Content

Introductory Welcome to the 15th BNF-Non Legume Symposium in Budapest

We cordially welcome you to the 15th BNF-NL-Symposium, held on **23rd/24th August 2016** as satellite symposium before the 12th European Nitrogen Fixation Conference (ENFC) and the Genomics Satellite Workshop (25th to 28th August) at Budapest, the capital city of Hungary. The venue is the very nice Danubia Hotel Helia right on the banks of the Danubius river in Budapest.

The BNF-Non-legume Symposia have a long tradition since the 1980s. In the years 2008 and 2012, the 11th and 13th BNF-NL-symposia were already organized successfully as satellite meetings of the ENFCs in Ghent and Munich, respectively. The 14th BNF-NL-Symposium was organized by our Chinese colleagues as satellite to the Asian Congress of Plant-Microbe Interaction in Cheng-Du in October 2014.

The BNF-NL-Symposia provide the forum for the presentation and discussion of current hot issues of Non-Legume nitrogen fixation research (associative and endophytic diazotroph – plant interactions, actinorhizal and cyanobacterial symbioses, environmental nitrogen fixation, applied and agronomic aspects). Since the topic "BNF with Non-Legumes" can only be presented in a quite limited time frame within the ENFC, the BNF-NL satellite symposium provides additional time for the presentation and discussion of these aspects. The participation in both meetings will give full insights in all fields of nitrogen fixation research.

This time, the Diamond Congress Service, Budapest, is taking care of the registration, payment and hotel room organization for all participants of ENFC and the satellite meetings (please see the technical instructions below).

For participants of oral and poster presentations, please follow strictly the guidelines presented below.

We are very happy, that for this 15th BNF-NL-symposium around 80 participants have registered. They represent 16 countries around the world. We cordially thank the chairpersons of the 6 sessions of putting together an attractive program.

During the symposium, the scientific advisory board of this symposium series will also meet and discuss and decide about the location of the follow-up symposium in 2018.

We are very much looking forward to most interesting scientific presentations and lively discussions during the symposium. We hope that many of you will also join the symposium dinner with Hungarian Folklore on the evening of 24th August.

The scientific coordinators:

Toni Hartmann Barbara Reinhold-Hurek

Scientific Coordinators of the 15th BNF-NL-Symposium:

Anton Hartmann, Helmholtz Zentrum München, Research Unit Microbe-Plant Interactions, Ingolstaedter Landstrasse 1, D 85764 Neuherberg, Germany
Phone: 0049 89 31872852; e-mail: a.hartmann@helmholtz-muenchen.de

Barbara Reinhold-Hurek, University of Bremen, Department of Microbe-Plant Interactions, P.O.Box 330440 D-28334 Bremen, Germany
Phone: 0049 421 21862860; e-mail: breinhold@uni-bremen.de

International Scientific Advisory Board:

Ivo Baldani, Brazil

Yoav Bashan, USA

Fabricio Cassan, Argentina

Ray Dixon, UK

Claudine Elmerich, France

Claudine Franche, France

Adriana Hemerly, Brazil

Ann Hirsch, USA

Euan James, UK

Lin Min, China

Yaacov Okon, Israel

Fabio Pedrosa, Brazil

Lilly Pereg, Australia

Gary Stacey, USA

Jonathan Zehr, USA

General information

Venue

Danubius Thermal Hotel Helia****
Budapest
Kárpát Street 62-64
H-1133

GPS Coordinates: N 47°31'32" E 19°3'21"

The four-star **Danubius Thermal and Conference Hotel Helia spa**, wellness and conference hotel, built in Scandinavian style, was opened in 1990 and partially renovated in 2003. Natural thermal water is pumped to the hotel spa from springs on the nearby Margaret Island, which is famous in Budapest for its high quality medical and relaxation services.

Registration desk

Tuesday, 23 August 2016	10:00-19:00
Wednesday, 24 August 2016	8:00-19:00

Important phone numbers

English is usually spoken at the emergency numbers listed below.
In case English is not spoken, dial 112

Ambulance:	104
Police:	107
General enquiries:	197
International enquiries:	199
Hungarian Automobile Club help number:	188
Fire brigade:	105
Central help number:	112
Inland enquiries:	198

Time

Hungary is in the Central European Time Zone.
In the summer months clocks are set at GMT + 2 hours.

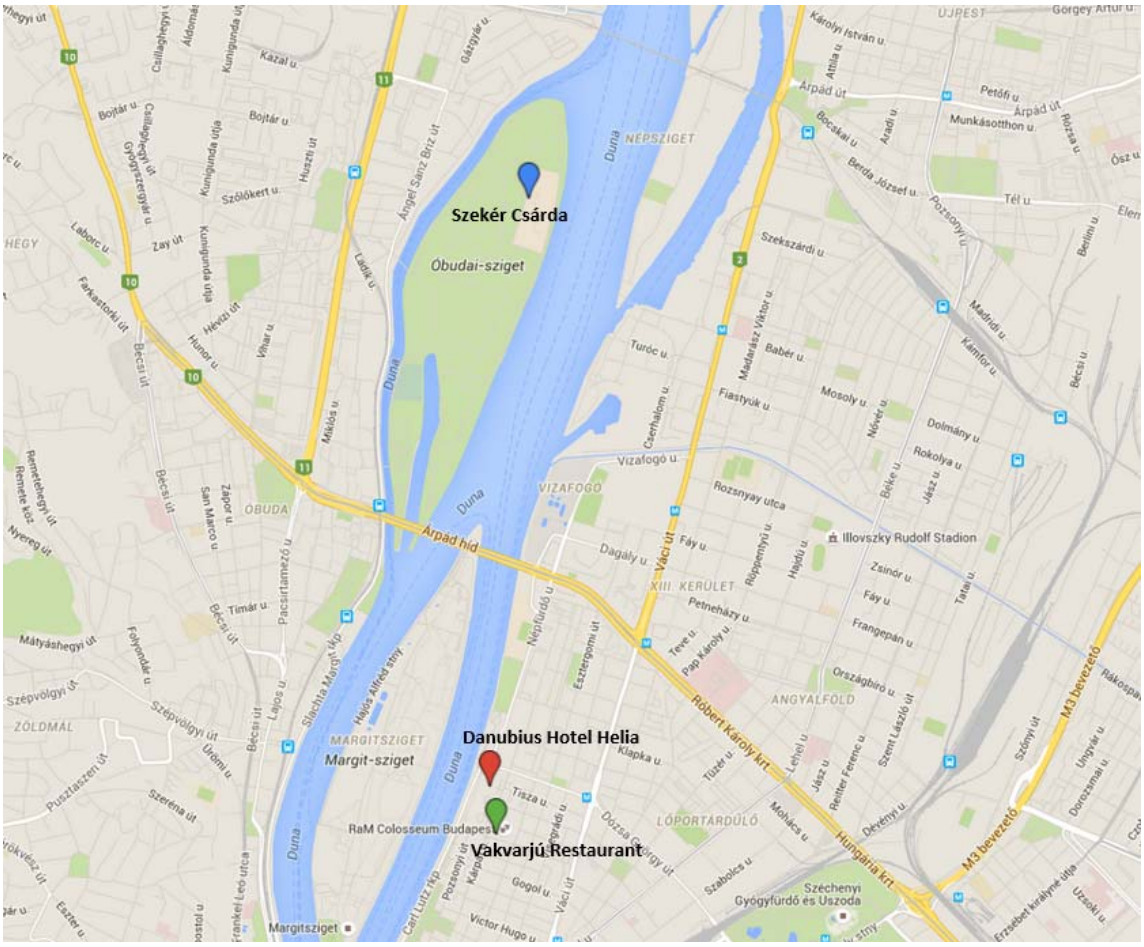
Social events

Banquet dinner

Venue: Szekér Csárda
Budapest, Hajógyári sziget 27796/16 hrsz.
H-1033

Wednesday, 24 August 2016 at 20:00 (Gathering in the hotel lobby at 19:15)

The Szekér Csárda is situated on the Óbuda Hajógyár island. The Csárda's forefront represents in its architectural the Hungarian rural feeling of the year 1800, the application tools visualize the rural traditions. Our aim is to give a taste of the Hungarian traditional gastronomy and folk culture. We welcome our guests for dinner with guaranteed folklore program. The Csárda's ground space has 840 square meters, it is air-conditioned, and dispose of a built-in stage, amplification and a cloakroom. The inner of the Csárda gives a very cozy feeling by the application of wood, cane, brick and with the everyday tools of the rural life.



Authors' guidelines

Oral presentations

Venue: Helia Hall (lecture hall)

The keynote lectures take 30 minutes (25 + 5 min discussion) and the symposium lectures take 20 minutes (15 + 5 min discussion). Please consider these time frames, appreciate your colleagues and audience by keeping the schedule.

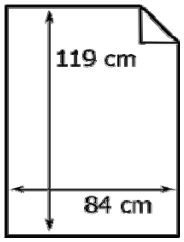
Technical instructions:
Please prepare your presentation in .ppt, .pptx (Microsoft Office PowerPoint 97-2013 format) or .pdf file. Please avoid using videos embedded in your show. If you wish to have a video, please contact the technician in the lecture hall in a break before your presentation (or preferably earlier) to check it in advance.

Please note that using your own notebook is NOT RECOMMENDED.

Poster presentations

Venue: Helia Hall (lecture hall)

Mounting: Tuesday, 23rd August, from 10:00
Removal: Wednesday, 24th August, before 19:00



The poster session (P-01.1 – P-06.2 according to the abstract number) will be from 23rd August 18:10 – 20:00. The posters may be shown during the whole symposium.

Posters left on the boards after the removal deadline will be dismantled by the organizers.

Technical instructions:
The posters should be prepared for standard STANDING (PORTRAIT) A/0 size (84 x 119 cm – 1 sqm). PLEASE DO NOT PRINT A LANDSCAPE POSTER! The organizers provide all equipment and tools (pins, adhesive tape, scissors) to mount your poster to the board at the symposium venue.

FLOORPLANS


MAIN ENTRANCE
COFFEE SHOP
ELEVATOR
RECEPTION
GROUND FLOOR
LOBBY
WINDOWS
'C' FLOOR
PLUTO
URANUS
112 m²
WINDOWS
SATURNUS
VENUS
RESTAURANT
630 m²
WINDOWS
WINDOWS
PANORAMA
140 m²
WINDOWS
HELIA CONFERENCE
360 m²
TERRACE
PRE-FUNCTION AREA
140 m²
ORION A
60 m²
ORION B
42 m²
MERCURE
140 m²
WINDOWS
TERRACE

Final program of the 15th BNF-NL-Symposium in Budapest 23rd/24th August 2016

Tuesday, 23rd August

10:00-13:00 Registration

13:00-13:15 Opening of the 15th BNF-NL-symposium
by Toni Hartmann & Barbara Reinhold-Hurek

13:15-15:30 **Session 1:** NL-associations with diazotrophs: bacterial side
Chairpersons: Emanuel Maltempi da Souza & Euan James

13:15-13:45 **Euan James, Scotland:**
Nitrogen fixation by non-legumes: from field measurements to bacterial genomes

13:45-14:15 **Emanuel da Souza, Brazil:**
Comparative molecular analysis of the interaction of diazotrophic PGPR with Gramineae

14:15-14:45 **Lin Min, China:**
Global search of small ncRNAs in root-associated nitrogen-fixing bacterium *Pseudomonas stutzeri* A1501 identifies a novel regulatory ncRNA that acts hierarchically to optimize nitrogen

14:45-15:05 **Florence Wisniewski-Dyé, France:**
Role of phosphorelays in *Azospirillum*: from genomics to functional analysis

15:05-15:25 **Andrea Krause, Germany:**
An ethanol responsive hierarchical signal cascade – important for the endophytic life of *Azoarcus* sp. BH72

15:30-16:00 **Coffee / Tea time**

16:00-18:30 Session 2: NL-associations with diazotrophs: the plant side

Chairpersons: Adriana Hemerly & Barbara Reinhold-Hurek

16:00-16:30 Adriana Hemerly, Brazil:

The role of plant receptors in the perception of beneficial diazotrophic associations

16:30-17:00 Barbara Reinhold-Hurek, Germany:

Exploring endophytic colonization mechanisms: Response to and control of rice infection by *Azoarcus* sp. strain BH72

17:00-17:30 Fernanda Plucani do Amaral, USA:

Mechanistic studies of bacterial plant growth promotion using the grass model plants *Brachypodium distachyon* and *Serratia viridis*

17:30-17:50 Jean-Michel Ané, USA:

The aerial root mucilage associated with an indigenous landrace of maize supports a diazotrophic microbiota

17:50-18:10 Paulo Ferreira, Brazil:

To be or not to be: miR408 regulation is critical for sugarcane sensing whether microorganisms are either beneficial or pathogenic

18:10-20:00 Poster Session

This session will start with optional max. 2 min presentations by the poster authors (elevator pitch).

Afterwards: Meeting of the scientific advisory board at Vakvarju Restaurant – across the street from Danubius Hotel Helia.

Wednesday, 24th August

8:30–10:30 Session 3: Frankia symbioses

Chairpersons: Claudine Franche & Katharina Pawlowski

8:30-8:50 Claudine Franche, France:

Fifteen years of research on the functional analysis of the actinorhizal symbiosis *Casuarina glauca-Frankia*

8:50-9:10 Katharina Pawlowski, Sweden:

Cluster II *Frankia* strains: symbioses with *Cucurbitales* and *Rosales*

9:10-9:30 Benjamin Billault-Penneteau, Germany:

Dryas: a model genus for root symbioses of the Rosaceae

9:30-9:50 Anna Ribeiro-Barros, Portugal:

A system biology approach to analyze salt stress tolerance in *Casuarina glauca* and the contribution of symbiotic *Frankia* bacteria

9:50-10:10 Guillaume Schwob, France:

Green alder (*Alnus viridis*, Chaix, DC) encroachment shapes differently fungal and bacterial communities in subalpine soils

10:10-10:30 Louis S. Tisa, USA:

Genes, genomes and genetics of *Frankia* and the actinorhizal symbioses

10:30-11:00 Coffee/Tea time

11:00-13:00 Session 4: Cyanobacterial symbioses

Chairpersons: Jon Zehr & Rachel Foster

(funded by the Gordon & Moore Foundation)

11:00-11:20 Jon Zehr, USA:

Marine unicellular symbiotic cyanobacteria: significance in global ecology, physiology and evolution

11:20-11:40 Ulla Rasmussen, Sweden:

Moss symbiosis

11:40-12:10 Eric Webb, USA:

Marine symbiotic assemblages with *Trichodesmium*

12:10- 12:30 Sophie Rabouille, France:

Exploring diazotrophic growth processes in marine cyanobacteria using combined, experimental and modeling

12:30-13:00 Himadri Pakrasi, USA:

Nitrogenase and photosystem II: the Ying and Yang in cyanobacterial nitrogen fixation

13:00-14:00 Lunch break

14:00-16:00 **Session 5:** Microbial Ecology of root-associated diazotrophs

Chairpersons: Thomas Hurek & Toni Hartmann

14:00-14:30 Neung Teaumroong, Thailand:

Non-photosynthetic *Bradyrhizobium* as rice endophyte and its molecular interaction and application aspects

14:30-14:50 Luc F.M. Rouws, Brazil:

Bradyrhizobium isolates from sugarcane roots represent a new phylogenetic group with potential for the development of inoculants

14:50-15:10 Thomas Hurek, Germany:

Microbial nitrogen cycling in rice fields

15:10-15:30 Juan Imperial, Spain:

How does sugarcane fix nitrogen? A rich and diverse microbiome suggests that complex bacterial and fungal assemblages contribute to sugarcane growth and sustainability

15:30-15:50 André Martinz-Oliveira, Brazil:

Use of trap plants to isolate PGPB strains from soils under different land uses and its application as inoculant for non-legumes

15:50-16:10 Nicholas D. Ayub, Argentina:

Robust biological nitrogen fixation in wheat and maize under soil conditions

16:10-16:30 Coffee/Tea time

16:30-18:30 Session 6: Application of NL-diazotroph associations

Chairpersons: Yaacov Okon & Toni Hartmann

16:30-17:00 Yaacov Okon, Israel:

The use of *Azospirillum brasilense* inoculants

17:00-17:30 Solon C. Araujo, Brazil:

Use of *Azospirillum* in Brazilian agriculture: a case of success

17:30-18:00 Sharon Doty, USA:

Nitrogen fixation in *Populus*: Characterization of the key diazotrophs

18:00-18:30 José Ivo Baldani, Brazil:

