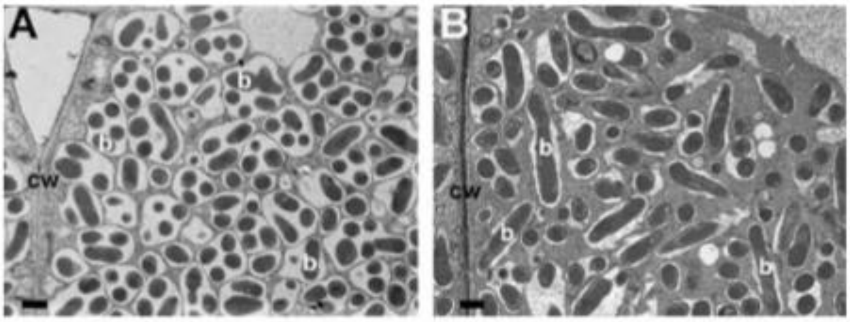
Research Prospects for Potential Solutions to Nitrogen Crisis in Agriculture

The North American Conference on Symbiotic Nitrogen Fixation (NACSNF) was founded over 40 years ago and gathers researchers studying N_2-fixing symbioses in mainly rhizobial, algae and *Frankia* systems. This year the 23rd NACSNF was held in the beautiful Pacific Ocean resort Hotel Las Brisas in Ixtapa, México and was attended by around 100 scientists from North America as well as other countries. The talks focused on the evolution of the nitrogen fixation genes, its genomics, development and biochemical signalling involved in the symbiotic association of bacteria to their hosts and applications of symbiotic nitrogen fixation for agricultural purposes. Azotic Technologies was represented by Professor Ted Cocking, David Dent and myself with David giving an oral presentation entitled "Establishment of cereal and non-legume crop symbiotic nitrogen fixation by *Gluconacetobacter diazotrophicus*".

The use of genomics, proteomics and transcriptomics were shown to have been of major importance for the findings presented at the conference. The genomics talks also emphasised the many molecular techniques for the studies of gene function, including the use of gene knockout, site-specific nuclease technologies, multiple gene deletions, hairpin knockdown and Tnt1 insertions. Talks on rhizobium plasmids demonstrating their involvements during quorum sensing potentially opens up research with similar interests in other nitrogen-fixing bacteria carrying plasmids. A very interesting talk was given by Professor Louis Tisa from the University of New Hampshire, USA whose research interests focused on the actinorhizal symbiosis between the non-nodule forming *Frankia* and various different species of plants. He demonstrated that the use of comparative genomics and RNASeq approaches to report the findings of secondary metabolites gene clusters coding for tolerance against salt stress, resistance to heavy metals and other toxic hydrocarbons. This makes *Frankia* a good candidate for nitrogen-fixation in plants growing under harsh environmental conditions. Professor Ann Hirsh from the University of California reported interesting findings about the bacteria *Burkholderia* which despite its nodule forming and nitrogen-fixing properties, has been dismissed as potential plant inoculant due to its pathogenicity to humans. By making use of MultiLocus Sequence Analysis and 16S RNA analyses, Professor Hirsh and her group reported that four plant-associated and environmental *Burkholderia* species are distinct from the pathogenic ones. The bacteria were shown to lack the virulence determinants and to not be pathogenic towards *Caenorhabditis elegans*, hence, are unlikely to be pathogenic to mammals, making *Burkholderia* another potential inoculant for safe agricultural applications, especially for promoting crop growth in acidic, arid soils (Angus *et al*., 2014).

In order to establish a successful symbiosis with its host plants, the rhizobial bacterium *Sinorhizobium meliloti* (used as the model bacteria during much of the research), must be enclosed within a curled root hair and induce the formation of an infection thread. In addition to producing Nod factor, the bacteria must also produce the acidic exopolysaccharide succinoglycan to initiate infection thread formation and sustain infection thread elongation. The importance of the distinct components of the bacterial membrane surface at the host-microbe interface and the significant implications involved for the establishment of a successful symbiosis on its host plants were clearly the focus of much research. Kathryn Jones from State University, USA as well as many other speakers, demonstrated the relationship between the production of succinoglycan (Jones *et al*., 2008) and other major membrane phospholipids and the establishment of symbiosis. Transcript profiles studies conveyed by Otto Geiger from the Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, using mutants unable to form major membrane phospholipids were reported to have lost the ability to form flagella which in return affected their symbiosis with host plants. It was also shown that a decrease in pH affects the formation of flagella which also had a negative impact on symbiosis in *Sinorhizobium meliloti*, emphasising the importance of the major phospholipids during symbiosis.

