepacia* complex strains is about 3% in CF patients; (mean age of acquisition, 14) ; *B. multivorans* and *B. cepacia* genomovar (gv) III are the main species involved (49 and 45% respectively). Fourty four percent of *B. multivorans* isolates, and 30% of *B. cepacia* gv III isolates are unique. Five highly transmissible clones (2, *B. multivorans* ; 3 *B. cepacia* gv III) have been identified ; septicemias equally due to *B. multivorans* and *B. cepacia* gv III occur at an annual rate : of 0,8 to 2,6 %) and are nearly always followed by death. The *B. cepacia* ET12 clone (gv III) highly transmissible causing CS is not present in France ; however an epidemic *CblA* negative strain of *B. multivorans* causing CS has recently been shown to adhere to epithelial cells as described in ET12 *B. cepacia* strains.

Genomic islands and evolution of catabolic pathways

Sentchilo, V. and **J. R. van der Meer (CH)**

Transfer of genetic material between different individual bacterial cells (i.e., horizontal gene transfer) is often mediated by highly sophisticated elements, like plasmids or bacteriophages. Recently, conjugative elements were discovered which normally reside on the chromosome but can excise under certain conditions, be transferred to a new host cell and reintegrate. They have been named gene islands, or, depending on their function, pathogenicity islands, symbiosis or degradation islands. The excision and integration processes of these gene islands are similar to bacteriophages. In our laboratory we have been studying the gene island for chlorocatechol degradation in Pseudomonas and Ralstonia spp. (named the 'clc element'). The clc element is 105 kb in size and is integrated in the gene for glycine tRNA. Interestingly, excision activity of the clc element is essentially non-homogeneous throughout a population, which means only some cells (1%) will contain the excised form. This can be seen from cells containing a transcriptional fusion between the promoter for the integrase and the green fluorescent protein. Furthermore, induction of excision of the clc element is promoted during growth on chlorinated compounds and in stationary phase. This suggests that excision and transfer of the clc element are subject to strong control and perhaps, external signalling molecules.

Adaptative plasmid-based strategies

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Bacterial plasmids provide a means for spread of phenotypic traits between bacteria without the need for establishment by homologous recombination in the recipient. Although plasmids are known in *Ralstonia* sp. and *Burkholderia* sp. the analysis of plasmid diversity both at the level of type of replication, maintenance, transfer systems and the phenotypes conferred, within these three genera has mainly been carried out with plasmids of *Pseudomonas* species. We are currently involved in a project to sequence and characterise the major plasmid groups of Pseudomonas by sequencing archetypes of each group and using this information as the basis for assessing the distribution of plasmids of each type and the genetic load carried by different members of each family. Pseudomonas plasmid groups are designated IncP-1, IncP-2 etc. The IncP-1 plasmids are moderately large, self-transmissible plasmids that have a very broad host range among all Gram negatives and are certainly capable of gene spread from environment to clinic. The IncP-1β plasmids in particular seem to be associated with recent evolution of degradative traits among soil bacteria, while the whole group is associated with dissemination of antibiotic resistance. The IncP-9 plasmids are larger self-transmissible plasmids with a more limited host range, being unstable outside the Pseudomonas species. Again they can carry both degradative and resistance determinants, and illustrate well how plasmids are generally organised as a core of maintenance and spread genes plus one or more regions that have been subject to multiple insertions by transposable elements carrying phenotypic traits and also facilitating mobilisation of chromosomal genes. A recent survey of clinical isolates in Poland demonstrated that 80% of multiple resistant *P.aeruginosa* strains carried large self-transmissible plasmids and a significant proportion of these belonged to the IncP-1 group. This illustrates (a) the continuing importance of these mobile genetic elements among these species and (b) the value of molecular tools in characterising them.