BNF in the bioenergy plant *Pennisetum purpureum*

18:30-18:40 Closing of the 15th BNF-NL-symposium

Announcement of the 16th BNF-NL-symposium in 2018

20:00 Symposium´s dinner with Hungarian Folklore and Music at Szekér Csárda

19:15 Gathering in hotel lobby for bus transfer

List of Posters

No.	Presenting author	Title
P-01.1	José Ivo Baldani	*In vitro* study suggests that stem apoplastic liquid of sugarcane modulates the chemotaxis and defense mechanism of the diazotrophic *Burkholderia tropica* strain PPe8
P-01.2	Sudhir Singh	Molecular analysis of the role of two paralogs of OxyR regulators in peroxide mediated oxidative stress response in *Azospiriilum brasilense* Sp7
P-01.3	San-Feng Chen	Using synthetic biology to increase nitrogenase activity
P-01.4	C. J. Waite	The regulation of nitrogen fixation and assimilation in the associative diazotroph *Klebsiella oxytoca* M5a1
P-01.5	Jörg Schumacher	Synthetic rebalancing of nitrogen fixation and nitrogen assimilation in diazotrophs
P-01.6	Vijay Shankar Singh	Identification and functional characterization of genes involved in carbon source utilization in *A. brasilense* Sp7
P-01.7	R. Wassem	Functional analysis of genes involved with nitrate metabolism in *Herbaspirillum seropedicae*
P-02.1	Kevin Garcia	Delivery of fixed nitrogen to cereal crops using *Salmonella typhimurium* carrying the refactored and native *Klebsiella nif* cluster
P-03.1	*Didier Bogusz*	Developing nitrogen-fixing symbioses in cereal crops: what can we learn from actinorhizal symbioses?
P-03.2	Meriem Benabdoun	The salinity tolerance of *Casuarina equisetifolia* in symbiosis with *Frankia* relation
P-03.3	Louis S. Tisa	Developing a Genetic System for *Frankia*
P-04.1	Denis Warshan	Functional genomics of cyanobacteria in symbiosis with boreal feather mosses
P-05.1	Wiebke Bünger	Cultivation of plant-associated bacteria belonging to the phylum *Verrucomicrobia*
P-05.2	Hannes Schmidt	Diversity and activity of diazotrophs associated with micro-environments of wetland rice
P-05.3	Nicolas Ayub	Effect of PHB production on nitrogen fixation in *Pseudomonas* inoculants
P-05.4	Elisete Pains Rodrigues	Composition and effects of maize exudates on chemotaxis of *A. brasilense* and its performance in maize development
P-05.5	E. M. Souza	Maize-driven selection of rhizobacteria
P-05.6	E. M. Souza	A single spontaneous GS mutation is responsible for the phenotype of an *Azospirillum brasilense* ammonium-excreting strain
P-06.1	Veronica M. Reis	Growth promotion and nitrogen metabolism of two sugarcane varieties inoculated with a mixture of five diazotrophs

Abstracts

Oral presentations

O-01.1

Nitrogen fixation by non-legumes: from field measurements to bacterial genomes

Euan K. James

The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK

It has long been known that symbiotic Biological Nitrogen Fixation (BNF), such as that conducted by symbioses between legumes and rhizobia are capable of contributing all of their hosts N-requirements. However, in recent years there has also been considerable interest in (non-nodulating) N_2-fixing bacteria that interact positively with non-legumes, such as maize (*Zea mays*), sorghum (*Sorghum bicolor*) and rice (*Oryza sativa*), as well as bioenergy crops, such as sugarcane (*Saccharum* sp.), and elephant grass (*Pennisetum purpureum*), which are capable of obtaining large amounts (up to 80%) of their N-requirements via BNF (1). As there are no nodules evident on any of these non-legumes it has been suggested that the bacteria responsible for the measured BNF are the diazotrophs that are loosely associated with them; these bacteria may be rhizospheric, epiphytic, endophytic, or a combination of all of these (2, 3).

Although many putative "endophytes" have been described on the basis that they have been isolated from surface sterilized plant material, an essential prerequisite for the description of a genuinely endophytic bacterium is that it be observed within the host plant via high resolution light and/or transmission electron microscopy. Arguably the most intensively studied endophytic bacterium is *Azoarcus* sp. strain BH72, and it has been suggested that this strain can fix considerable quantities of N within the roots of rice and C4-grasses in exchange for carbon provided by the host in the form of ethanol and/or malic acid. Similarly, *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp. are also known to fix N_2 in association with rice, sugarcane and *Miscanthus*. However, although BNF is one of the mechanisms by which these bacteria promote growth of grasses it is not actually known if they perform BNF within the plant tissues or on the root surface (or both), what form of nutrition they receive from their host plants, and if and how their genomic and metabolic profiles change when they are in close positive (quasi-symbiotic) association with their hosts. Such fundamental information is essential if N-fixing PGPRs are to be effectively exploited to reduce N-fertiliser inputs to both cereals and energy/biofuel crops.

(1) James & Baldani (2012) Plant Soil 356: 1–3
(2) James (2000) Field Crop Res 65:197-209
(3) Turner et. al. (2013) Genome Biol 14:209

O-01.2

Global gene expression profiling of rhizospherical *Herbaspirillum seropedicae* SmR1 shows extensive metabolic changes

M. Z. Tadra-Sfeir, V. C. S. Pankievicz, E. Balsanelli, L. C. C. Brusamarello-Santos, R. A. Monteiro, R. Wassem; L. Chubatsu, V. Baura, E. M. Souza, F. O. Pedrosa

Department of Biochemistry and Molecular Biology, Universidade Federal do Paraná, Curitiba, PR, Brazil

Herbaspirillum seropedicae readily colonizes epiphytically and endophytically the root system of diverse Poaceae. For the plant, the outcome of such interaction may be beneficial or neutral. The factors that make this bacterium highly effective as a rhizosphere colonizer are largely unknown. In this study we used high-throughput sequencing (RNA-Seq) for transcriptional profiling of rhizospheric *H. seropedicae* SmR1 from three different plants: rice, maize and wheat. Root-attached and planktonic bacteria were recovered 1 or 3 days after inoculation, total RNA was purified and mRNA enriched fractions were used for library construction. The results revealed in all the plants an analogous response of the bacteria to rhizospheric environment. The most prominent changes were related to metabolic adaptations, cell wall modifications and defence. In all plants, genes involved in microaerophilic growth, nitrogen fixation and polyhydroxybutyrate (PHB) synthesis were activated in epiphytic bacteria. Moreover, genes associated with polysaccharide biosynthesis, peptidoglycan turnover and outer membrane protein biosynthesis were differentially expressed, suggesting reorganization of cell wall envelope components. Several ABC transporter genes whose products are involved both nutrient uptake and drug efflux were also regulated in the bacteria attached to the roots. The results show a remarkable molecular adaptation of *H. seropedicae* SmR1 during interaction roots of different plants.

Financial support: INCT-FBN/CNPq/MCTI.

O-01.3

Global search of small ncRNAs in the root-associated nitrogen-fixing bacterium *Pseudomonas stutzeri* A1501 identifies a novel regulatory ncRNA that acts hierarchically to optimize nitrogen fixation

Yuhua Zhan[1], Yongliang Yan[1], Zhihong Xie[2], Qi Cheng[1], Claudine Elmerich[3], Min Lin[1]

[1]Biotechnology Research Institute/National Key Facility for Crop Gene Resources and Genetic Improvement, Chinese Academy of Agricultural Sciences, Beijing, China
[2]Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences/Key Laboratory of Coastal Biology and Bioresource Utilization, Yantai, China
[3]Institute Pasteur, Paris, France

To date very few small regulatory non-coding RNAs have been functionally characterized in nitrogen-fixing bacteria. The *Pseudomonas stutzeri* A1501 is a plant growth promoting rhizobacteria that carries the genetic information for nitrogen fixation on a 49-kb genomic island (1, 2). A search for RNA transcripts synthesized by *P. stutzeri* A1501 under nitrogen fixation conditions identified 53 ncRNAs exhibiting differential expression during a variety of stress conditions. Our study was focused on a novel ncRNA specific of *P. stutzeri*, named NfiS, whose synthesis is significantly increased under nitrogen fixation conditions or upon non-ionic osmotic stress induced by sorbitol. NfiS controlled nitrogenase activity directly by the post-transcriptional regulation of *nifK* mRNA, and also indirectly through the induction of the RpoN/NtrC/NifA regulatory cascade via unidentified mechanisms. Deletion of *nfiS* resulted in about 40% reduction in nitrogenase activity, while activity of the NfiS-overexpressing strain showed an increase to 150 % as compared to the wild type. Moreover, the production of the nitrogenase MoFe protein polypeptides (NifK and NifD) was decreased in the Δ*nfiS* mutant strain. This suggested a correlation between the NfiS level and the nitrogenase synthesis and activity. Analysis of the secondary structure of NfiS led to the hypothesis that an 11-nt sequence was critical in the control of nitrogen fixation, particularly in the putative pairing with the mRNA of *nifK*. Mutational analysis and microscale thermophoresis experiments support the hypothesis that NfiS was recruited for nitrogen fixation and the conclusion that NfiS plays a role in increasing the translation efficiency and the half-life of *nifK* mRNA. Structural and functional divergences of NfiS, in diazotrophic or non-diazotrophic *P. stutzeri*, may reflect the functional divergences of NfiS evolution under diazotrophic or non-diazotrophic backgrounds. The acquisition of the *nif*-island and the recruitment of NfiS by *nifK* mRNA are evolutionary events that appear to contribute to fine-tuned regulation of nitrogenase activity in *P. stutzeri*. This study provides a new regulatory pathway mediated by an ncRNA for optimal nitrogen fixation that may operate in other diazotrophs.

(1) Yan et al. (2008) PNAS 105: 7564-7569
(2) Yan et al. (2010) BMC Genomics 11:11

O-01.4

Role of phosphorelays in *Azospirillum*: from genomics to functional analysis

S. Borland, C. Prigent-Combaret C., and F. Wisniewski-Dyé

UMR-CNRS 5557, Ecologie Microbienne, Université de Lyon, Université Lyon 1, 69622 Villeurbanne, France

Azospirillum is a plant growth-promoting rhizobacterium (PGPR) living in the rhizosphere of many important crops and grasses (1). Despite numerous studies about its plant beneficial properties (phytohormone production, nitrogen fixation…), little is known about how the bacterium senses and responds to its rhizospheric environment. Two component signal transduction systems (TCS) are widespread in prokaryotes and play critical roles in sensing and responding to environmental cues. Aside from the paradigm phosphotransfer pathway involving one histidine kinase (HK) and one response regulator (RR), more complex versions of TCS exist with multiple phosphotransfer reactions involving hybrid histidine kinases (HyHK). The aim of this study is to identify and characterize TCS encoding genes potentially involved in *Azospirillum*-plant adaptation. Interestingly, *Azospirillum* genomes harbour a very large number of genes encoding TCS and are especially enriched in HyHK genes compared to other plant-associated bacteria of similar genome sizes (Borland *et al.*, 2015). Analysis of HyHK structure and architecture further revealed an intriguing complexity of these systems. Phylogenetic analyses of the transmitter and receiver domains of *A. lipoferum* 4B HyHK indicate that expansion of this family mainly arose through horizontal gene transfer but also through gene duplications all along the diversification of the *Azospirillum* genus (2). A mutational approach was used to decipher the role of four complex HyHKs in *Azospirillum lipoferum* 4B. We identified an atypical three-component system which negatively regulates key rhizosphere competence traits, likely through the modulation of c-di-GMP balance.

(1) Wisniewski-Dyé F. *et al.* (2013) In "Beneficial plant-microbe interactions: ecology and applications" pp.237-269

(2) Borland S. *et al.* (2015) BMC Genomics 16: 833

O-01.5

An ethanol responsive hierarchical signal cascade - important for the endophytic life of *Azoarcus* sp. BH72

Andrea Krause, Theresa Harten, Henrike Julich, Manasee Mankar, Barbara Reinhold-Hurek

Department of Microbe-Plant Interactions, University of Bremen, FB2, P.O. Box 330440, 28334 Bremen, Germany

The habitat of the nitrogen-fixing endophyte *Azoarcus* sp. BH72 is the root apoplast of grasses grown under waterlogged conditions. Rice cultivated under these conditions produces and accumulates ethanol. Since ethanol diffuses through plant membranes, it would appear in the apoplast and could serve there as sole carbon source for endophytes. To metabolise ethanol, strain BH72 is with eight alcohol dehydrogenases (ADH) well equipped (1). For three ADHs, expression was ethanol-inducible under aerobic and microaerobic conditions, of which two (ExaA2, ExaA3) were also expressed inside rice roots. Disruption of these genes reduced growth of *Azoarcus* on ethanol-containing media and diminished competitiveness during endophytic colonisation (2). Similarly, the aldehyde dehydrogenase AldA is important for growth of *Azoarcus* on ethanol – an enzyme encoded by a gene whose expression profile was also ethanol dependent and detectable inside of infected rice roots.

ExaA2 and *exaA3* are clustered in the genome surrounded by genes encoding two two-component regulatory systems (TCS) termed ExaS-ExaR and ElmS-GacA. Functional genomics revealed (a) that expression of the corresponding genes was induced by ethanol under aerobic and microaerobic conditions, (b) that the genes were also expressed in close association with or even inside of rice roots, (c) that both TCSs were indispensable for growth on ethanol, and (d) that they were important for competitiveness during rice root colonisation. Both regulatory systems are forming a hierarchically organised ethanol responsive signal transduction cascade with ExaS-ExaR as highest level, essential for effective expression of the ethanol oxidation system based on ExaA2 and AldA. No influence of any TCS on the ethanol induced *exaA3* expression was detectable. In contrary, transcription and expression levels of *exaA3* increased when any of the *tcs* genes was deleted. An additional element of this regulatory signal cascade is RpoN. Disruption of *rpoN* inhibited *Azoarcus* to grow on ethanol and influenced the ethanol-induced expression of the ethanol oxidation system as well as of the *tcs* genes which coincides with a σ^{54}-dependent promoter prediction upstream of the corresponding genes.

All this together underlines the importance of ethanol for the endophytic lifestyle of *Azoarcus* sp. BH72 but indicates also a tight regulation of the ethanol oxidation system during root colonisation.

(1) Krause et al. 2006, Nat. Biotechnol. 24: 1385-1391

(2) Krause et al. 2011, Mol. Plant-Microb Interact. 24:1325-1332

O-02.1

The role of plant receptors in the perception of beneficial diazotrophic bacteria

Helkin G.F. Ballesteros, Thais L.G. Carvalho[1], Matheus Atella, Thayssa M.D. Fernandes, Pablo Hardoim, José Paulo M. Filho, João Luis Mendes, Paulo C. G. Ferreira, Adriana S. Hemerly

Laboratório de Biologia Molecular de Plantas, Instituto de Bioquímica Médica Leopoldo de Meis, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

The associations that occur between grasses and nitrogen-fixing associative and endophytic bacteria have raised a large interest in their use in agriculture, in view of the positive effects on biomass, productivity and tolerance against stresses. In Brazil, BNF plays a fundamental role in sugarcane cultivation by reduction of the use of nitrogen fertilizers, making Brazilian sugarcane culture more competitive in global markets.

An intriguing question in such associations is how plants perceive signals from other living organisms, thus sorting pathogens from beneficial ones, to transduce this information and activate proper responses that will finally culminate in plant adaptations to optimize their growth rates. Plants might recognize and differentiate beneficial endophytic microorganisms from pathogenic bacteria, allowing their colonization and plant growth promotion. On the other side, they might keep a stringent control over bacterial numbers to avoid pathogenicity.

Our group has been studying sugarcane genes involved in the establishment of this particular type of association with nitrogen-fixing bacteria, aiming to investigate how an endophytic and beneficial association is launched. By using differential transcriptome approaches, we identified several members of plant receptor families that have specific expression profiles for beneficial or pathogenic bacteria. Functional analyses of selected genes are being performed in an heterologous beneficial association system that was developed with *Arabidopsis thaliana*. The data suggests that specific plant receptors sense beneficial and pathogenic associations, possibly participating in immune signal transduction pathways that balance the activation/repression of defense responses to regulate the proper bacterial numbers inside the plant.

Supported by INCT, CNPq, FAPERJ and CAPES

O-02.2

Exploring endophytic colonization mechanisms: Response to and control of rice infection by *Azoarcus* sp. strain BH72

Xi Chen, Marta Marszałkowska, Florian Schnurrer, and Barbara Reinhold-Hurek

Institute of Microbe-Plant Interactions, Faculty of Biology and Chemistry, University of Bremen, Germany

Azoarcus sp. strain BH72, a mutualistic endophyte of rice and other grasses, is of agro-biotechnological interest because it supplies fixed nitrogen to its host Kallar grass and colonizes plants in remarkably high numbers without eliciting disease symptoms. This raises the question of mechanisms of compatible interactions between host and bacterium (1). The complete genome of strain BH72 is sequenced (2), and the rice genome is also available. This allows application of functional genomic analyses of both partners during interaction.

As we have developed a pathosystem for flooded rice roots, applying *Xanthomonas oryzae* pv. oryzae (Xoo) (3), we compared the transcriptomic response of rice roots to endophyte and pathogen infection by transcriptome microarray experiments. Using the 44K Agilent microarray system, rice root gene expression profiles responding to *Azoarcus* colonization were analyzed, and genes with over 1.5-fold different expression in three biological replicates were taken for further analysis. In case of *Azoarcus* infection, 260 rice roots genes were up-regulated and 263 down-regulated. Noticeably, in *Xoo* infection, there were over twice as many of up-regulated genes (543) and less than half of down-regulated ones as in *Azoarcus* (116). Moreover, the up-regulated genes upon *Xoo* infection showed much higher overall fold change than upon *Azoarcus* colonization, and the down-regulated genes in *Azoarcus* case were more strongly down-regulated than in *Xoo* infection. It indicates the strategy as an endophyte to keep a harmony with its host might be shutting down the defense of the host plants. Furthermore, only 14 genes showed a similar regulation in both infections and 42 the opposite one. Functional analysis of the differently regulated genes revealed that *Azoarcus* was able to induce the complete defense machinery even as an endophyte. Genes coding for pattern recognition receptors, MAPKs, resistance proteins, WRKY transcription factors, Myb transcription factors, enzymes for producing reactive oxygen species, pathogenesis-related proteins and proteins involved in producing secondary metabolites related to pathogen resistance, especially phytoalexins, were induced. In all these categories, *Xoo* induced a much stronger reaction than *Azoarcus*. Among several metabolic adaptations, the *Azoarcus* induced mainly jasmonic acid and to lesser extent salicylic acid-inducible immune responses. Therefore, rice mutants that are affected in salicylic or jasmonic acid-dependent responses were tested for colonization efficiency by *Azoarcus* sp. and *Xanthomonas*. Endophyte and pathogen colonization of the root interior were differentially affected, suggesting that hormonally controlled defense pathways can control the density of endophytes in roots.