Jesús Montiel from Hungary reported some very fascinating findings on the terminal bacteroid differentiation and their loss of reproduction ability within nitrogen-fixing root nodules. His research involved the use of six inverted repeat-lacking clade (IRLC) legumes which were inoculated with their respective rhizobial partners. The data presented showed remarkable differences in the morphology of a large number of the bacteroids following symbiosis as a result of terminal differentiation characterized by cell enlargement (Figure 1), genome amplification and loss of reproductive ability.
The production of small plant-derived nodule-specific cysteine-rich (NCR) peptides from the IRLC legumes used in this research were shown to mediate this terminal differentiation of the bacteroids, resulting in the reproductive capacity of bacteroids being drastically reduced (Van de Velde, W., *et al*. 2010). These particular findings could prove to be of great importance especially if proliferation of the bacteria is affected, which might result in lower nitrogen-fixation in nodules or during systemic distribution of the diazotrophs within each plant cell.



Expression of NCR genes in *Lotus japonicus* leads to features of terminal bacteroid differentiation. A and B show the TEM of a symbiotic nodule cell expressing GUS (A) and NCR035 (B) demonstrates an increase in bacteroid size in the case of NCR035 expression. (Van de Velde *et al*., 2010)

Interests on the study of NCR peptides were also demonstrated at the molecular level by the research of Professor Dong Wang from the University of Massachusetts. By studying this differentiation process in the nodule of an alfalfa-clover, *Medicago truncatula,* Prof Dong discovered that one of the NCR peptides, DNF4, supports nitrogen-fixing bacteria when inside the plant, but its actions can kill free-living bacteria outside. The dual effect of DNF4 appear to reflect a mechanism to ensure that the rhizobia stay in a properly differentiated state whilst within in the plant, indicating a possible fitness benefit to the host plant. The findings would possibly suggest that the DNF4 peptides could be the key factor which helps maintain bacteria survival inside host cells and hence, could contribute significantly to efforts to improve legume crops without using more fertiliser (Dong *et al*., 2010; Kim *et al*., 2015). Dr. Paul Price from Brigham Young University reported on the recovery of a bacterial peptidase capable of degrading host NCR peptides which catalytic activity is required for the symbiotic effects in *M. truncatula*. This collection of studies by these three researchers and their groups demonstrate the importance and evolving nature of the NCR peptides in controlling bacterial differentiation in host-microbe symbiosis.

Professor Yaacov Okon from the Hebrew University of Jerusalem gave one of the few talks which put emphasis on the agricultural applications of nitrogen fixing bacteria; he discussed the plant growth promotion abilities and his research perspectives of the diazotroph *Azospirillum brasilense*. The latter elaborated on the pronounced morphological effects on roots as a result of the exogenous indole-acetic acid hormone production by the bacteria in several grasses, cereals and legumes when inoculated with *Azospirillum*. As a result, an increase in yield of 8-17% in plant biomass was observed which has led to this bacteria becoming an established inoculant which is still being used to optimise the yield in cereal crops and legumes.

Although nodulation is often considered to be a distinguishing characteristic of legumes, a large number of species are not known to nodulate and very little is known about the mechanisms of nitrogen acquisition in non-nodulated legumes. The non-nodulating legumes such as honey locust (*Gleditsia triacanthos*) were said to retain a possible precursor for symbiotic nitrogen-fixation indicating that these legumes could form successful symbiosis with nitrogen-fixing bacteria. In an effort to explore such a possibility, Dr. Gyaneshwar Prasad from the University of Wisconsin Milwaukee reported nodule like outgrowth on the roots of *G. triacanthos* growing in their natural environment. A few of these outgrowths were red/pink indicating presence of leghemoglobin. Bacterial strains were isolated that showed maximum similarity to Rhizobium/Agrobacterium clade based on 16S rDNA sequences. Rhizobium species marked with GUS and GFP were demonstrated to colonise *G. triacanthos* roots and was detected in the root hairs as well as in the epidermal and cortical cells. This is the first report showing that non-nodulating legume *G. triacanthos* can form symbiosis with rhizobia.

To summarise, it was made very apparent at the conference that there is increasing need to tackle the nitrogen crisis in agriculture and it was clearly demonstrated by the substantial amount of effort put into the research that there is a need to better understand the symbiosis between nitrogen-fixing bacteria and host, as well as to introduce the nitrogen fixation ability in non-legumes as an alternative to nitrogen fertiliser. Basic and applied research and development approaches discussed aim to reduce the use and environmental impact of industrial N-fertilisers, while maintaining or even increasing plant productivity. However, it appeared that most of the research dismissed anything which is not Rhizobium based and there was a general failure to acknowledge the symbiosis of nitrogen-fixing bacteria in non-legumes, but rather considering it as being an “associative interaction” rather than a genuine symbiosis.

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