New ways opened by entire genome sequences

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Ralstonia solancearume is a gram negative beta-proteobacterium responsible for bacterial wilt. Due to its unusually wide host range, covering over 200 plant species, and a large geographical distribution in all tropical and subtropical regions of the world, this bacterium is responsable for one of the worldwide most devastating bacterial plant disease. We recently achieved sequencing and annotation of the complete genome of strain GMI100, a recognized model organism commonly used for molecular dissection of pathogenicity process towards plants. This genome is composed of two replicons of 5.6 and 2.1 Mb pairs. Both replicon harbor a mosaic structure with numerous stretches of DNA which have a base composition significantly different from the average composition of the bacterium. This suggests that these regions could have been acquired through horizontal gene transfer. The type III protein secretion system encoded in this bacterium by *hrp* gene plays a key role in pathogenicity. Therefore the genome of strain GMI1000 has been carefully inspected for genes candidate pathogenicity effector proteins to be translocated through this pathway into plant cells. Based on three complementatary prediction processes, over 50 genes for candidtes effectors were predicted. So far, 35 of these have been shown to be transcriptional coregulated with *hrp* genes that encode structural components for the type III secretion machinary. Functional analysis of these genes is in progress and already established that three of them control host specificity by restricting host range of the strain. A preliminary analysis of the distribution three of the candidate genes established that they are not found in every *R. solanacearum*. This analysis will be extended to all genes for candidate effectors through the used of microarrays in order to establish their contribution to biodiversity in the species and to tentatively develop means ofprediction for the the host-range of other strains.

The chaperone/usher pathways of *Pseudomonas aeruginosa*: identification of fimbrial gene clusters (cup) and their involvement in biofilm formation.

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Pseudomonas aeruginosa, an important opportunistic human pathogen, persists in certain tissues in the form of specialised bacterial communities, referred to as biofilm. The biofilm is formed through series of interactions between cells and adherence to surfaces, resulting in an organised structure. By screening a library of Tn5 insertions in a non-piliated *P. aeruginosa* strain, we identified genes involved in early stages of biofilm formation. One class of mutations identified in this study mapped in a cluster of genes specifying the components of a chaperone/usher pathway that is involved in assembly of fimbrial subunits in other microorganisms. These genes, not previously described in *P. aeruginosa*, were named *cupA1-A5*. Additional chaperone/usher systems (CupB and CupC) have been also identified in the genome of *P. aeruginosa* PAO1; however, they do not appear to play a role in adhesion under the conditions where the CupA system is expressed and functions in surface adherence. The identification of these putative adhesins on the cell surface of *P. aeruginosa* suggests that this organism possess a wide range of factors that function in biofilm formation. These structures appear to be differentially regulated and may function at distinct stages of biofilm formation, or in specific environments colonised by this organism.

The expression of the *cup* genes appeared tightly controlled and is repressed by the MvaT transcriptional regulator. MvaT belongs to a novel family of regulator that is only found in the *Pseudomonas* genus. A *mvaT* mutant shows an increased capacity in forming biofilm that could be correlated to the de-repression of the *cup* genes. Global transcriptional profiling using *P. aeruginosa* microarrays revealed that among the *cup* clusters, the *cupA* genes are the most highly de-repressed. Understanding the function of MvaT and its contribution to the lifestyle of *P. aeruginosa* may uncover new opportunities for controlling biofilm associated infections.

Pseudomonas in cystic fibrosis: past, present, future

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Most patients with cystic fibrosis (CF) suffer from recurrent and chronic endobronchial P aeruginosa infections. The accumulated knowledge shows that it is possible to prevent or delay the onset of these chronic infections in most CF patients by eliminating cross-infection and by early aggressive antibiotic treatment of intermittent colonization. The lung tissue damage is caused by activation of the immunologically specific inflammatory defense mechanisms of the lungs initiated by the antibody response and dominated by polymorphonuclear neutrophil leukocytes and their proteolytic and oxidative products. This inflammation induces a phenotypic shift from non-mucoid to mucoid, alginate producing phenotypes of P aeruginosa which then grow as a biofilm endobronchially. Such biofilms are impossible to eradicate by antibiotics. By using chronic suppressive antibiotic maintenance therapy and anti-inflammatory drugs it is, however, possible to maintain the lung function of the patients for years. Further improvement of the prophylaxis and therapy may include methods used by environmental microbiologists to prevent and remove biofilm.