(1) Reinhold-Hurek, B., et al. 2011. Curr. Opin. Plant Biol. 14: 435-443
(2) Krause A, et al. 2006. Nat. Biotechnol. 24:1385-1391
(3) Chen X, et al. 2015. New Phytologist: doi: 10.1111/nph.13458

O 02.3

Mechanistic studies of bacterial plant growth promotion using the grass model plants *Brachypodium distachyon* and *Setaria viridis*

Fernanda Plucani do Amaral[1], Vania Carla S. Pankievicz[2], Ana Arisi[3], Emanuel M. de Souza[2], Fabio Pedrosa[2] and Gary Stacey[1]

[1] Divisions of Plant Science and Biochemistry, C.S. Bond Life Science Center, University of Missouri, Columbia, Missouri, USA

[2] Department of Biochemistry and Molecular Biology, Federal University of Parana, Curitiba, PR, Brazil

[3] Department of Food Science and Technology, Federal University of Santa Catarina, Florianopolis, SC, Brazil

Plants interact with a wide range of soil microorganisms. In some cases, this interaction can result in significant benefits to both microbe and plant host. It is well known that some soil bacteria can promote the growth of plants increasing crop yield. The effects of such 'plant growth promoting bacteria' (PGPB) have been well documented with a variety of plant species. One lesson from such studies is that both the bacterial and plant genotype are critical in order to induce significant growth promotion. However, the molecular mechanisms behind this growth promotion are still largely unclear. We believe that the adoption of a suitable model bacterial-plant system could significantly accelerate research to increase our mechanistic understanding of these important associations. Our previous studies demonstrated significant plant growth promotion in the model grass species *Setaria viridis* (1) and *Brachypodium distachyon* (2) in association with *Azospirillum brasilense*, *Herbaspirillum seropedicae* and/or *Azoarcus olearius*. We are now expanding on these initial studies to discover and characterize important bacterial and plant genes essential for the interaction, as well as for plant growth promotion. For example, we are using RNA-seq to identify genes induced within the host species in response to bacterial inoculation. The use of a plant model species provides the ability to further explore the function of these genes using transgenic and/or genetic methods. As a complement to these studies, we are also using TnSeq, a high-through-put method for transposon mutagenesis, to identify and then subsequently characterize Azoarcus genes essential for root colonization. The results of this experiment identified over 90 candidate genes whose presence appears to be essential to successful root association. Further investigation of the plant and bacterial genes identified through our studies should increase our mechanistic understanding of these associations. Our ultimate goal is to utilize this information to manipulate the plant-PGPB association to maximize the utility to agriculture, including the growth of bioenergy grass species on marginal land.

(1) Pankievicz et al. (2015) The Plant Journal
(2) Amaral et al. (2016) Plant Mol. Biol.

O-02.4

The aerial root mucilage associated with an indigenous landrace of maize supports a diazotrophic microbiota

Jean-Michel Ané[1], Pablo Zamora[2], Pierre-Marc Delaux[1], Dhileepkumar Jayaraman[1], Jonathan A. Eisen[3], Bart C. Weimer[4], Howard-Yana Shapiro[2,5], Alan B. Bennett[2]

[1]Department of Bacteriology and Agronomy, University of Wisconsin, Madison
[2]Department of Plant Sciences, University of California, Davis
[3]Genome Center, University of California, Davis
[4]Department of Population Health and Reproduction, University of California, Davis
[5]Mars Incorporated

Plants have evolved with a complex microbiome comprising diverse microbes and biochemical pathways that have been proposed to play important roles in nutrient acquisition, plant growth and plant defense. We hypothesized that isolated indigenous landraces of maize grown using traditional practices with little or no fertilizer may have evolved strategies to improve plant performance under low fertility levels. We studied one such indigenous landrace of maize (Totontepec maize) grown in nutrient depleted fields near Oaxaca, Mexico, that is characterized by the extensive development of aerial roots that secrete a carbohydrate-rich mucilage. Analysis of the microbiota associated with the roots, stems and mucilage indicated that the mucilage was enriched in taxa for which many known species are diazotrophic and the mucilage was enriched for homologs of genes encoding nitrogenase subunits. Assays of nitrogenase activity (acetylene reduction and $^{15}N_2$ incorporation) in various maize plant organs indicated that the mucilage, but no other plant parts, harbored diazotrophic activity. A major question of whether the fixed N was transferred from the mucilage to the plant was addressed by assays of natural abundance of ^{15}N in plant tissue samples by mass spectrometry. These assays suggested that the mucilage-localized N_2 fixation contributed to the nitrogen nutrition of maize grown in the field but this conclusion requires confirmation by more direct methods.

O-02.5

To be or not to be: miR408 regulation is critical for sugarcane sensing whether microorganisms are either beneficial or pathogenic

Flavia Thiebaut, Adriana S. Hemerly, Paulo C. G. Ferreira

Laboratório de Biologia Molecular de Plantas, Instituto de Bioquímica Médica Leopoldo de Meis, UFRJ, Rio de Janeiro, Brazil

Plants have a complex mechanism of gene expression regulation that influences their development, adaptation and response to biotic and abiotic interactions. One of these mechanisms involves small RNAs (sRNAs), and can act silencing genes at transcriptional or post-transcription level. In order to understand the roles of sRNA during interaction with endophytic diazotrophic bacteria in grasses, maize was inoculated with *Herbaspirillum seropedicae* and *Azospirillum brasilense*, while sugarcane was inoculate during *Gluconacetobacter diazotrophicus* and the expression of microRNAs was analyzed. The analysis uncovered a dynamic regulation of known miRNA in plants inoculated with diazotrophic bacteria. In particular, expression analysis in has shown that four Copper-regulated miRNAs – miRNA397, miRNA398, miRNA408 and miRNA528 had their expression increased in the presence of the bacteria. Targets of those microRNAs are involved in copper homeostasis and in defense pathways against pathogenic microorganisms, suggesting that maize and sugarcane colonization by diazotrophic bacteria is facilitate by attenuation of defense mechanisms.

To gain knowledge on the physiological responses to pathogens mediated by miRNAs, we have screened the sRNA transcriptome of sugarcane plants infected with *Acidovorax avenae* and detected 25 conserved miRNAs families that have their expression modulated in the presence of this pathogen. In contrast to what was observed in plants inoculated with beneficial bacteria, pathogenic infection leads to decreased expression of Copper-regulated miRNAs. Interestingly, mature miR408 was also down-regulated in samples infected with other sugarcane pathogen, the fungus *Puccinia kuehnii.* Accordingly, RT-PCR and 5'RACE showed that targets of miRNA408 are up-regulated in upon infection with both pathogens.

Our results suggest that regulation of miR408 in sugarcane is a key step in sensing whether microorganisms are either pathogenic or beneficial, triggering specific miRNA-mediated regulatory mechanisms accordingly.

Supported by INCT, CNPq, FAPERJ and CAPES

O-03.1

Fifteen years of research on the functional analysis of the actinorhizal symbiosis *Casuarina glauca-Frankia*

Emilie Froussart, Didier Bogusz, Claudine Franche

Equipe Rhizogenèse, UMR DIADE (IRD-UM), Institut de Recherche pour le Développement (IRD), 911 avenue Agropolis, BP 64501, 34394 Montpellier cedex 5, France
claudine.franche@ird.fr

Because nitrogen is a critical element for tree growth and development, the understanding of the molecular basis of actinorhizal root nodule symbiosis is a major issue. The development of genomics both in *Frankia* and in some actinorhizal plants such as the tropical tree *Casuarina glauca*, together with the possibility to obtain transgenic actinorhizal plants, has offered valuable tools to achieve significant progress in the understanding of the actinorhizal symbiosis (1).

Transcriptomic data were used to identify genes potentially important in actinorhizal nodulation process and the function of candidates genes was then examined in *C. glauca* by RNAi-based gene silencing and promoter studies. So far more than 10 plant genes expressed during the interaction with *Frankia* have been characterized and about 15 transcriptional fusions between promoters from symbiotic genes and the reporter genes *GUS* or *GFP* have been studied in transgenic *Casuarinaceae* plants (2).

We have established that:

- the Common Symbiotic Signaling Pathway (CSSP) required for endosymbiosis with arbuscular mycorrhizal fungi and rhizobia is also involved in actinorhizal nodulation (3)
- key symbiotic genes expressed in nodule cortical cells infected by *Frankia* are also expressed at the prenodule stage
- two promoters were shown to be induced by diffusible *Frankia* factors, thus providing a biological test for the isolation and further characterization of *Frankia* molecules perceived by the root system (2,4)
- the hormone auxin plays an important role during plant cell infection in actinorhizal symbiosis (5)
- although the actinorhizal nodule is comparable with a symbiotic lateral root, the molecular mechanisms involved in primordia initiation in lateral roots may differ from those in actinorhizal nodules.

The major challenges in the coming years will be 1) the biochemical characterization of *Frankia* symbiotic signal(s); 2) the isolation of the Frankia symbiotic signal(s) receptor(s); 3) understanding how *Frankia* triggers lateral root program to initiate actinorhizal primordium.

This work was supported by the Institut de Recherche pour le Développement (IRD) and the Agence Nationale de la Recherche (ANR).

(1) Zhong et al. (2013) J. Biosci. 38 : 815-823
(2) Froussart et al. (2016) Symbiosis DOI 10.1007/s13199-016-0400-4
(3) Svistoonoff et al. (2013) PLoS ONE 8: e64515
(4) Clavijo et al. (2015) New Phytol. doi: 10.1111/nph.13506
(5) Perrine-Walker et al (2010). Plant Physiol. 154: 1372-80

O-03.2

Cluster II *Frankia* strains: symbioses with Cucurbitales and Rosales

Katharina Pawlowski[1], Marco Salgado[1], Thanh Van Nguyen[1], Irina V. Demina[1], Tomas Persson[1], Brian Vanden Heuvel[2], Kai Battenberg[3], Daniel Wibberg[4], Jörn Kalinowski[4], Christoph Ziegenhain[5], Wolfgang Enard[5], Max Griessmann[5], Martin Parniske[5] and Alison M. Berry[3]

[1]Stockholm University, Department of Ecology, Environment and Plant Sciences, Stockholm, Sweden
[2]Department of Biology, Colorado State University, Pueblo, CO, USA
[3]Department of Plant Sciences, University of California, Davis, CA, USA
[4]CeBiTec, Bielefeld University, Bielefeld, Germany
[5]Faculty of Biology, University of Munich (LMU), Martinsried, Germany

Two types of root nodule symbioses exist, legume/rhizobia and actinorhizal symbioses. In the latter, nitrogen-fixing soil actinobacteria of the genus *Frankia* induce the formation of root nodules on a diverse group of dicotyledonous plants from eight different families from three different orders, Fagales, Cucurbitales and Rosales. Phylogenetically, symbiotic frankiae can be divided in three clusters. Cluster II is basal[1]; these strains show strong genome reduction and with one exception[2] could never be cultured. The strains of cluster II can enter symbioses with host plants from four different families present on four different continents, actinorhizal Rosaceae and and one rhamnaceous genus, *Ceanothus*, from the order Rosales and Coriariaceae and Datiscaceae from the order Cucurbitales. Among the host plants of cluster II, the first group by far to speciate was *Coriaria* (Coriariaceae), suggesting that the Cucurbitales symbioses preceded the Rosales symbioses.

Actinorhizal nodules from different host plant genera show great diversity regarding anatomy, *Frankia* differentiation in infected cells and nodule metabolism. We analyszd specific features of the Cucurbitales symbioses using the host species *Datisca glomerata.* E.g., since the infection pathway in Cucurbitales has not been characterized yet, the transcriptomes of infected and uninfected cortical cells of *D. glomerata* nodules in order to identified infected cell-specific genes. Then, the plant and bacterial transcriptomes of from nodules of *D. glomerata* were compared to those from *Ceanothus thyrsiflorus* (Rosales).

(1) T. Persson et al. (2015) PLoS One 10:e0127630
(2) M. Gtari et al. (2015) Scientific Reports 5:13112

O-03.3

Genes, genomes and genetics of *Frankia* and the actinorhizal symbiosis.

Louis S. Tisa[1], Rediet Oshone[1], Arnab Sen[2], Faten Ghodhbane-Gtari[3], and Maher Gtari[3]

[1]Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, Durham, NH USA, louis.tisa@unh.edu

[2] NBU Bioinformatics Facility, Department of Botany, University of North Bengal, Siliguri, 734013, India

[3] Laboratoire Microorganismes et Biomolécules Actives Université Tunis El Manar (FST) & Université Carthage (INSAT). Campus universitaire, 2092, Tunis, Tunisie

Frankia forms nitrogen-fixing symbioses with 8 different Angiosperm families, commonly known as actinorhizal plants. *Frankia* sp. strains are generally classified into one of four major phylogenetic groups that have distinctive plant host ranges. Symbiotic interactions between *Frankia* and the host plant are not well understood and very little is known about the initial molecular interactions in the rhizosphere. The nature of the chemical signals exchanged between the two partners of actinorhizal symbioses is still unknown. *Frankia* has the ability to bind and sequester several toxic heavy metals and it has potential bioremediation and phytoremediation applications especially on heavy-metal-contaminated land. Actinorhizal plants are able to tolerate harsh environmental conditions including salt stress and exposure to toxic metals, metalloids and aromatic hydrocarbons. Their symbiotic association with the actinobacteria *Frankia* has been proposed to play a role in the ability of these plants to survive under harsh environmental conditions. Due to the absence of genetic tools for *Frankia*, we have also pursued new genomic approaches toward studying these bacteria. Over twenty-five *Frankia* genomes have been sequenced providing opportunities to use bioinformatics approaches and other new technologies. A positive correlation between genome size and plant host range was suggested from these data: Larger genomes had broader host ranges. The absence of obvious nodulation genes similar to those found in *Rhizobia* genomes suggests that the actinorhizal symbiosis uses novel signal compounds during the infection process. Analysis of the *Frankia* genomes also demonstrated the presence of unexpected numbers of secondary metabolite gene clusters and potential novel natural products as candidates. RNASeq results for three broad-host-range *Frankia* strains (EAN1pec, EuI1c and EUN1f) grown under nitrogen-replete (NH_4) and nitrogen-deficient (N_2) conditions will be presented. Lastly, we will discuss our efforts at developing a genetic system for *Frankia* and first successful introduction of plasmid into these bacteria.

O-03.4

Dryas: a model genus for root symbioses of the *Rosaceae* family

B. Billault-Penneteau[1], K. Pawlowski[2], D. Reinhardt[3], M. Parniske[1]

[1] Faculty of Biology, Genetics, University of Munich (LMU), Martinsried, Germany.
[2] Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm, Sweden
[3] University of Fribourg, Department of Biology, Fribourg, Switzerland

The Root Nodule Symbiosis (RNS) evolved several times independently but exclusively in the *Fabales*, *Fagales*, *Cucurbitales*, and *Rosales* (the *FaFaCuRo* clade) based on a putative genetic predisposition acquired by a common ancestor.[1,2] More than 200 species of dicotyledonous plants, mostly trees and shrubs, belonging to eight families and 24 genera can enter actinorhizal symbioses with nitrogen-fixing soil actinomycete of the genus *Frankia*. Among these actinorhizal plants, the *Rosaceae* family represents a particular attractive family to test evolutionary hypotheses related to nodulation, because RNS in this family a single genus, *Dryas*, contains closely related nodulating and non-nodulating species. This important agronomical plant family is well studied and is subject to several genomic approaches but there is a lack of data on the four genera *Cercocarpus*, *Chamaebatia*, *Dryas*, and *Purshia* engaged in RNS[3].

Compared to other actinorhizal plants, relatively few investigations focussed on nodulating *Rosaceae*. Nitrogen-fixation nodules were observed on roots of the North American *Dryas drummondii*[3,4], but not on the European *D. octopetala*[5]. This latter species is also used in ectomycorrhiza studies[6].These features make the genus Dryas ideal for the study of the evolution of RNS and of root symbioses in general. The two Dryas species are the subject of a *de-novo* genome-sequencing project. We have established techniques and protocols for these plants allowing transcriptomic and genetic approaches. These tools have already enabled novel discoveries regarding root symbioses within the genus *Dryas.*

(1) J.J. Doyle (2011) MPMI 24:1289
(2) G.D.A. Werner et al. (2014) Nat. Commun. 5:4087
(3) S.J. Kohls et al. (1994) Plant and Soil 162: 229
(4) W. Newcomb (1981) Can. J. Bot. 59:2500
(5) G. Bond (1976) Cambridge University Press, pp. 443–474
(6) M.F.M. Bjorbaekmo et al. (2010) BMC Plant Biology 10:244

O-03.5

A system biology approach to analyze salt stress tolerance in *Casuarina glauca* and the contribution of symbiotic *Frankia* bacteria

Ana I. Ribeiro-Barros[1, 2], Inês Graça[1], Tiago Jorge[2], Paula Alves[1], Katharina Pawlowski[3], Carla António[2], José C. Ramalho[1]

[1]PlantStress&Biodiversity Group, Instituto Superior de Agronomia (ISA), Universidade de Lisboa (ULisboa), Lisboa, Portugal
[2]Plant Metabolomics Laboratory, ITQB NOVA, Instituto de Tecnologia Química e Biológica António Xavier
[3]Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm, Sweden

Soil salinization is a major land degradation problem and is increasing steadily in many parts of the world. It is estimated that the continuous salinization of arable land, associated with low rainfall, high evaporation, saline irrigation water and poor water management, will result in losses of 30% of agricultural land over the next 25 years, increasing to 50% by 2050 (1). Thus, salinity is one of the most important challenges for agricultural systems.
The actinorhizal tree *Casuarina glauca* tolerates extreme environmental conditions, such as high salinity. This species is also able to establish a root-nodule symbiosis with N_2-fixing bacteria of the genus *Frankia*. In order to analyse the mechanisms underlying salt stress tolerance in *C. glauca*, we have examined the impact of increasing NaCl concentrations (200, 400 and 600 mM) on leaves and nodules of symbiotic and non-symbiotic plants. The symbiosis with *Frankia* Thr was turned off at 200 mM NaCl (2). Even so, the first stress symptoms (e.g. leaf chlorosis and necrosis) were observed only at 600 mM NaCl in both plant groups (3). The innate salinity tolerance was connected with photosynthetic adjustments, membrane preservation and a controlled oxidative state (2, 4).
Analysis of the branchlet transcriptomes by NGS identified ca. 180,000 contigs, with a great diversity of functions, including stress-related-, metabolic-, transport- and regulatory genes, with a marked differential expression at 400 and 600 mM NaCl. LCMS/MS and SWATH analysis identified 357 proteins, of which ca. 100 were differentially expressed in the presence of salt. Stress-responsive proteins were mainly associated with energy, amino acid and carbohydrate metabolism. GC-TOF-MS metabolite profiling of branchlet tissue revealed 26 primary metabolites, ranging from sugars to amino- and organic acids, most of which decreased under stress conditions. Altogether, the results of high-throughput analyses are in line with the morphological, physiological and biochemical data, reflecting the strong capacity of *C. glauca* to cope with salt stress.

Acknowledgements: Fundação para a Ciência e Tecnologia (FCT), project PTDC/AGRFOR/4218/2012 (AIRB), FCT Investigator Program IF/00376/2012/CP0165/ CT0003 (CA), ITQB research unit GREEN-it BBioresources for sustainability^ (UID/Multi/04551/2013), and grant PD/BD/113475/2015 (TFJ).

(1) Wang et al. (2003) Planta 218:1-14
(2) Duro et al. (2016) Plant Soil 398:327-337
(3) Batista-Santos et al. (2015) Plant Physiol. Biochem. 96:97-109
(4) Scotti-Campos et al. (2016) J Plant Physiol. 196:60-69

O-03.6

Green alder (*Alnus viridis*, Chaix, DC) encroachment shapes differently fungal and bacterial communities in subalpine soils

Guillaume Schwob[1], Mélanie Roy[2], Thomas Pommier[1], Maria P. Fernandez[1]

[1] Laboratoire Ecologie Microbienne, UMR CNRS 5557 / UMR INRA 1418, Université Claude Bernard Lyon I, Villeurbanne, France
[2] Laboratoire Evolution et Diversité Biologique, UMR 5174, Toulouse, France

Since the abandonment of human agropastoral activities in Alps mountains, subalpine grasslands undergo a rapid and progressive biological invasion of *Alnus viridis* shrubs. Thanks to its stolons, its nitrogen fixing symbiose with the actinobacteria *Frankia* and its ectomycorhizal cohorts, green alders outcompete others subalpine tree species and quickly evolve from a mosaic stage of some patchy individuals to a thick and closed forest associated with a loss of biodiversity (1). While impacts of *Alnus viridis* encroachment on soil properties, plant, arthropod and ornithological diversities are well described (2,3,4), little is known about its density effect on soil microbial communities, its symbionts (bacterial and fungal) detection and distribution in soils and on the functional modifications in response to this ecological succession in subalpine ecosystems. The 3 colonization stages (grassland, mosaic and forest) were studied in two different sites in the Vanoise National Park (Savoie, France). Symbionts distribution, microbial richness and communities structure in both soils and nodules were analyzed by metabarcoding of 3 bacterial (*16S rDNA*, *nifH* and *amoA*) and 1 fungal (ITS1) genes. Pedological analysis were performed and functional diversity of N cycle bacteria evaluated by nitrification and denitrification enzyme assays. *Frankia*, and *Alnus*-specific ectomycorhizal fungi, were detected in all samples regardless of the ecological stage. While *Frankia* abundance and diversity didn't vary significantly, ectomycorhizal fungi abundance increased together with the increase of the host density. Site and colonization stage both shape fungal communities structure. In the case of bacteria, one site harboured ecological stage effect on both community structures and functional activities. Indeed, each colonization stage revealed specific ammonia oxidizing and N2 fixing communities and different nitrification activity levels.
These results show that (i) *Alnus viridis* drives differently bacterial and fungal communities, (ii) its encroachment impacts both structural and functional diversity of bacteria related to the N cycle, (iii) the distribution in soils of its bacterial symbionts (*Frankia*) is weakly influenced by the host density. These results are discussed in the light of the possible status of obligate symbiont of *Frankia* strains associated to green alder (5). This study provides a better understanding of the impact of *Alnus viridis* encroachment on abandoned subalpine pastures and the seek for optimal solutions of management.

(1) Anthelme et al. (2003) Arct. Antarct. Alp. Res. 35:48-55
(2) Bühlmann et al. (2014) Alp Bot 124(2):187-191
(3) Anthelme et al. (2001) Forest Ecol Manag 145:57-65
(4) Hiltbrunner et al. (2014) Oecologia, 176: 11-24
(5) Pozzi et al. (2015) Environ Microbiol, 17:3125-3138

O-04.1

Marine unicellular symbiotic cyanobacteria: significance in global ecology, physiology and evolution

Jonathan P. Zehr, Maria del Carmen Munoz-Marin, Hanna Farnelid and Kendra Turk-Kubo

Department of Ocean Sciences, University of California, Santa Cruz, CA 95064 USA

Nitrogen fixation is an important source of fixed nitrogen for open ocean oligotrophic gyre ecosystems. Historically, nitrogen fixation was believed to be primarily due to the filamentous cyanobacterium *Trichodesmium*. Flow cytometry and metagenomics led to the discovery that an uncultivated, widespread nitrogen-fixing unicellular cyanobacterium living in symbiosis with a small single-celled alga, the haptophyte *Braarudosphaera bigelowii* (1). This organism has significance for ocean ecology, physiology of nitrogen fixation in cyanobacteria, and has implications as a model for evolution of symbiosis and organelles. The cyanobacterium (called UCYN-A) has a 1.4 Mb streamlined genome and it is unusual because lacks photosystem II, Rubisco and the entire TCA cycle, while it retains photosystem I. It is now known that there are divergent, but closely-related strains with the same metabolic deletions (2), and it is unclear how this simple single-celled symbiosis functions. The cyanobacterium still has not been cultivated and cannot be easily visualized. Moreover, its abundance and activity cannot be easily quantified in the ocean. Despite this, the cyanobacterium has been detected using cultivation-independent molecular methods in large areas of the oceans, and even has been detected in the sequences obtained in the global TARA and MALASPINA expeditions (2). The large geographic distribution shows that UCYN-A is likely to be of quantitative importance in the ocean nitrogen cycle. The ecological significance of the recently described genetic diversity is not yet understood, but, fluorescent in situ hybridization probes for the subgroups has provided information on their cellular structure; there are multiple UCYN-A cells per haptophyte in UCYN-A2, compared to the single cell in UCYN-A1. There may be depth or spatial differences in niches, and certainly there are physiological differences in how molecules and energy are transferred in the symbiosis. Evolutionarily, the close interaction of the cells and extensive streamlining is reminiscent of what must have been the early events leading to the evolution of organelles, such as the chloroplast. In summary, this unique and globally-important organism is an intriguing model of evolution in action, that presents intriguing questions regarding the physiology of nitrogen fixation.

(1) Thompson, Foster et al. (2012) Science 337: 1546-1550
(2) Bombar, Heller et al. (2014) ISME J. 8:2530-2542
(3) Cornejo-Castillo et al. (2016) Nat. Comm. | DOI: 10.1038/ncomms11071

O-04.2

Cyanobacterial communities associated with boreal feather mosses – from gene to ecosystem

Ulla Rasmussen[1], Guillaume Bay[2], David A. Wardle[2], Marie-Charlotte Nilsson[2], Christopher L. Dupont[3], Philip D. Weyman[3], Denis Warshan[1]

[1]Department of Ecology, Environment and Plant Sciences, Stockholm University, Sweden
[2]Department of Forest Ecology and Management, SLU, Umeå, Sweden
[3]J. Craig Venter Institute, La Jolla, CA, USA

Diazotrophs in symbiosis with plant hosts constitute an important input of nitrogen into many terrestrial ecosystems. In the nitrogen limited boreal forest the input of nitrogen from cyanobacteria living in symbiosis with pleurocarpous feather mosses (e.g. *Pleurozium schreberi* and *Hylocomium splendens*) represent the main pathway of biological nitrogen input into the ecosystem (1). Studies on the cyanobacterial communities living in symbiosis with the feather mosses have shown a high host specificity (2). Given the ecological importance of the cyanobacteria-moss interaction little is known about the diversity of the cyanobacterial community - its structure, seasonal and host specific changes and functional diversity. We have by using specific *nifH* primers targeting four major cyanobacterial clusters and quantitative PCR, investigated how community composition, abundance and *nifH* expression varied by moss species and over the growing seasons. We demonstrate a temporal and host-dependent dynamic of the cyanobacterial communities (i.e. composition, *nifH* abundance and *nifH* expression). Variation in N_2-fixation rates are greatly explained by temporal changes in cyanobacterial *nifH* expression, especially of the genus *Stigonema* which implies that this cyanobacterial genus might - although not dominating - be the most influential N_2-fixer in this ecosystem (3). Our findings provide insights on N_2-fixation rate variations but also highlight the lack of knowledge about factors controlling the establishment, maintenance, composition and activity of the moss-cyanobacteria symbiosis and its eventual consequences for N input into boreal forests. Knowing, that the cyanobacteria and its host are communicating prior to infection (4) we are by using a genomic and transcriptomic approach currently searching for genes which might be of importance for the establishment of the moss-cyanobacteria symbiosis.

(1) DeLuca et al. (2002) Nature 419:917–920
(2) Ininbergs et al. (2011) New Phytol. 192:507–517
(3) Warshan et al. (2016) ISME doi:10.1038/ismej.2016.17
(4) Bay et al. (2013) New Phytol. 200:54–60

O-04.3

Defining the diversity, importance and activity of microbial communities associated with *Trichodesmium* colonies

Eric A Webb[1], Michael Lee[1], Nathan G Walworth[1], Feixue Fu[1], David A Hutchins[1], Tracy Mincer[2]

[1]Marine and Environmental Biology, University of Southern California, Los Angeles, CA 90089,USA

[2]Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Woods Hole, MA 02543 USA

Marine N_2 fixation is an important source of new nitrogen to the oligotrophic oceans. For example the combined nitrogen produced via the activity of N_2 fixing (diazotrophic) organisms can exceed NO_3 flux into the euphotic zone, and thus can fuel over half of new primary production (1). The colony-forming, cyanobacterium *Trichodesmium* has been shown to be a critically important diazotroph in the tropics and subtropical oceans, but there is still much that we do not know about their activity, evolution and diversity. The *Trichodesmium* colony environment is an oasis of fixed N and C in the oligotrophic oceans and contains an assemblage of metabolically diverse organisms (or epibionts). Recently, using cultured representatives, metagenomes and RNAseq, it was shown that the *Trichodesmium* genus has a unique gene-sparse genome littered with large, conserved, expressed intergenic spaces (2) - a finding that stands in contrast to its sympatric cyanobacteria (and all other free living bacteria, e.g., (3, 4)). It is known that cohabitating organisms develop relationships that can alter the course of their genomic evolution (i.e., genomic minimization, inflation etc) and affect the activity of both taxa (*4*, *5*). Using both field and laboratory experiments, our data support the importance of interaction between *Trichodesmium* and specific colony-inhabiting, methylotrophic bacteria in modulating *Trichodesmium* N_2 fixation as well as genome evolution in both taxa.

(1) J. A. Sohm, E. A. Webb, and D. G. Capone, (2011) Nat Rev Micro. 9, 499–508
(2) N. Walworth et al., (2015) P.N.A.S. 112, 4251–4256
(3) B. K. Swan et al., (2013) P.N.A.S. 110, 11463–11468
(4) S. J. Giovannoni, J. C. Thrash, and B. Temperton, (2014) ISME J. 8, 1553–1565
(5) J. J. Morris, R. E. Lenski, and E. R. Zinser, (2012) mBio. 3, e00036–12–e00036–12

O-04.4

Exploring diazotrophic growth processes in marine cyanobacteria using combined, experimental and modeling approaches

Sophie Rabouille

srabouille@obs-vlfr.fr

The discovery of new diazotrophic organisms in the world's ocean has widened the puzzle of the oceanic, nitrogen budget. Experiments performed on the planktonic, cyanobacterial strains cultured to date have shed some light on their respective physiological properties and growth efficiencies. Even though these strains are only a few amongst the wide diversity of nitrogen fixers, they do take a share in the global marine primary production. But how significant is this share in the world ocean? Global scale modeling approaches are best suited to tackle this question; yet current biogeochemical models fail in predicting observed rates. This inaccuracy is likely to be related to the very simplistic representation of nitrogen fixation. With the aim to analyze and discuss what level of information matters that should be incorporated in biogeochemical models, we developed models of nitrogen fixation at the organism scale to describe growth processes in response to environmental conditions. These models are used as virtual laboratory and compared to culture data to gain better understanding of the metabolic controls of nitrogen fixation.

Keywords: nitrogen fixation, physico-chemical forcing, cultures, modeling

O-04.5

Nitrogenase and Photosystem II: the Ying and the Yang in cyanobacterial nitrogen fixation

Himadri B. Pakrasi, Deng Liu, Michelle Liberton, Maitrayee Bhattacharyya-Pakrasi

Department of Biology, Washington University, St. Louis, MO 63130, USA

Cyanobacteria are unique among prokaryotes for their ability to perform oxygenic photosynthesis and for their circadian lifestyle. Unicellular cyanobacteria like *Cyanothece* are fascinating organisms that actively use both phases of the diurnal cycle to separate and carry out antagonistic metabolic processes. The intracellular environment oscillates between aerobic and anaerobic states during the day-night period, allowing oxygen-sensitive processes like nitrogen fixation to occur at night while photosynthetic oxygen evolution takes place during the day. This diurnal periodicity endows a highly dynamic profile to these organisms, which have been captured at the genome, transcriptome, proteome and ultrastructural levels. Whole genome analysis of six *Cyanothece* strains revealed that this group of unicellular nitrogen fixing cyanobacteria specialize in metabolic compartmentalization and energy storage, concomitantly accumulating metabolic products in inclusion bodies that are later mobilized as part of a robust diurnal cycle (1, 2). Transcriptomic analyses showed that the existence of these incompatible processes in the same single cell depends on tightly synchronized expression programs involving ~30% of genes in the genome, a phenomenon that also extends to a large extent at the protein level (3). To examine the proteome of *Cyanothece,* we used a high-throughput accurate mass and time (AMT) tag approach (4). We have also examined the dynamic profiles of all of these proteins during a diurnal period (5). Three-dimensional tomographic reconstructions showed that the thylakoid membranes in *Cyanothece* form a dense and complex network that extends throughout the entire cell (6). In particular, the organization of these membranes and various intracellular bodies profoundly change between day and night periods. Oxygen evolution in cyanobacteria is catalyzed by photosystem II (PSII), a large membrane bound molecular machine. Since, PSII activity severely impairs the nitrogen fixation activity in *Cyanothece* cells, the protein composition of PSII undergoes subtle modifications that prevent PSII to oxidize water to molecular oxygen during the nitrogen fixation period (7). Together, these studies have provided a comprehensive picture of how a physiologically relevant diurnal light-dark cycle influences the metabolic behavior of a diazotrophic photosynthetic bacterium.