Population genetic structure, the case of *Staphylococcus*

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The population structures of bacterial species are complex and often controversial. To a large extent, this is due to uncertainty about the frequency and impact of recombination in bacteria, information that is crucial to the understanding the emergence of clones of clinical significance such as those with elevated virulence or drug resistance, and to the subsequent management of such clones. The existence of clones within bacterial populations, and of linkage disequilibrium between alleles at different loci, is often cited as evidence for low rates of recombination. However, clones and linkage disequilibrium are almost inevitable in species that divide by binary fission and can be present in populations where recombination is frequent. In recent years, it has become possible to directly compare rates of recombination in different species. These studies indicate that in many naturally transformable bacterial species such as *Neisseria meningitidi* and *Streptococcus pneumoniae*, homologous recombination is a more significant evolutionary force than point mutation for clonal diversification within neutral (housekeeping) loci. In contrast, the frequency of recombination appears to be lower in *Staphylococcus aureus*, although phylogenetic analysis has revealed that recombination still plays a role over the long-term evolution of this species.

Metal resistant *Ralstonia*, inhabitants of industrial and anthropogenic biotopes

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Metal-resistant *Ralstonia* belonging to the species *R.metallidurans*, *R.campinensis* or *R.basilensis* carry multiple heavy metal resistance genes and specifically colonize industrial soils or sediments highly contaminated by heavy metals. Therefore, any knowledge about the genetics and the microbial ecology of these strains adapted to harsh biotopes, may be of importance in the perspective of possible relationships with nosocomial infections of environmental origin. The best known strain and a qualified representative of the group, *R.metallidurans* CH34, is a facultative chemolithotroph and displays large plasmids (pMOL28 (180kb) and pMOL30 (260kb)) carrying a variety of genes for resistance to heavy metals ions as those of cadmium, cobalt, chromate, copper, mercury, nickel, lead, thallium and zinc.

The strain is a also a good recipient of foreign genes, displays an interesting mutator phenotype as well as a variety of mobile genetic elements that might play a role in its genetic versatility, its adaptation to difficult environments or in gene dissemination. To assess the natural genetic engineering in the genus *Ralstonia* is of importance with respect to the diversity of specialised functions and of biotopes that is so characteristic of the genus.

The genome of *R.metallidurans* is now available at a draft level and has been compared with the fully annotated genome of the plant pathogen *R.solanacearum.* The genome of *R.solanacearum* includes a 3.6 MB chromosome and a 2.1 MB megaplasmid. Up to 60 % of the chromosomal genes and 30 % of megaplasmid genes have high identity with *R.metallidurans* genes. The genome of *R.solanacearum* contains many metal resistance genes displaying similar organisation and high identity with those of *R.metallidurans*: all these genes are located on the *R.solanacearum* megaplasmid. On the other hand, the genome of *R.metallidurans* does not have the type III secretion sytem that is essential for plant pathogenesis in *R.solanacearum*.

Exploitation of genomic data also shows that the *R.metallidurans* genome is very rich in metal resistance genes , and especially in tricomponent metal efflux systems (HME-RND) of the RND multidrug resistance family and in P-ATPase-mediated efflux of cations. At least 12 RND operons were listed in *R.metallidurans* (to be compared with 0 to 3 in 60 other procaryotic genomes and 4 in R.solanacearum): most of them are located on the chromosome.In the same way, 7 genes encoding putative P-ATPases were listed in *R.metallidurans*, 5 of them being lonked to plasmid pMOL30 (to be compared with an average of 1 to 4 in other sequencd genomes).