(1) Welsh, E. A., et al. (2008) Proc Natl Acad Sci U S A 105, 15094-15099
(2) Bandyopadhyay, A., et al. (2011) mBio 2, doi: 10.1128/mBio.00214-11
(3) Stöckel, J., et al. (2008) Proc Natl Acad Sci U S A 105, 6156-6161
(4) Stöckel, J., et al. (2011) PLoS One 6, e16680
(5) Aryal, U. K., et al. (2011) BMC Syst. Biol. 5, 105
(6) Liberton, M., et al. (2011) Plant Physiol 155, 1656-1666
(7) Wegener, K. M. et al. (2015) J. Biol. Chem. 290, 3764-3774

[These studies have been supported by United States Department of Energy and National Science Foundation]

O-05.1

Non-photosynthetic *Bradyrhizobium* as rice endophyte and its molecular interaction and application aspects

Pongdet Piromyou[1], Pongpan Songwattana[1], Teerana Greetatorn[1], Takashi Okubo[3], Kaori Chiba Kakizaki[3], Janpen Prakamhang[2], Panlada Tittabutr[1], Nantakorn Boonkerd[1], Kiwamu Minamisawa[3], Neung Teaumroong[1]

[1]School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, 30000, Thailand
[2]Department of Applied Biology, Faculty of Sciences and Liberal Arts, Rajamangala University of Technology-Isan, 30000, Thailand
[3]Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan

Plant associations by bradyrhizobia are not only found in leguminous plants but also in non-leguminous species including rice. Samples were collected from various rice (*Oryza sativa*) tissues and crop rotation systems. Six bradyrhizobial strains were obtained exclusively from rice with crop rotational system, not from rice monoculture system. The isolates were separated into photosynthetic bradyrhizobia (PB) and non-photosynthetic bradyrhizobia (Non-PB). Thai bradyrhizobial strains promoted rice growth of Thai rice cultivars better than the Japanese strains. This implies that the rice cultivars have the factors governing rice-bacteria associations. However, strain SUTN9-2 promoted highest total rice (*Oryza sativa* L. cultivar Pathum Thani 1) dry weight among endophytic bradyrhizobial strains tested. Therefore, SUTN9-2 was selected for further conducting in draft genome sequencing. Some known bacterial genes involved in bacteria-plant interaction were selected. Expression of type III secretion component (*rhcJ*), type IV secretion component (*virD4*) and pectinesterase (*peces*) genes of the bacterium were found up-regulated when the rice root exudate was added in the culture. When SUTN9-2 was inoculated into rice seedlings, genes *peces*, *rhcJ*, *virD4* and exopolysacharide production (*fliP*) were highly expressed in the bacterium during 6-24 hours after inoculation. In addition, gene for glutathione-*S*-transferase (*gst*) was slightly expressed after 12 hours after inoculation. To examine whether T3SS is involved in bradyrhizobial infection to rice plants, wild-type SUTN9-2 and T3SS mutant strains were inoculated into the original host plant (*A. americana*) and rice plant (cultivar Pathum Thani 1). T3SS mutants reduced their ability to invade rice tissues as compared with wild-type strain, although their phenotypes in *A. americana* were not changed by T3SS mutations. These results suggested that T3SS is one of the important determinants to modulate the rice infection, although T4SS and pectinesterase are also likely to be responsible for the early steps of rice infection. In addition, the strain SUTN9-2 was used as rhizobium inoculum for mung bean in the form of rice stubble. The results revealed that SUTN9-2 still persisted in rice tissues until rice-harvesting season. After harvesting the rice, mung beans were planted to the same pot for 3 weeks. The results showed nodulation of GUS-tagging bradyrhizobia SUTN9-2 in mung bean. Therefore, it is possible that rice stubble can be used as inoculum in the rice-legume crop rotation system.

O-05.2

Bradyrhizobium isolates from sugarcane roots represent a new phylogenetic group with potential for the development of inoculants

Gustavo F. de Matos[1], Jerri E. Zilli[2], Jean L. Simões de Araújo[2], Veronica M. Reis[2], José I Baldani[2], Luc F. M. Rouws[2]

[1]Pós-Graduação em Agronomia (Fitotecnia), Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, Brazil

[2]Embrapa Agrobiologia, Seropédica, RJ, Brazil

Molecular biological studies have demonstrated the presence of *Bradyrhizobium*-related *nifH* mRNA phylotypes in sugarcane, suggesting a role of this bacterial genus, which is well known as legume microsymbiont, in biological nitrogen fixation (BNF) associated with sugarcane, a Poaceae family member (1, 2). In a follow up study, the use of cowpea as a 'trap plant' allowed the cultivation of a diverse collection of *Bradyrhizobium* spp. endophytes from sugarcane roots (3). In this collection of 109 isolates, 36 isolates were highly similar as determined by BOX-PCR analysis. A greenhouse growth promotion experiment was carried out and the representative strain, P9-20, of this group showed a significant positive effect on sugarcane root volume after 30 days growth. In addition, the acetylene reduction assay demonstrated that strain P9-20 had a low but consistent nitrogenase activity *in vitro* in a semi-solid culture medium. Based on these results, four representants of the group with 36 isolates were characterized in more detail. Multilocus sequence analysis (MLSA) placed these isolates in the *B. japonicum* superclade, on an independent branch near *B. huanghuaihaiense*, a soybean microsymbiont. A similar phylogeny was observed for the *nodC* gene, although inoculation assays showed that the novel strains were unable to nodulate soybean. Sequencing and annotation of the whole genome of strain P9-20 revealed the presence of 43 genes involved with nitrogen fixation including two copies of *nifHDK.* Phylogenetic analyses of the *nifH*1 and *nifH*2 genes showed that they were divergent in terms of nucleotide sequence, which is unusual in other studied species/strains of the genus *Bradyrhizobium*. Possibly this result is related with the specific lifestyle of these new isolates. Here, the most recent findings of this ongoing study will be presented.

(1) Fischer et al. (2012) Plant Soil. 356:83-99
(2) Thaweenut et al. (2011) Plant Soil. 338: 435-449
(3) Rouws et al. (2014) Env. Microbiol. Rep. 6:354–363

O-05.3

Microbial nitrogen cycling in rice fields

Mugdha Sabale, Mukta Chinchwadkar, Barbara Reinhold-Hurek and Thomas Hurek

Institute of Microbe-Plant Interactions, Faculty of Biology and Chemistry, University of Bremen, Germany

With maize and wheat, rice is globally one of the most important crops for food production. The area under rice and maize cultivation covers more than 155 million hectares for each, moreover the largest number of people depends on rice as a staple food. In Asia, where 90% of the rice is produced and consumed, the most productive cultivation systems are flooded paddy fields. Traditionally, two to three rice harvests per year can be gained in irrigated systems. Due to water consumption, many lowland rice areas in Asia are undergoing a transition that involves adoption of new management strategies, with crop rotations encompassing a non-flooded crop, including maize. Shifting from flooded to non-flooded cropping is likely to affect microbial nitrogen cycling. To analyze consequences of these shifts, large-scale field plots with triplicate repetitions were set up in a cooperative ICON project (Introducing non-flooded crops in rice-dominated landscapes: Impact on carbon, nitrogen and water budgets) at the International Rice Research Institute in the Philippines. Major processes comprising the N cycle - denitrification, nitrification, biological N_2-fixation (BNF) - strongly depend on the redox situation that is likely to change in soil and root environments with altered management regimes.

Therefore, cultivation-independent methods were developed to detect populations and transcriptional activities for key processes. To assess the abundance of a key-enzyme-coding marker gene in a bacterial population, Real-time-PCR can be used on DNA, or be coupled to a step of reverse transcription to detect the transcripts and thus activity. For both applications, appropriate positive controls and standards are required for quantitative PCR. Thus we cloned and sequenced respective gene fragments from samples of ICON experiments, and used these plasmid DNAs as standards to calculate DNA copy numbers. For RNA samples, also reverse transcription reactions should be taken into account for accurate quantifications.

Thus we developed *in vitro* - transcribed, known templates for all primer systems used. For the following genes, quantitative RT-PCR reactions were established: *amoA* (nitrifying *Bacteria*), a*moA* (nitrifying *Archaea*), *nirK* (denitrifying *Bacteria*), n*irS* (denitrifying *Bacteria*), n*osZ* (denitrifying *Bacteria*), *nifH* (nitrogen-fixing *Bacteria, Archaea, nif/anf/vnf*). The methods were applied to analyze microbial communities during the dry season on ICON plots. We compared soil and root compartments and also different management regimes: We analyzed different fertilizer applications (zero and conventional N-fertilizer application) in fields without crop rotation (only flooded rice grown), non-flooded rice management, and maize. Depending on the management regimes, different parts of the microbial nitrogen-cycle were active, also depending on the compartment.

O-05.4

How Does Sugarcane Fix Nitrogen: A Rich and Diverse Microbiome Suggests That Complex Bacterial and Fungal Assemblages Contribute to Sugarcane Growth and Sustainability

Rafael S.C. de Souza,[1] Vagner K. Okura,[1] Jaderson S.L. Armanhi,[1] Beatriz Jorrín,[2] Márcio José da Silva,[1] Manuel González-Guerrero,[2] Laura M. de Araújo,[1] Natália F. Verza,[1] Juan Imperial[2,3] and Paulo Arruda[1,4]

[1] CBMEG, Unicamp, Campinas, Brazil
[2] CBGP, UPM-INIA, Madrid, Spain
[3] Consejo Superior de Investigaciones Científicas, Madrid, Spain
[4] Dept. Genética e Evolução, Unicamp, Campinas, Brazil

Sugarcane fields in Southeastern Brazil continuously produce yields in the neighborhood of 150 Tm / Ha with little fertilization. After the initial observations carried out by Joanna Döbereiner and the continued work of her team and former associates, it is widely accepted that the plant benefits from bacterial nitrogen fixation. A large number of remarkable sugarcane endophytes, some of them with the ability to fix nitrogen, have been isolated and characterized. However, their role in sugarcane nutrition has not been unequivocally substantiated. Having in mind that the intensive sugarcane cropping regime should impose a burden not only on the plant N economy but also on the supply of other nutrients, and that sugarcane-associated microorganisms could play a role in alleviating these burdens, we undertook the characterization of the sugarcane microbiome, along its development and in its different organs. Here we describe a comprehensive inventory of the structure and assemblage of the bacterial and fungal communities associated with sugarcane. Our analysis identified 23,811 bacterial OTUs and an unexpected 11,727 fungal OTUs inhabiting the endosphere and exosphere of roots, shoots and leaves. These communities originate primarily from the native soil around plants and colonize plant organs in distinct patterns. We identified core bacterial and fungal communities composed of less than 20% of the total microbial richness but accounting for over 90% of the total microbial relative abundance. The roots showed 89 core bacterial families, 19 of which accounted for 44% of the total relative abundance. Stalks are dominated by a diverse group of yeasts that represent over 12% of total relative abundance. The core microbiome described here comprises groups, whose biological role underlies important traits in plant growth and fermentative processes that will be discussed.

Supported by Repsol (Spain) and by Repsol / Sinopec (Brazil).

O-05.5

Use of trap plants to isolate PGPB strains from soils under different land uses and its application as inoculant for non-legumes

André L. Martinez Oliveira, Mónica Y. Alzate-Zuluaga, Karina M.Lima Milani, Odair José A. P. Santos, Amanda Aleixo, Danielle Cristina Ferreira, Karita dos Reis Costa

Departamento de Bioquímica e Biotecnologia, Universidade Estadual de Londrina - UEL, Londrina, Brazil

The soil microbiome is strongly modulated by the quality and quantity of root exudates, a phenomenon known as 'rhizosphere effect' that induces increase in the population density of few bacterial taxa, while lowers the bacterial diversity found in the non-rhizospheric soil (1). This phenomenon was explored by growing maize or tomato in soils from different land uses following the isolation of nitrogen-fixing bacteria able to colonize these plants in population densities in excess of 1 x 10^5 cells g^{-1} root+rhizosphere. Representatives of the culturable pool from the root+rhizosphere residing bacteria were characterized (biochemical traits) and phylogenetically positioned (16S rRNA gene sequencing) to identify potential plant growth-promoting bacteria (PGPB). Inoculation trials under greenhouse and field conditions were performed to confirm the growth-promoting ability of selected strains. The bacterial community accessed from maize plants comprised 107 diazotrophic isolates, mainly from the Proteobacteria domain (87% of isolates). From these collection, 54 isolates promoted the initial development of maize plants (10 days of growth, seed-inoculated plants) while 8 isolates decreased the plant development. 17 isolates improved the initial growth of maize plants in up to 30% (compared to uninoculated plants), following the evaluation under greenhouse conditions. These strains were used to prepare liquid and solid inoculant formulations, this last using as vehicle a biodegradable foam prepared by extrusion of a starch/cane bagasse-based mixture. From the isolates tested under field conditions so far, three strains showed consistent results as PGPB for maize: *Rhizobium* sp. strain 8121, *Pseudomonas* sp. strain 4331, and *Azomonas* sp. strain 4311. Among other effects observed in the physiology and development of plants, maize inoculation with the selected strains could substitute up to 70% of maize N-fertilizer demand without decrease the productivity. In this same sense, the use of tomato as trap-plant to access the soil bacterial community with plant growth-promoting potential resulted in 49 bacteria isolates comprising 8 different genera: *Rhizobium* (32 isolates), *Variovorax* (5 isolates), *Burkholderia* (3 isolates), *Pseudomonas* (3 isolates), *Caulobacter* (1 isolate), *Herbaspirillum* (1 isolate), *Massilia* (1 isolate) and *Roseateles* (1 isolate). Although the use of tomato as trap-plant resulted in a lower diversity of isolates as compared to maize, we found 16 bacterial strains that increased the dry weight of tomato seedlings in up to 30%, from which four bacterial strains (*Pseudomonas* sp. strain 17T, and *Rhizobium* sp. strains 4T, 21T and 41T) were selected and are under evaluation in a greenhouse trial to confirm its growth-promoting ability. In conclusion, the approach adopted to access soil bacteria by the use of trap-plants expanded the knowledge of the PGPB diversity in Brazilian soils, indicating new potential strains to produce commercial inoculants for maize and highlighting the putative role of Rhizobium as associative bacteria in non-leguminous plants as tomato.

Acknowledgements: CNPq; Fundação Araucária, CAPES, INCT-FBN

(1) Philippot et al. (2013) Nat. Rev. Microbiol. 11:789-799

O-05.6

Robust biological nitrogen fixation in wheat and maize under soil conditions

Nicolas A. Ayub

Instituto de Genética Ewald A. Favret (CICVyA-INTA), Buenos Aires, Argentina

Plant growth promotion and in some cases detectable levels of biological nitrogen fixation (BNF) have been reported for various non-legume plant species inoculated with diazotrophic bacteria. In model systems, in which plants were inoculated with the diazotrophs under gnotobiotic conditions, significant BNF and contribution to plant nitrogen have been observed, when the bacteria were mutated or engineered to excrete ammonium. However, agronomic exploitation of these findings necessitates that the inoculant bacteria are able to compete with diverse microbial communities resident in the plant rhizosphere of major crops under non-gnotobiotic soil conditions. Recently, we were able to construct a *nif*-cosmid X940, derived from the *nif*-gene cluster of *Pseudomonas stutzeri* A1501, which upon transformation of *Pseudomonas protegens* Pf-5 as chassis to engineer a nitrogen fixing, ammonium-excreting bacterium, caused constitutive nitrogen fixation and ammonium excretion in *P. protegens* Pf-5 X940 (1). Recently, we also could show that heterologous production of PHB polymer efficiently inhibits heterologous nitrogenase activity in the genus *Pseudomonas*, suggesting that the transferred PHB production is competing for the costs necessary to drive heterologous acquired nitrogen fixation (2). Finally, we could demonstrate that the use of the efficient non-PHB-producing strain *Pseudomonas protegens* Pf-5 X940 as inoculum for major cereal crops transferred the nitrogen fixed by BNF to maize and wheat under non-gnotobiotic soil conditions which completely covered the nitrogen needs of the plants under N-limiting conditions(3).

In this lecture, I propose an integral revision of the approach to implement BNF in non-leguminous crops and a discussion of the perspectives of dysregulation of BNF in recombinant (“transgenic”) diazotrophic bacteria for commercial application of BNF in major crops.

(1) Setten et al. (2013) PLoS One 8: e63666
(2) Pascuan et al. (2015) J. Mol. Evol. 81:84-89
(3) Fox et al. (2016) Env. Microbiol. doi:10.1111/1462-2920.13376

O-06.1

The use of *Azospirillum brasilense* inoculants

Fabricio Dario Cassan, Martin Diaz-Zorita and Yaacov Okon

Rio Cuarto. UBA and Department of Plant Pathology and Microbiology, Faculty of Agriculture, Food and Environment, Rehovot Campus, The Hebrew University of Jerusalem

Following inoculation of several grasses, cereals and legumes with *Azospirillum*, there are pronounced morphological effects on roots, beginning with the proliferation of root hairs. The morphological effects are generally dependent on the inoculum concentration and are consistent with the exogenous indole-acetic acid (IAA) levels secreted by *Azospirillum.* The IAA/cytokinin ratio and nitric oxide (NO), a key signalling molecule involved in a wide range of functions in plants are also important.

One of the most important achievements obtained from this research is the utilization of *Azospirillum* commercial inoculants mainly in South America. About 3.5 million ha of wheat, maize, sorghum and legumes are inoculated (2015) and the use continues to increase. Results from field experiments became more consistent with the commercial use of successful liquid inoculants containing osmoregulators for maintaining 10^9 viable cells/ml after 1 year storage. The regulation of products (REDCAI) in the last decade has contributed to the system success. Another important development is the study of signals-dialogue between the bacterium and the plant (IAA, NO). *A. brasilense*, when exposed to IAA, adapts itself to the plant rhizosphere, by changing its arsenal of transport proteins and cell surface proteins, the regulatory machinery of the bacterium is changing. Some mechanisms and signaling for growth and nodulation improvement following co-inoculation of legumes with *A*. *brasilense* and rhizobia have been elucidated. There are induced flavonoids production by legume roots inoculated with *Azospirillum, nod* genes transcription in rhizobia as response to flavonoids produced by the plant and nod factor (LCO's) production by rhizobia as a response to flavonoids produced by the plant. There are advances in understanding the roles of storage materials polyhydroxyalkanoates (PHB) and the cell surface components-EPS, LPS and surface proteins leading to resistance of cells to stress conditions during storage of inoculants and following their application.

O-06.2

Use of *Azospirillum* in Brazilian Agriculture: a successful case

Solon C.de Araujo

Consultant of ANPII

The research with *Azospirillum* in Brazil began in the 70´s with the first works of Dra. Johanna Dobereiner and her team. A strong research initiated with the discovery of *Azotobacter paspali* and *Azospirillum brasilense*, both as nitrogen fixers by Embrapa and Universities, looking for agronomical application of this bacteria as a potential increaser of productivities in cereals and other crops. Corn, wheat, sorghum and rice were the first crops in field tests with selected strains of the bacteria. In 2004, results of field tests with corn and wheat with the demonstration of the agronomical efficacy of the strains AbV 5 and AbV 6, both selected by the Paraná University and tested by EMBRAPA (the most important entity of agricultural research in Brazil), was presented in the meeting of RELARE (a net with researchers, industries and regulatory authorities).

With these results, the strains were authorized for use in inoculant production and the private companies began the product development: cultivation in large scale, formulation that gives large shelf life and easy use by the farmers, packages and storage conditions. Now, in Brazil, we have eight companies producing inoculant with *Azospirillum*.

The Ministry of Agriculture demands four field tests for two years, in two different geographic regions, conducted by official entities. The results need to present statistical differences in comparison with the control treatment. The minimum standard of the product is 1 $X10^8$ C.F.U. per gram or mL. Following the tradition in use of soybean inoculant, with 30 million hectares using inoculants, the new inoculant for gramineous had a good receptivity by the farmers.

Now there are eight companies producing *Azospirillum* inoculants in Brazil, the same product used for corn (around 70% of the total produced), wheat and rice (this last in a small quantity). Last year the total doses was around 2.500.000 (1 dose=100 mL=1 ha). An increase in harvest due to inoculation in corn, in a wide average, is around 500 kg per hectare. Of course, it depends on many factors: climate, nutrients in the soil, pH, correct application, variety of seeds, and others.

The product is positioned both as nitrogen fixer and PGPR. We believe that it is impossible to separate the effects, because both are interdependent. The effect in increase of the root system is very clear, and also that of nitrogen uptake. In farms the product can be applied on seeds, in furrow and by foliar pulverization, with similar results in the improvement of productivity.

As far as challenges are concerned, we need more researches: long shelf life, survival on the seeds, resistance to hydric stress, increase in the rate of nitrogen fixation, tests in other crops than gramineous plants. Many of these targets will be developed by the industries and the research institutions, in collaborative projects.

In marketing we need more effective activities so as to demonstrate the effect of the product to the farmers, cooperatives and consultants. Now, the inoculant is used in 15% of the area of corn and wheat and we need to increase the area with *Azospirillum* in high levels.

O-06.3

Nitrogen fixation in *Populus*: Characterization of the key diazotrophs

Sharon L. Doty[1], Pierre M. Joubert[1], Andrew W. Sher[2] and Zareen Khan[1]

[1] University of Washington, Seattle
[2] University of California, San Diego

Just as research on the human microbiome has demonstrated the profoundimportance of our microbiota on human health, plants are also strongly influenced by theecosystem within them. To improve the environmental and economic sustainability ofbiomass production, it is essential that the biological interactions between plants andassociated beneficial microbiota be more fully understood. Nitrogen is an essentialmacronutrient for plant growth. The use of N fertilizer for bioenergy plantations, however, is incompatible with the goals of climate change mitigation since its production requires fossil fuels, and soil bacteria convert the excess N fertilizer into nitrous oxide, a potent greenhouse gas. It has long been assumed that only legumes and actinorhizal plants can benefit from symbiotic N_2-fixation; however, it has become clear that N_2-fixation by associated and internal (endophytic) microorganisms can provide substantial N to the host plant without requiring root nodules. Poplar (*Populus*) trees are an early successional pioneer plant species able to colonize nutrient-limited, cobble-dominated riparian zones. Native poplar plants have a diverse microbiota including strains that can fix dinitrogen gas, and promote plant growth and health under abiotic stresses including nutrient limitation and drought. Our lab demonstrated using the $^{15}N_2$ incorporation assay and the acetylene reduction assay that N_2 is fixed at high levels in wild poplar by endophytes, the microorganisms that live within plants 1. Although other research groups have reported on endophytes from poplar, no others have demonstrated endophytes with the high levels of N_2-fixation, broad host range, and dramatic growth enhancements under stress that we have seen with endophytes from wild poplar in challenging environments 2-4. Through genomic analysis, transposon mutagenesis, fluorescent in situ hybridization, and NanoSIMS, we seek to more fully understand the plant-microbe interactions involved in endophytic symbiosis, specifically in regards to N_2-fixation, with the ultimate goal of optimizing this technology towards increasing biomass yields on marginal lands with reduced nutrient and water inputs.

(1) Doty, S.L. et al. (2016) PLoS One, in press
(2) Khan, Z. et al. (2012) ISRN Agronomy doi: 10.5402/2012/890280
(3) Kandel, S et al. (2015) Crop Science 55:1765-1772
(4) Khan,Z. et al. (2015) Forests 6:3582-3593

O-06.4

BNF in the bioenergy plant *Pennisetum purpureum*

[1]José Ivo Baldani, [2]Sandy Sampaio Videira, [3]Diego Quesada, [1]Vera Lucia Divan Baldani, [1]Marcia Soares Vidal, [1]Stefan Schwab, [1]Jean Luiz Simões Araújo, [1]Segundo Urquiaga

[1]Embrapa Agrobiologia, BR465, Km 07, 23891-455- Seropédica, RJ, Brazil
[2] Centro de Tecnologia Mineral - CETEM, 21941-908- Rio de Janeiro – Brazil
[3]Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, Brazil

The fast-growing C4 grass *Pennisetum purpureum,* commonly known as elephant grass, produce large amount of biomass and grows very well in the semi-humid or humid tropics. Several genotypes are highly enriched with protein and respond almost linearly to N-fertilizer application while others show low forage quality but produce very well in poor soils, and do not demand large quantities of N-fertilizer. A long-term study carried out in concrete cylinders with many *Pennisetum* genotypes showed a large variation in dry-matter production (81 Mg to 15 Mg/ha/year) and total N accumulation by plants (393 kg N to 75 kg N/ha/year). The BNF quantification showed that N derived from the biological process varied from 14 to 36% and confirmed one year later. A field experiment carried out with the three of the most productive *Pennisetum* varieties (Gramafante, Cameroon, Merker x 239DA-2) and the variety Roxo (as a low productive control) showed that the dry matter production was around 60 Mg ha/harvest and accumulated up to 400 kg N/ha in seven months. The contribution of BNF in these genotypes varied from 25 to 40%, equivalent to 106 and 165 kg N/ha/harvest, respectively.

A detailed study to explore the diazotrophic bacterial community associated with these genotypes allowed the isolation of several nitrogen-fixing bacteria including *Herbaspirillum*, *Azospirillum, Gluconacetobacter, Burkholderia, Klebsiella* and *Enterobacter*. A culture independent approach from the genotypes Cameroon, Gramafante, BAG 02, Roxo, and CNPGL91F06-3 showed that the clone libraries (16S rRNA sequences) were dominated by sequences affiliated to members of *Leptotrix* (12.8 %) followed by *Burkholderia* (9 %) and *Bradyrhizobium* (6.5 %), while most of the *nifH*-clones were closely related to *Bradyrhizobium* (26 %).

A field inoculation experiment with five diazotrophic strains, isolated from different genotypes, showed that strain LP343 (*G. diazotrophicus*) increased the biomass up to 30% in the genotype Cameroon compared to no-inoculated plants in the first year crop and confirmed in the second cut (12 months later). A draft genome sequence from strain LP343 was carried out and an automatic annotation showed a genome size quite similar to *G. diazotrophicus* PAL5 strain. Genes involved in the nitrogen metabolism, nitrogen fixation and nitrosative stress but no denitrification were found. In addition, high number of genes involved in the stress response, defense mechanism and cofactors, pigments and vitamins were also detected.

Poster presentations

P-01.1

In vitro study suggests that stem apoplastic liquid of sugarcane modulates the chemotaxis and defense mechanism of the diazotrophic *Burkholderia tropica* strain PPe8

[1]Paula Renata Alves da Silva, [1]Marcia Soares Vidal, [2]Michele Zibetti Tadra-Sfeir, [1]Jean Luiz Simões-Araújo, [2]Emanuel Maltempi de Souza, [1]José Ivo Baldani

[1]Embrapa Agrobiologia, BR465, Km 07, 23891-455- Seropédica, Rio de Janeiro, Brazil
[2]Universidade Federal do Paraná, Curitiba, Paraná, Brazil

The stem apoplastic fluid of sugarcane contains different sugars, organic acids and amino acids that may supply the demand for carbohydrate by the endophytic colonizing bacteria. Nitrogen-fixing bacteria such as *B. kururiensis* and *G. diazotrophicus* have been detected in the apoplast of rice and sugarcane plants, respectively (1, 2). On the other hand, *B. tropica* strain Ppe8, isolated from sugarcane stem, is part of the bacterial consortium inoculant recommended to sugarcane. However, there is very little information regarding this plant-bacterium interaction considering that it can colonize the internal sugarcane tissues. Here, we made use of the RNA-Seq transcriptomic analysis to study the influence of sugarcane apoplastic fluid on expression of genes of strain Ppe8. The bacterium was grown in JMV liquid medium until log phase and then the culture was divided in two parts: one part received 50% of fresh JMV medium while the other received 50% of apoplastic fluid from sugarcane variety RB867515. Total RNA was extracted two hours after growth the bacteria in the new medium, the rRNAs were removed, enriched mRNAs and cDNA libraries made and sequenced using Illumina MiSeq plataform. Data analysis provided by the CLC program showed that 503 genes were repressed and 157 genes were induced in the presence of apoplastic fluid compared to the fresh JMV medium. These expressed genes belonged to different functional classes in the genome of PPe8 strain. It was observed that genes related to chemotaxis and movement were repressed in the presence of apoplastic fluid suggesting that the bacteria do not have an active motion drive in the apoplast. Another important point was the repression of genes that could induce the defense responses in the plant, suggesting that strain Ppe8 may recognizes the fluid of the apoplast. Among these genes, we highlight those involved in lipopolysaccharide biosynthesis (LPS), exopolysaccharides (EPS) and peptidoglycan.

(1) Mattos et al. (2008) An. Acad. Bras. Cienc. 80:477-493
(2) Dong et al. (1997) An. Bot. 80:147–158

P-01.2

Molecular analysis of the role of two paralogs of OxyR regulators in peroxide mediated oxidative stress response in *Azospiriilum brasilense* Sp7

Sudhir Singh, Susheel Dwivedi, and Anil Kumar Tripathi

School of Biotechnology, Banaras Hindu University, Varanasi, INDIA

Azospirillum brasilense Sp7 is Gram negative, plant growth promoting diazotrophic bacterium (1-2). During root colonization, it has to face a natural defense mechanism of the plant including ROS released by the roots, which are used to restrict invasion by pathogenic microorganism (3). A. brasilense Sp7 protects itself from the natural defense of the plant with the help of two paralogs of oxidative stress regulator oxyR (oxyR1 and oxyR2), which are global transcriptional regulators that activate expression of various gene involved in responding to oxidative stress (4). While oxyR1 is divergently oriented to katA (catalase), oxyR2 is divergently oriented to ahpC (alkylhydrperoxide reductase) in the genome of A. brasilense Sp7.

Inactivation of oxyR1 and oxyR2 via insertion of kanamycin resistance gene cassette resulted in reduced growth of oxyR1::km mutant compared to the parental strain. Growth of oxyR2::km mutant in minimal malate medium in presence of tert-butyl hydrperoxide (t-BOOH), however, was better than that of the parental strain and the oxyR1::km mutant. Reporter lacZ fusions of katA and ahpC promoters revealed that katA promoter is ten times less active in oxyR1::km mutant than that in the parent or oxyR2::km mutant. But, ahpC promoter is five times more active in oxyR2::km mutant than that in the parent or oxyR1::km mutant. These results indicated that slow growth of oxyR1::km mutant is due to reduced catalase activity, whereas increased tolerance to t-BOOH in oxyR2::km mutant was due to increased alkylhydroperoxide reductase activity. Determination of transcriptional start site by 5'RACE for katA and ahpC suggested that GGATAA and TTGGAT were used as -10 and -35 elements for katA gene, and TAACTT and TTCCAT were used as -10 and -35 elements for ahpC gene. Biological significance of OxyR2 gene was shown through interaction of GFP-tagged A. brasilense Sp7 and oxyR2::km mutant with rice roots showing that rice root colonization by oxyR2 mutant was severly compromised indicating the importance of OxyR2 in rice root colonization.

(1) Bernhard et al. (1995) Appl. Environ. Microbiol 61:1013-1019
(2) Tarrand et al. (1978) Can. J. Microbiol. 24:967-980
(3) Vanessa and Mary (2014) Front Microbiol. 5:368
(4) Sang et al. (2015) Cell Rep. 12:1589-1599

P-01.3

Using synthetic biology to increase nitrogenase activity

Xin-Xin Li, Xiao-Meng Liu, Hao-Wen Shi and San-Feng Chen

State Key Laboratory for Agrobiotechnology and Biological Science College, China Agricultural University, Beijing 100193, P. R. China

Nitrogen fixation has been established in protokaryotic model *Escherichia coli* by transferring a minimal *nif* gene cluster composed of 9 genes (*nifB, nifH, nifD, nifK, nifE, nifN, nifX, hesA and nifV*) from *Paenibacillus* sp. WLY78 (1). However, the nitrogenase activity in the recombinant *E. coli* 78-7 is only 10% of that observed in wild-type *Paenibacillus* (1-3). Thus, it is necessary to increase nitrogenase activity through synthetic biology.

In order to increase nitrogenase activity in heterologous host, a total of 28 selected genes from *Paenibacillus* sp.WLY78 and *Klebsiella oxytoca* were placed under the control of *Paenibacillus nif* promoter in two different vectors and then they are separately or combinationally transferred to the recombinant *E. coli* 78-7. Our results demonstrate that *Paenibacillus suf* operon (Fe-S cluster assembly) and the potential electron transport genes *pfoAB, fldA* and *fer* can increase nitrogenase activity. Also, *K. oxytoca nifSU* (Fe-S cluster assembly) and *nifFJ* (electron transport specific for nitrogenase) can increase nitrogenase activity. Especially, the combined assembly of the potential *Paenibacillus* electron transporter genes (*pfoABfldA*) with *K. oxytoca nifSU* recovers 50.1% of wild-type (*Paenibacillus*) activity. However, *K. oxytoca nifWZM* and *nifQ* cannot increase activity.

(1) Wang et. al. (2013) PLoS Genet. 9:e1003865
(2) Zhang L. and Chen S. (2015) World J. Microbiol. Biotechnol. 31:921–927
(3) Zhang et al. (2015) Biotechnol. Letter 37:1999–2004

P-01.4

The regulation of nitrogen fixation and assimilation in the associative diazotroph *Klebsiella oxytoca* M5a1

C. J. Waite, V. Elsin, R. Berchtold, T. Tanny, J. Gan, N. Sanguankiattichai, S. Amartay, M. Buck, J. Schumacher

Division of Cell and Molecular Biology, Department of Life Sciences, Imperial College Road, Imperial College, London, SW7 2AZ, UK

Engineering free-living and associative diazotrophic bacteria for the release of fixed nitrogen (N) into the rhizosphere represents one promising strategy for meeting the global demand for agricultural fertiliser sustainably (1, 2). As part of an ongoing collaborative project (BB/N003608/1) aiming to rationally re-engineer the cell signalling and metabolism of model diazotrophs for the supply of surplus reduced N (e.g. ammonium) to plants, we present a preliminary model for the regulatory interplay between N fixation and N assimilation in the associative, soil-dwelling bacterium *Klebsiella oxytoca* M5a1 (*Ko*). In most bacteria the rate of N assimilation is coupled to the cellular N status (glutamine/α-ketoglutarate ratio) by a regulatory cascade involving post-translational uridylylation of PII type proteins (GlnB and GlnK) and the σ^{54}-type transcriptional activator NtrC (3). In diazatrophs such as *Ko*, these proteins also regulate the expression of the *nif* gene cluster, encoding the nitrogenase complex and its associated factors, via a second, downstream σ^{54}-type transcriptional activator NifA (4). Nitrogenase expression is coupled tightly to internal N status (via NtrC), anaerobiosis (via NifL, the negative regulator of NifA) and import of exogenous fixed N (via interactions between GlnK and the primary ammonium transporter, AmtB (5)). Ultimately, the co-regulation of the *gln* (N assimilation) and *nif* (N_2-fixation) regulons by multifunctional regulatory proteins affords highly economical nitrogen metabolism, according to supply and demand, in which a surplus of fixed N compounds is minimised.

Klebsiella oxytoca M5A1 provides a suitably characterised model diazotroph in which to develop an integrated systems-level understanding of N economy management, including identification of key nodes of control and robustness, whether catalytic or regulatory, that prevent excess fixed N production and export. In preparation for omics analyses (RNA-seq, targeted MRM-MS proteomics and LC-MS metabolite profiling) we have characterised multiple key parameters during the diazatrophy transition that follows ammonium run-out including (a) cell growth rate, (b) transcription of key N regulons, (c) nitrogenase activity and (d) critical O_2 concentrations. We have developed an O_2-independent fluorescent gene reporter system and a library of regulatory mutants (both gene deletions and CRISPR-targeted substitutions) which together reveal novel control mechanisms at play in this organism. We report initial findings employing synthetic transcription factors, for instance a chimera of the O_2-sensitive *Bradyrhizobium japonicum* and native *Ko* NifA homolgoues, to redirect/tune *nif* gene expression and thereby uncouple the N demand of the cell from N fixation activity.