Genomics shows also that most of the plasmid-bourne metal resistance operons have a simplified chromosomal counterpart. It suggests a duplication of a basic operon followed by the acquisition by the plasmid of additional genes , which allow the bacteria to reach a higher resistance level.

Proteomics and , in some extent, transcriptomics have much helped in the detection of these additional genes or functions. The corresponding resistance proteins are frequently unique and , up to now, have not be found in other genomes.

Genomic data helped also to evaluate the diversity of mobile genetic elements in the genome of *R.metallidurans*, and especially to the detection of at least two MGE similar to the catabolic transposon Tn*4371*found in a biphenyl degrader, *R.oxalatica* A5. In the same perspective, B. Tuemmler has shown , during the same meeting, the presence in *R.metallidurans* CH34 of a genomic island that is characteristic of clinical isolates of *P.aeruginosa* ("clone C"). The percentage of identity between the genomic islands found in both bacteria is of 100%.

The reported genomic data , sustained by first proteomic and transcriptomic studies, support the idea that *R.metallidurans* has an evolutionary history of adaptation to heavy metals or to harsh and toxic biotopes, and also, that its genetic capabilities may offer to some genes a route from industrial biotopes to clinical environments.

Pseudomonas aeruginosa: Recombinations and epidemiology

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The 5 – 7.5 Mb large *Pseudomonas aeruginosa* genome is made up of a mosaic of a conserved core genome and of a 10 – 30% large portion of strain- and clone-specific DNA. The gene pool that is common to all *P. aeruginosa* is characterized by linkage equilibrium and a low average sequence diversity of less than 0.5%, the major exceptions being the O-antigen, pyoverdine, flagella and pilin biosynthesis regions. Genomic diversity is mainly caused by insertions and deletions. The *P. aeruginosa* chromosome possesses three hypervariable regions close to the *pilA*, *phnAB* and *lipH* loci with a pronounced intra- and interclonal genomic variability that is mainly caused by about 100 kb large gene islands that are inserted into the 3‘ end of tRNA genes. 106 kb large plasmids carrying multiple fertility and virulence determinants have been shown to reversibly integrate in either one of the two $tRNA^{Lys}$ genes of the chromosome. The strain –specific gene islands adjacent to the *lipH* gene are integrated into conserved $tRNA^{Gly}$ genes and have a bipartite structure. The first part adjacent to the tRNA gene consists of strain-specific ORFs encoding metabolic functions and transporters, the majority of which has homologs of known function in other eubacteria. The second part is mostly made up of ORFs of yet unknown function that exhibit an amino acid sequence identity of 35 - 100% and conserved gene order in all gene islands of this type analyzed so far. These potentially transmissible islands seem to be rather common amongst metabolically versatile proteobacteria that initially had been classified as pseudomonads by physiology-oriented taxonomists. Islands of very high sequence homology were found in the *Xylella fastidiosa, Ralstonia metallidurans, Burkholderia cepacia* and other *Pseudomonas* genomes. We hypothesize that this novel type of gene island derives from mobile elements which, upon integration, endow the recipient with strain-specific metabolic properties, thus possibly conferring on it a selective advantage in its specific habitat.

Phase variation and pathogenicity in *Ralstonia solanacearum*

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Ralstonia solanacearum is a plant pathogenic bacterium that undergoes a spontaneous phenotypic conversion (PC) from a wild-type pathogenic (ON) to a non-pathogenic (OFF) form. PC is often associated with mutations in *phcA* which is a key virulence regulatory gene. Until now, reversion to the wild type pathogenic form was never observed for PC (*phcA*) variants and the biological significance of PC has been questioned. In this study, we characterised various DNA rearrangements (IS element insertions, tandem duplications, deletions and a base substitution) in 19 PC mutants affected in *phcA* from the model strain GMI1000. For five of these variants, reversion to the pathogenic form was observed in the presence of tomato host plant. No reversion was ever observed in the absence of plant, but reversion was observed in presence of tomato root extract for a 64 pb tandem duplication. This is the first report showing a complete phase variation cycle in a plant pathogenic bacterium and a parallel can be drawn with phase variation in human and animal pathogens. A model for *R. solanacearum* life cycle is proposed.