(1) Brewin et al. (1999) J. Bac. 181:7356–7362
(2) Setten et al. (2013) PLoS One, 8:e63666
(3) Schumacher et. al. (2013) mBio. 4:e00881-13
(4) Temme et al. (2012) PNAS 109:7085–7090
(5) Gruswitz et al. (2007) PNAS 104:42–7

P-01.5

Synthetic rebalancing of nitrogen fixation and nitrogen assimilation in diazotrophs

Ana Bonnatto, Adam Gosztolai, Christopher Waite, Michal Komorowski, Volker Behrends, Mark Bennet, Michael Stumpf, Mauricio Baraona, Martin Buck, Jörg Schumacher*
j.schumacher@imperial.ac.uk

Division of Cell & Molecular Biology, Imperial College London, London, UK

PII proteins play a central role in regulating the cellular nitrogen economy, perceiving the nitrogen status (glutamine/α-ketoglutarate ratio)(1), regulating adaptive responses of nitrogen assimilation and fixation in soil, and are also important during nodule development (2, 3). A low nitrogen status triggers post translational uridylylation of PII proteins by the uridylyl-transferase/removase (UT/UR, *glnD*). Most diazotrophs encode for both the canonical PII (*glnB*) and its highly homologous GlnK (*glnK*), which is generally co-transcribed with the ammonium transporter (*amtB)*. In *E.coli*, PII and GlnK have overlapping functionalities in regulating both nitrogen assimilation gene expression (via NtrB/NtrC) and glutamine synthetase activity (GS), but GlnK may play more specific roles in ammonium transport and in diazotrophs in regulating nitrogen fixation via *nifL-nifA* systems. In contrast to the constitutively expressed *glnB*, *glnK* expression is generally subject to nitrogen status. A dynamic integrative systems understanding of the PII regulated nitrogen economy is currently lacking.

In order to gain a quantitative understanding of PII responsiveness to environmental changes in *E.coli,* we accurately determined the absolute intracellular concentrations of PII, GlnK and GS, as well as their post translational states, and in relation to changing glutamine and α-ketoglutarate levels, using MRM-MS targeted proteomics and NMR metabolomics, respectively. Our models show that both *glnB* and *glnK* are required for maximal growth under ammonium limiting conditions, explained by altered GS adenylylation kinetics and that the post translational states of PII and GlnK both depend on sequestration of GlnK (to AmtB) away from UT/UR. Broadly speaking, we found that PII is the major control point for NtrB/NtrC mediated transcription regulation, while both PII and GlnK can effectively act on GS enzyme activity, allowing for rapid adaptation to ammonium shock. When tracing the information flow through the coupled PII-GS post translational system we observed a perhaps unprecedented inherent information capacity, suggesting a fine metabolic tuning capacity of the central nitrogen assimilation pathway. We present ongoing work on (BB/N003608/1) how we exploit these insights to synthetically rewire cell signalling in diazotrophic *Klebsiella oxytoca*, rebalancing N-assimilation and N_2-fixation for surplus ammonium secretion.

(1) J. Schumacher et al. (2013) mBio 4:e00881
(2) R. Dixon et al. (2004) Nat. Rev. Microbiol. 2:621 (2004)
(3) T. Arcondeguy et al. (1997) Genes & Development 11:1194

P-01.6

Identification and functional characterization of genes involved in carbon source utilization in *A. brasilense* Sp7

Vijay Shankar Singh, Ashutosh Prakash Dubey, Ankush Gupta, Sudhir Singh, and Anil Kumar Tripathi.

School of Biotechnology, Banaras Hindu University, Varanasi, India

Azospirillum brasilense is nitrogen fixing, non-photosynthetic, plant growth promoting ά-proteobacteria found in the close vicinity of various plant roots (1) including C3 cereals and C4 grasses. Organic acids (mainly C4-dicarboxylates) as well as trace amount of sugars and sugar alcohols of root exudates act as preferred source of carbon and energy for the nitrogen fixation and for the growth (2). To search the genes and proteins involved in C4-dicarboxylates utilization, SDS-PAGE and 2D gels of *A. brasilense* grown in different C4-dicarboxylates supplemented medium have been resolved and observed that a DctP protein of 14 paralogs of TRAP transport system is continuously upregulated in malate grown culture while nearly constant expression has been observed in succinate and fumarate grown cultures of *A. brasilense*. Insertional inactivation of induced *dctP* and double knock-out mutant of *dctP* and another C4-dicarboxylates transporter *dctA* have shown that DctPQM is a major transporter (~75% growth retardation in *dctP::km* mutant) while DctA (~25% growth retardation in *dctA::gm* mutant) is a minor C4-dicarboxylates transporter in *A. brasilense*. Enhanced promoter activity of *dctP* and *dctA* at µM and mM range of substrates, respectively have been shown that DctPQM is high affinity while DctA is low affinity transporter and enhanced promoter activity of *dctP* in *σ54::km* while zero activity of *dctA* promoter have been shown that σ54 positively regulate the expression of *dctA* gene. In addition to C4-dicarboxylate transporters we observed a PQQ dependent quinoprotein alcohol dehydrogenase (ExaA) protein has been upregulated in glycerol as well as fructose grown cultures of *A. brasilense* and belongs to Type-I of PQQ-ADH. 5' RACE study predicted the σ54 binding site and it has been demonstrated that σ54 regulate positively and another RpoH2 sigma factor regulate negatively the expression of *exaA* gene. Role of divergently organized two component system as well as regulator binding site have been predicted by mutations and it has been shown that LuxR type of regulator EraR regulate the expression of *exaA* by showing positive interaction with promoter bounded σ54-RNA Polymerase complex without having GAFTGA domain.

(1) Bashan et al. (1990) Can. J. Microbiol. 36:591-608

(2) Del Gallo et al. (1994) Okon Y (ed) *Azospirillum*/plant associations. CRC, Boca Raton, Fla., pp 57-75

P-01.7

Functional analysis of genes involved with nitrate metabolism in *Herbaspirillum seropedicae*

P. Bonato[1]; M. B. Batista[1]; D. Camilios-Neto[3]; V. C. S. Pankievicz[1]; M. Z. Tadra-Sfeir[1]; R. A. Monteiro[1]; L. U. Rigo[1], F. O. Pedrosa[1]; E. M. Souza[1], L. S. Chubatsu[1]; R. Wassem[2]

[1]Department of Biochemistry and Molecular Biology and
[2]Department of Genetics, Universidade Federal do Paraná, Curitiba, PR, Brazil
[3]Department of Biochemistry and Biotechnology, Universidade Estadual de Londrina, Londrina, PR, Brazil

Herbaspirillum seropedicae is a nitrogen-fixing β-proteobacterium that associates with roots of gramineous plants. *In silico* analyses revealed that *H. seropedicae* genome has genes encoding a putative respiratory (NAR) and an assimilatory nitrate reductase (NAS). To date, little is known about nitrate metabolism in *H. seropedicae*, and, since this bacterium cannot respire nitrate, the function of NAR remains unknown. RNA-seq transcriptional profiling allowed us to pinpoint genes important for nitrate metabolism in *H. seropedicae,* including nitrate transporters and regulatory proteins. Additionally both RNA-seq data and physiological characterization of a mutant in the catalytic subunit of NAR (*narG* mutant) showed that NAR is not required for nitrate assimilation but is required for: (i) production of high levels of nitrite, (ii) production of NO, and (iii) dissipation of redox power, which in turn may increase carbon consumption. In addition, wheat plants showed an increase in shoot dry weight only when inoculated with *H. seropedicae* wild type, but not with the *narG* mutant, suggesting that NAR is important to *H. seropedicae*-wheat interaction. We also show that nitrate assimilation in this organism is regulated by NtrBC, the master nitrogen regulatory two-component system, and by NtrYX, which seems to specifically regulate nitrate metabolism. An *ntrY* mutant of *H. seropedicae* showed wild type fixing nitrogen phenotype, but the nitrate dependent growth was abolished. Gene fusion assays indicated that NtrY/NtrX is required for expression of genes coding for the assimilatory nitrate reductase as well as the nitrate-responsive two-component system NarX/NarL. The purified NtrX protein was capable of binding the *narK* and *narX* promoters, and the binding site at the *narX* promoter for the NtrX protein was determined by DNA footprinting, supporting the role of the NtrY/NtrX system in regulating nitrate metabolism in *H. seropedicae*.

P-02.1

Delivery of fixed nitrogen to cereal crops using *Salmonella typhimurium* carrying the refactored and native *Klebsiella nif* cluster

Min-Hyung Ryu[1], Miryoung Song[1], Kevin Garcia[2], Shanmugam Rajasekar[2], Taylor Wahlig[2], Jean-Michel Ané[2] and Christopher Voigt[1]

[1]Synthetic Biology Center, Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[2]Departments of Bacteriology and Agronomy, University of Wisconsin-Madison, Madison, WI 53706, USA

Most cereal crops require a significant amount of nitrogen fertilizer to reach their yield potential. Acquiring nitrogen fertilizers is difficult for poor farmers in developing countries. In contrast, the excessive use of nitrogen fertilizers in developed countries leads to significant environmental and sustainability problems. Unfortunately, unlike legumes, most cereals cannot obtain a significant amount of nitrogen through biological nitrogen fixation. Low oxygen levels (micro-aerobic conditions) and carbon availability are two prerequisites for efficient biological nitrogen fixation with most aerobic bacteria. We hypothesized that these two features may be more available deep inside of root tissues than on their surface. Bacterial endophytes can colonize plant tissues internally without eliciting significant host defense responses. These endophytic bacteria enter root tissues through cracks caused by lateral root formation or wounds. Thus, we engineered endophytic bacteria residing in cereal roots such that they are capable of fixing nitrogen efficiently and providing nitrogen to the plant. We found that some enteric bacterial pathogens including *Salmonella typhimurium* strains behave as excellent root endophytes that can colonize alfalfa, rice and maize efficiently. As a potential model for endosymbiosis, we have transferred several refactored and native *Klebsiella nif* clusters to *Salmonella* and shown activity *in vitro*. Preliminary evidence shows the activity of nitrogenase after the colonization of maize B73 with some engineered *S. typhimurium* strains. These clusters are therefore ready to be placed under synthetic control, for example, of plant-derived signals in chassis organisms.

P-03.1

Developing nitrogen-fixing symbioses in cereal crops: what can we learn from actinorhizal symbioses?

Didier Bogusz and Claudine Franche

Rhizogenesis, UMR DIADE, IRD Montpellier, France; e-mail: didier.bogusz@ird.fr

Most plants including cereals form an endosymbiosis with arbuscular mycorrhizal fungi (the AM symbiosis). Studies on legumes and more recently on actinorhizal plants have shown that the establishment of symbioses depends on plant genes of the Common Symbiotic Signaling Pathway (CSSP) which are also required for the establishment of this more ancient AM endosymbiosis. This work suggests that nitrogen fixing symbioses have evolved from the AM symbiosis through the acquisition by rhizobia and *Frankia* of the ability to activate the CSSP. The CSSP then activates programs for the preparation of infection by compatible microbes and, in the case of the legume-Rhizobium and actinorhizal symbioses, for the initiation of nodulation. Several strategies are being pursued to investigate whether the CSSP in cereals can be activated by nitrogen-fixing bacteria to improve symbiotic responses, thus mimicking the process which led to the evolution of the legume-*Rhizobium* and actinorhizal symbioses.

Efforts towards engineering symbiotic nitrogen fixation in cereals have predominantly focused on transferring knowledge acquired on model legume systems. Less specialized forms of root nodule symbioses (RNS), including actinorhizal associations with *Frankia* bacteria, combine features that render them more promising models for potential RNS transfer to new hosts.

These features include the following:

- Actinorhizal plants appeared in the fossil records long before Legumes (1) thus, any insights obtained about these symbioses are relevant to understand both symbioses
- Genome sequencing of several *Frankia* strains belonging to Cluster I and Cluster III failed to reveal orthologues of rhizobial common *nod* genes required for LCO biosynthesis (2). Thus, the structure of *Frankia* symbiotic factors might be more basic that the rhizobial Nod factors.
- In actinorhizal plants belonging to subclade C, *Frankia* induces cell divisions in the cortex, leading to the formation of a small external protuberance called the prenodule that represents a very simple symbiotic organ (3). Therefore, obtaining a prenodule in cereals rather than mature nodule may be an easier goal to achieve.
- Because they originate from cell divisions in the pericycle cells in front of a xylem pole, and because their root like structure, actinorhizal nodule lobes have been considered as modified lateral roots. Therefore, how *Frankia* trigger lateral root program could be a clue to initiate nodule primordium in non-legume crop.

(1) Swenson & Benson (2008) K. Pawlowski and W.E. Newton (eds). Nitrogen fixing actinorhizal symbioses, pp. 73-104
(2) Normand et al. (2007) Genome Research 17:7-15
(3) Laplaze et al. (2000) Molecular Plant-Microbe Interactions 13:107-112

P-03.2

The salinity tolerance of *Casuarina equisetifolia* in symbiosis with *Frankia* relation

Meriem Benabdoun[1,2], Zeineb Benabdoun[2], Abdelhamid Djekoun[1,2] & Nadia Ykhlef[1,2]

[1] Université des Frères Mentouri, Route Ain-El-Bey, Constantine, Algérie
meriem-benabdoun@hotmail.fr
[2]Laboratoires de Génétique, Biochimie, et Biotechnologies Végétales, Chaabat-Erssas, Constantine, Algerie.

The salty land occupies about 7% of the world's surface. In arid and semi-arid areas, especially around the Mediterranean regions, soil salinity constitutes one of the major abiotic factors that reduce yields of several crops (1). The introduction of salt-tolerant plants, in association with bacteria, is one of the most used for the valuation of land in these technical areas. Actinorhizal plants constitute the second largest group of nitrogen-fixing plants. Among them, we find *Casuarina* forming a symbiotic root with soil bacteria called *Frankia* (2, 3).

The actinorhizal tree *C. equisetifolia* shows a high tolerance to salt (4), but it is likely to form efficient actinorhizal nodules in the presence of a high concentration of NaCl, however, the mechanisms of this tolerance are unknown to this day. The aim of this work is to analyze the effect of salinity on the symbiosis *C. equisetifolia-Frankia* through the study of morphological, physiological and biochemical processes of the plant.

For this, seven NaCl concentrations have been used (from 0 to 500 mM).The results obtained, show that salt affects nodulation. Moreover, the total chlorophyll content and the relative water content (RWC) have been reduced under the effect of salt stress, while proline and soluble sugars are significantly accumulated in the leaves under the effect of salt. These participate in the phenomena of osmotic adjustment. It appears from this study that *C. equisetifolia* is a halophyte tolerant to high concentrations of NaCl.

(1) Middleton and Thomas (1997) World Atlas of Desertification. In: Edward Arnold, London. p. 69
(2) Baker and Mullin (1992) Actinorhizal symbioses. In: Biological Nitrogen Fixation, Eds. Stacey G., Burns R.H. & Evans H.J. Chapman and Hall, New York. pp. 259–292
(3) Hocher et al. (2009) Cahiers Agricultures. 18:498-505
(4) Tani and Sasakawa (2006) Soil Science and Plant Nutrition 52:21-25

P-03.3

Developing a Genetic System for *Frankia*

Rediet Oshone[1], Sheldon G. Hurst[1], Celine Pesce[1] and Louis S. Tisa[1]

University of New Hampshire, USA

Frankia are soil-dwelling actinobacteria that form a nitrogen-fixing symbiotic association with 8 different families of angiosperms. Despite over two decades of research in the area, the genetics of *Frankia* is still in its infancy. There are no known systems for gene transfer in *Frankia*. For an increased understanding of *Frankia* physiology and plant-microbe interactions, it is essential that transformation and targeted mutagenesis protocols are established. Here, we report the introduction of a plasmid (pHTK1) that could stably replicate and express both an antibiotic resistance gene and the green fluorescent protein marker (GFP) in *Frankia*.

Frankia sp. CcI3 was conjugated with *Escherichia coli* BW29427 (DAP^-) containing the pHTK1 plasmid. The conjugants were plated on minimal growth medium containing tetracycline (20 µg/ml) and kanamycin (50 µg/ml). After three weeks, single colonies of transformants were inoculated into broth growth medium containing both antibiotics. The plasmid was extracted using a modified alkaline lysis method and visualized on an agarose gel. A PCR approach was used to confirm the plasmids by amplifying the REP and the *tetA* genes. The Transformed *Frankia* cells expressing the GFP protein were examined under UV light.

The filter mating between *Frankia* sp. CcI3 and *E. coli* BW29427 containing pHTK1 produced a conjugation frequency of 10^{-4} to 10^{-5} transconjugants/recipients. Single colonies of transformed *Frankia* cells were isolated and propagated in minimal growth medium containing tetracycline and kanamycin. Isolated transconjugants were stably maintained in culture suggesting that the pHTK1 plasmid was replicating in *Frankia* and the *tetA* gene on the plasmid was being expressed. The plasmid was re-isolated from *Frankia* transconjugants and visualized on an agorose gel. PCR amplification of the *tetA* and REP regions confirmed the presence of the plasmid in transconjugants. The copy number of the plasmid in *Frankia* was determined to be two per genome. Transconjugants fluoresced under UV light indicating the expression of the GFP protein in *Frankia*.

This is the first report on the induction of a plasmid that could stably replicate in *Frankia* and is a major breakthrough in *Frankia* genetics. We are currently trying to use the pHTK1 plasmid to engineer a codon-optimized CRISPR/Cas9 system for site specific gene disruption in *Frankia*.

P-04.1

Functional genomics of cyanobacteria in symbiosis with boreal feather mosses

Denis Warshan[1], Christopher L. Dupont[2], Philip D. Weyman[3], Ulla Rasmussen[1]

[1]Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm, Sweden

[2]Microbial and Environmental Genomics Group, Craig Venter Institute, La Jolla, CA, USA

[3]Synthetic Biology and Bioenergy Group, Craig Venter Institute, La Jolla, CA, USA

The nitrogen cycling in boreal forest ecosystems is largely determined by a symbiotic association between feather mosses (*Pleurozium schreberi* and *Hylocomium splendens*) and diazotrophic cyanobacteria that fix majority of nitrogen flowing into boreal ecosystems (1). Because nitrogen is often limiting in boreal forests, the interaction between the cyanobacteria and the mosses greatly affects the productivity of this ecosystem that makes up almost 30% of Earth's forested land (2-3). We seek to understand the genetic diversity of the cyanobacteria associated with the mosses and the molecular steps leading to the moss-cyanobacterial symbiosis.

We sequenced the genomes of five different *Nostoc* spp. that are able to form symbiotic associations with feather moss. As a control, we also sequenced the genome of one *Nostoc* sp. that is unable to form symbioses with the mosses. Comparative genome analysis of these cyanobacterial species allowed us to probe the genomic diversity of moss-associated *Nostoc* strains and identify a set of 32 genes differentially retained in genomes of symbiotic competent cyanobacteria compared to the non-symbiotic competent strain.

We also obtained transcriptomic and proteomic data for *Nostoc* grown in isolation, together, or with chemical contact with the moss only through filter separation. Transcriptomic and proteomic data revealed that differentially retained genes and their neighborhood are upregulated in chemical contact (gas vesicles, chemotaxis related genes), during both condition (pyrroloquilonine-quinone and exopolysaccharide production) and together with the moss partner (taurine catabolism and aliphatic sulfonate transporter). Thus, we hypothesize that cyanobacteria symbiotic gene clusters are essential to establish the symbiosis with feather moss.

(1) DeLuca et. al. (2002) Nature 419:917–920

(2) Anderson et al. (1991) Ecol. Appl. 1:326–347

(3) Goodale et al. (2002) Ecol. Appl. 12:891–899

P-05.1

Cultivation of plant-associated bacteria belonging to the phylum *Verrucomicrobia*

Wiebke Bünger, Jana Müller, Thomas Hurek, Barbara Reinhold-Hurek

Institute of Microbe-Plant Interactions, Faculty of Biology and Chemistry, University Bremen, Postbox 33 04 40, 28334 Bremen, Germany

Cultivation–based studies of microorganisms living in association with plants are essential and important tools in understanding the roles and functions of these organisms in their natural niche. During this study bacteria were isolated from the rhizoplane as well as the endosphere of rice roots. Along the way, the efficiency of root- surface sterilization for cultivation-independent approaches was evaluated. We found that a surface sterilization by the commonly used ultrasonication is not sufficient to remove the majority of surface bacteria. In contrast, a chemical treatment with sodium hypochlorite was highly efficient (1).
By cultivation-dependent approaches, a broad phylogenetic range of about 100 different bacterial isolates were brought to pure culture. Amongst other bacterial phyla, four putatively novel species of the phylum *Verrucomicrobia* were isolated from rice roots and further characterized. Members of this phylum are widely distributed, rather abundant in soils and highly diverse. This phylum can be divided into 7 subdivisions, consisting of cultured and uncultured strains (2). A phylogenetic assignment by sequencing of the partial 16S rDNA sequences showed that three of our strains could be classified to subdivision 2 and subdivision 4, whereas the fourth strain could not be confidently assigned to any subgroup because of high phylogenetic distance.
So far, there is a lack of knowledge about the interaction of *Verrucomicrobia* with plants due to low cultivation rates and consequently a low number of cultivated strains. Only few studies so far dealing with the rhizosphere of leek and potato described the abundance and interaction of *Verrucomicrobia* subdivision 1 with plants (3+4).
Thus, the four novel strains were characterized with respect to major characteristics like plant growth promoting capabilities, growth, and morphology. Inoculation of rice seedlings in gnotobiotic culture followed by fluorescence microscopy showed that the bacteria formed tight biofilms along the roots without influencing plant growth negatively, however they varied in colonization density. Some strains showed the capability to produce indole-acetic acid and to solubilize phosphate, as putatively plant growth-promoting characteristics. Hence, our strains may help to get new insight into this yet sparsely characterized bacterial phylum.

(1) Reinhold-Hurek et al. (2015) Annu. Rev. Phytopathol. 53:403-4023
(2) Schlesner et al. (2006) Prokaryotes 7:881-896
(3) Nunes da Rocha et al. (2011) FEMS Microbiol. Ecol. 78:297-305
(4) Nunes da Rocha et al. (2010) J. Soils Sediments 10:326-339

P-05.2

Diversity and activity of diazotrophs associated with micro-environments of wetland rice

Hannes Schmidt, Dagmar Woebken

Division of Microbial Ecology, University of Vienna, Austria
hannes.schmidt@microbial-ecology.net

Rice is one of the world's most important crop plants. The production is strongly limited by nitrogen (N), which is typically supplied by industrial fertilizers that are costly and hazardous to the environment. It is known that *Biological Nitrogen Fixation* through N_2-fixing bacteria and archaea (diazotrophs) can alleviate the N-shortage in rice cultivation. However, our knowledge on the micro-sites of N_2 fixation, as well as the diversity and *in situ* N_2 fixation activity of diazotrophs in the soil-microbe-plant interface (i.e. rhizosphere) of flooded rice fields is still rudimentary.

Greenhouse studies were performed to identify key factors that control rice-diazotroph association and related N_2 fixation activities. Paddy soils of different geographical origin were cultivated with two agriculturally used genotypes of wetland rice. Samples were separated into bulk soil, rhizosphere soil, rhizoplane, and roots at flowering stage of rice plant development. These samples were subjected to functional assays and various molecular biological techniques to identify the inhabiting diazotroph community.

Based on Illumina amplicon sequencing of 16S rRNA and *nifH* genes and transcripts, we will present insights into the diversity and potential activity of bacterial/diazotroph communities associated with the different rice micro-environments. Emphasis will be put on comparatively discussing the influence of (a) the soil microbial "seed bank" and (b) plant genotype in shaping the microbiomes. Actual N_2 fixation activities of soil-genotype combinations and micro-environments will be shown on the basis of incubation assays using $^{15}N_2$-containing atmospheres. Areas of potential N-transfer between diazotrophs and rice roots will be presented via the detection and visualization of spatial colonization patterns of selected diazotrophic groups on rice rhizoplanes.

P-05.3

Effect of PHB production on nitrogen fixation in *Pseudomonas* inoculants

Gabriela Soto, Romina Fox, Lorena Setten, Matteo Mozzicafreddo, Nicolas Ayub

Instituto de Genética Ewald A. Favret (CICVyA-INTA), Buenos Aires, Argentina

We recently transferred a genomic island harboring nitrogen fixation genes from PHB-producing strain *Pseudomonas stutzeri* A1501 to the non-nitrogen-fixing non-PHB producing bacterium *Pseudomonas protegens* Pf-5 via transformation with the cosmid X940. The resulting strain Pf-5 X940 showed an uncommon phenotype of constitutive nitrogen fixation and ammonium excretion. Interestingly, inoculation of dicots and monocots with Pf-5 X940 increased the inorganic nitrogen concentration in soil and plant productivity under nitrogen-deficient conditions. Here, we showed that similar to *Pseudomonas protegens* Pf-5, others non-PHB producing *Pseudomonas* species (*P. putida*, *P. veronii* and *P. taetrolens*) transformed with cosmid X940 showed also constitutive nitrogenase activity, while PHB-producing *Pseudomonas* strains (*P. balearica* and *P. stutzeri*) containing cosmid X940 showed a typical derepression phenotype of nitrogen fixation observed commonly in natural diazotrophic bacteria. These results suggest that constitutive nitrogenase activity may generally depend on the absence of PHB biosynthesis.

(1) Setten et al. (2013) PLoS One 8:e63666
(2) Pascuan et al. (2015) J. Mol. Evol. 81:84-89

P-05.4

Composition and effects of maize exudates on chemotaxis of *A. brasilense* and its performance in maize development

Elisete Pains Rodrigues, Mayara Silva Barbosa, César Augusto Tischer, André Luiz Martinez de Oliveira

Departamento de Bioquímica e Biotecnologia, Universidade Estadual de Londrina - UEL, Londrina, Brazil

The root exudates produced by different plant species are known to induce chemotactic responses on soil microflora and to mediate plant-bacteria interactions. The soil microbiome is selectively affected by root exudates according to its qualitative and quantitative composition, which also has shown to be variable according to plant age, edapho-climatic conditions and microbial diversity of soil. On the other hand, bacterial inoculants containing plant growth-promoting bacteria (PGPB) such as *Azospirillum brasilense* has shown variability in its efficiency according to, among other factors, the plant genotype.

In this sense, we evaluated the composition of root exudates of two commercial maize hybrids: DKB 240 and DKB 390 (Dekalb, Monsanto) with contrasting responses to *A. brasilense* inoculation, its chemotaxis effect and influence in plant colonization and development when used as inoculant addictive. The composition of root exudates varied mainly in the amount and classes of flavonoids, although differences in other compounds were also evident. The root exudate of maize genotype with better response to *A. brasilense* inoculation (DKB 390) showed a higher chemotaxis activity over bacterial cells and improved the early colonization of heterologous maize genotypes (DKB 240 and P30F53H, this last from Pioneer) by *A. brasilense* in a factor of 10. In addition, the composition of root exudates from DKB 390 had also a larger number of different flavonoids (including the presence of isoflavones) when compared to DKB 240 genotype. The root exsudates of maize have also influenced the early development of heterologous maize plants, by improving the number of secondary roots, root volume and plant dry mass, even when no bacteria were applied to the plants. We expect that the identification of active compounds in the exudate of responsive plant genotypes may led to the development of additives that can improve the efficiency of commercial inoculant formulations.

Acknowledgements: UEL, CNPq; Fundação Araucária, CAPES, INCT-FBN

P-05.5

Maize-driven selection of rhizobacteria

E. Balsanelli[1], V. A. Baura[1], F. O. Pedrosa[1], A. Oliveira[2], R. A. Monteiro[1], E. M. Souza[1]

[1]Department of Biochemistry and Molecular Biology, UFPR, Curitiba, PR, Brazil
[2]Universidade Estadual de Londrina, Londrina, PR, Brazil

The understanding of the ecological interactions that influence plant growth and productivity under field conditions is a precondition for a sustainable agriculture. In recent years the importance of the rhizospheric microbial community for plant health and development has emerged. To understand the influence of the plant genotype on the rhizopheric microbial community we used two maize cultivars, cv. 2B512 that was responsive to *Azospirillum brasilense* FP2 inoculation, and cv. 30A37, which was not responsive, in a succession of re-inoculation experiments.

Soil from the Brazilian Atlantic Forest, one of world´s biodiversity hotspots, was used as inoculum for the maize cultivars grown in pots under greenhouse conditions. After thirty days, the mixture of soil and roots from this first maize cycle was used as inoculum for a second cycle of maize cultivation, and the process was repeated 4 times, yielding a total of 5 cycles. Bacteria from each cycle were isolated from bulk soil, rhizosphere soil and roots using seven different media. A total of 1705 isolates were recovered from cv. 30A37, and 1747 from cv. 2B512 over the five cycles. Partial 16SrRNA sequencing of over 80% of the isolates from the last three cycles showed predominance of Beta- and Gamma-proteobacteria. Interestingly, several sequences were attributed to putative new species.

A chemotactic trap experiment designed to further select effective PGPRs was performed with a pool of all isolates from the fifth cycle. 21 different bacteria species preferentially colonized the trap maize plants. Some isolates increased lateral root number and root dry weight when inoculated in axenic maize. Early plant growth promotion seems to be cultivar-specific. Massive parallel sequencing of 16SrRNA amplified from bulk and rhizospheric soil DNA showed a switch in the phyla profile of rhizospheric soil over the cycles for both cultivars, with enrichment of Actinobacteria, Bacteroidetes and Archaea, but reduction of Acidobacteria. Although 16SrRNA sequences from both cultivars had similar proportions, enriched bacteria at genus and species levels were statistically different between the two cultivars. In conclusion, maize plants seemed to select over time specific groups of bacteria from the pool present in the soil, several of them with plant-growth-promoting capacity.

Supported by INCT/FBN/CNPq, PVE-SWB.

P-05.6

A single spontaneous GS mutation is responsible for the phenotype of an *Azospirillum brasilense* ammonium-excreting strain

Karina F. D. N. Santos, Vivian R. Moure, V. Hauer, A. R. S, Santos, L. Donatti, Carolina W. Galvão, F. O. Pedrosa, E. M. Souza, R. Wassem, M. B. R. Steffens

Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná,
e-mail: souzaem@ufpr.br

A. brasilense is a well-known Plant-Growth Promoting Rhizobacteria (PGPR). This microorganism is capable to fix nitrogen and to produce phytohormones. The nitrogen fixation genes (*nif*) are subjected to tight transcriptional regulation while the nitrogenase activity is post-translationally controlled by reversible ADP-ribosylation of dinitrogenase reductase under high ammonium or energy depletion.

HM053 is a spontaneous mutant strain derived from *A. brasilense* FP2 and it is able of excreting ammonium and fixing nitrogen in the presence of high concentrations of NH_4^+, and has very low glutamine synthetase activity (GS). HM053 GS was not adenylylated in response to an ammonium shock or under any condition tested. Genomic sequencing revealed a single base substitution in the *glnA* gene leading to substitution of a proline residue by a leucine at position 347 of the GS. No other mutation was found in genes related to nitrogen metabolism in HM053. This strain was capable of colonizing the surface of wheat roots and increased by 30% and 49% the shoot and root dry weight, respectively, when compared with un-inoculated plants, and by 30% and 31% when compared with the parent strain FP2. Although HM053-36 (*nifH::gusA*) and FP2-7 (*nifH::gusA*) showed GusA activity located mainly at lateral root emergence points, HM053-36 consistently showed stronger signals and expressed the *nifH* gene at a level 278 fold higher than strain FP2 *in planta*.

In conclusion, a spontaneous single mutation in GS of *A. brasilense* HM053 seems responsible for the constitutive nitrogen fixation and an ammonium excreting phenotype. HM053 ability to excrete ammonium and fix nitrogen even in the presence of high NH_4^+concentration could explain why this mutant performed better than FP2, and suggests that HM053 may be a better nitrogen biofertilizer. The results suggest that discrete site-directed changes in the genome may allow construction of new and more effective *Azospirillum brasilense* strains.

Keywords: Plant-growth-promoting rhizobacteria, wheat, glnA

Supported by INCT/FBN/CNPq.

P-06.1

Growth promotion and nitrogen metabolism of two sugarcane varieties inoculated with a mixture of five diazotrophs

Silvana G. dos Santos[1], Flaviane da S. Ribeiro[1], Camila S. da Fonseca[1], Willian Pereira[1], Leandro A. Santos[1] and Veronica M. Reis[2]

[1]Universidade Federal Rural do Rio de Janeiro, Dept. de Solos, Seropédica, RJ, Brazil
[2]Embrapa Agrobiologia, Seropédica, RJ, Brazil. E-mail: veronica. massena@embrapa.br

The objective of this study was to evaluate the initial development and nitrogen metabolism (N) in seedlings of two sugarcane varieties inoculated with diazotrophs. Experiments were performed using three kinds / stages of plant cultivation: (i) sprouting in boxes with sterile sand and vermiculite substrate, (ii) cultivation in plastic tubes containing commercial substrate Multiplant® (not sterile), and (iii) cultivation in hydroponic systems with modified Hoagland solution. As inoculation treatment, a mixed inoculant consisting of five strains of diazotrophs: *Gluconacetobacter diazotrophicus* (Gd - strain BR11281^T - PAL-5^T) *Herbaspirillum seropedicae* (Hs - BR11335 - HRC54), *Herbaspirillum rubrisubalbicans* (Hr - BR11504 = HCC103), *Burkholderia tropica* (Bt - BR11366^T = PPe8^T) and *Azospirillum amazonense* (Aa - BR11145 = CBAMc), and two sugarcane varieties, RB867515 and IACSP95-5000 were used. The hydroponic cultivation was evaluated under two N rates [high (3 mM) and low (0.3mM)] and also after 72h restriction of N nutrients. At the end of each cultivation experiment, different evaluations were performed. After the sprouting phase, the shoot (SDM) and root dry mass (RDM), volume and area of roots, root length by thickness class and numbers of bifurcations were measured. After the tube cultivation, the shoot dry matter (SDM) and root dry matter (RDM), height and stem diameter and length and width of the leaves were measured. During and after the hydroponic cultivation, the activity of the nitrate reductase and glutamine synthase enzymes in the soluble fraction, and the dry biomass and nutrient content were estimated.

In inoculated plants, the RDM after sprouting increased by 50%. This effect was also observed with the aid of WinRHIZO® Arabidopsis software as significant increase in volume and root area. Moreover, inoculation increased the fine root length by approximately 30% in the variety RB867515. After cultivation in tubes, inoculated seedlings of both varieties had higher RDM and SDM. In hydroponic cultivation it was observed that the inoculation increased the nitrate reductase activity (NRa) in leaves and roots and glutamine synthetase (GS) in the leaves. The effect of NRa was found only in the variety RB867515 and after N restriction treatment, while there was no variety distinction for GS. Inoculation increased the N-nitrate content in the leaves and reduced levels in roots. The inoculated plants also showed an increase in N-amino content in the roots; the largest effects were observed after the N restriction treatment. At the end of hydroponic cultivation, inoculated plants of the variety RB867515 showed higher RDM, dry mass of secondary tillers, total dry matter and N content in the stem.