Polychlorinated biphenyl-degrading microbial communities in soils and sediments.

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Recent advances in the degradation of polychlorinated biphenyls (PCBs) have focussed on the use of experimental enrichment cultures to obtain PCB-degrading communities, and the use of culture-independent approaches to characterize natural and experimental PCB-degrading communities and to identify the key members in this process. PCB-degrading communities can be surprisingly diverse. Novel types of composite bacteria-mineral biofilm communities have been described. Community metabolism of PCBs may lead to the formation of protoanemonin, a dead-end product in some instances but, in others, a seemingly productive intermediate. Analysis of isotope fractionation and preferred enantiomer degradation has provided new information on degradation of PCBs in anaerobic settings. The first defined community capable of dehalorespiration of PCBs has been described, and important community members identified. Here, we provide an overview of the current knowledge of aerobic and anaerobic degradation of PCBs in microbial consortia and in the environment, including novel approaches to determine in situ PCB degradation.

Ecological, Physiological and Genetic Determinants of Stress-Induced Mutagenesis in Bacteria.

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Using molecular, genetic, physiological, ecological, and evolutionary approaches we have studied mutagenesis in aging colonies and revealed its high variability in a large number of *Escherichia coli* natural isolates. Mutagenesis increases in aging colonies as a consequence of carbon source starvation and oxidative shock. This mutagenesis is genetically controlled by two major global regulons, RpoS and CRP-cAMP. A down-regulation of the mismatch-repair system seems to be responsible for the fixation of mutations in aging colonies resulting largely from the error-prone activity of DNA polymerase II. The polymorphism of mutagenesis in aging colonies phenotypes among natural isolates seems to be a consequence of the diversity of selective pressures in different environments from which different strains were isolated rather than to their phylogenetic relationship. For example, human pathogens have lower mutagenesis in aging colonies and higher constitutive mutation rates than human commensals. Using computer simulations we show that stress-induced mutagenesis, like mutagenesis in aging colonies, may rapidly evolve via hitchhiking with adaptive mutations and thereby limit the selection of constitutive mutator alleles. These simulations suggest also that stress-induced mutagenesis may have an important impact on the rates and modes of adaptation of bacteria independently whether it is selected as a mutator phenotype or is just a stress-induced pleiotropic effect.

Physiological status of specific bacterial populations introduced into soil

Maraha, Ninwe, Backman, Agneta and **Jansson, Janet K. (SE)**

(Poster)

The physiological status of specific bacterial populations inoculated into soil was measured using *gfp*-tagged strains and flow cytometry in combination with different viability staining techniques. We studied two model bacteria: one gram negative PGPR strain, *Pseudomonas fluorescens* SBW25, and one gram positive 4-chlorophenol degrading strain, *Arthrobacter chlorophenolicus* A6. The soil was inoculated with these strains and the bacterial community was extracted on nycodenz density gradients. The isolated cells were stained with either CTC (cyano-ditolyl-tetrazoliumchloride) or PI (propidium iodide), to stain viable or dead cells, respectively. The total number of *gfp*-tagged cells was quantified by flow cytometry. Furthermore, the proportion of dead and viable cells in the specific populations were monitored by gating the CTC stained or PI stained cells within the *gfp* fluorescent region. We also compared CFU counts to the number of viable fluorescent cells measured by flow cytometry to estimate the number of viable but non-culturable cells of each model strain in the soil. Results indicated that the physiological status of these specific populations could be monitored in soil over time. The large majority of the cells were neither metabolically active, nor dead, but in a dormant or resting state. This is the first demonstration that the physiological status of specific bacterial populations could be monitored in soil using flow cytometry. This approach has potential for monitoring the physiological status of bacteria of interest in both environmental and clinical settings.

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