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STSM Topic: Characterisation of interactions between Scots pine and the meristematic endophyte *M. extorquens*.

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1. Background of the STSM

We have identified a novel plant-endophyte association in the bud tissues of Scots pine (*Pinus sylvestris* L.) (Koskimaki et al., 2015a; Pirttila et al., 2000). Bacteria of the genera *Methylobacterium* and *Pseudomonas*, and a yeast, *Rhodotorula minuta*, live in the meristematic cells of the buds (Pirttilä et al. 2000, 2003). The *Methylobacterium* spp. are the most common endophyte throughout the year (Pirttila et al., 2005). Our results have shown that one of the meristematic endophytes, *Methylobacterium extorquens* DSM13060, colonizes the pine host by active penetration of the epidermis in the roots or stem, or through the stomata. The bacterium forms infection threads and infection pockets similar to the stem-nodulating rhizobia, and colonizes the shoot via the xylem vessels (Koskimäki et al. manuscript). Once colonized, the bacterium dwells inside the cells, near the nucleus (Koskimaki et al., 2015a, Koskimäki et al. manuscript). After two months of colonization, the bacterium significantly increases growth and development of pine seedlings, to the same extent as mycorrhizal fungi (Pohjanen et al., 2014).

Plant-associated microbes have been thought to increase plant growth mainly through phytohormone production (Holland and Polacco, 1994; Holland, 1997; Ivanova et al., 2001; Ivanova et al., 2008), but additional mechanisms exist (Koenig et al., 2002). The Scots pine endophytes do not produce the most common phytohormones, but we have identified a number of new growth-promoting compounds, *e.g.* adenine derivatives (Pirttilä et al., 2004). Of particular interest are several fatty acid analogs (FAA) that increase viability of Scots pine buds *in vitro* (Koskimäki et al. 2015b). FAAs are potent antioxidants and they are synthesized during endophyte infection (Koskimäki et al. 2015b). A genome analysis indicates that genes required for FAA biosynthesis are common to both endophytes and rhizobia, and these compounds could have a role of enabling intracellular life (Koskimäki et al. 2015b). The FAA compounds have potential for use as therapeutics in many pharmaceutical applications.

2. Purpose and workplan for the STSM

In COST Action FA1103 Working Group 4, the focus is “New industrial products in life sciences”. The described project fell finely within this theme, as the FAAs are products identified from the endophyte of Scots pine, *M. extorquens* DSM13060, and the STSM project aims for development of industrial products from FAAs. For pre-clinical studies, high quantities (from milligrams to grams) of FAAs need to be produced. We have tested several methods in the past (Koskimäki et al. 2015b) and selected the method below for obtaining sufficient amounts of the molecules for the *in vitro* and *in vivo* studies. The aim of the STSM was to produce high quantities of FAAs for studying the activity further in various potential industrial applications.

3. Description of the work carried out during the STSM

Optimization of culture conditions for FAA production

Factors for optimal cell growth were determined for *M. extorquens* DSM13060 and *A. lata* DSM1123. Carbon and nitrogen consumption were analyzed for both bacterial species to estimate the initiation of nitrogen-limitation and secure availability of carbon source during the bioreactor cultures for effective production of FAAs. After optimization of FAA production in small scale cultures (< 1000 mL) the larger bioreactor cultures were initiated. Setting up the bacterial fermentation process together with the optimization of the various culture parameters took considerable proportion of time planned for the STSM visit. Despite we managed to determine all FAA-production parameters for *M. extorquens* DSM13060, the slow growth rate and low cell densities in optimized conditions in bioreactor cultures forced us to focus on *A. lata* DSM1123 strain, which grew significantly faster and accumulated more FAAs. This was made to keep up with the STSM time frame and to reach the set goals.

Optimization of degradation conditions for FAA production

Bacterial cells were collected from the bioreactor via centrifugation and after washes transferred to degradation solution in small bioreactor for easy control of pH, dissolved oxygen and temperature. For efficient synthesis of FAAs, several parameters of degradation were optimized from cell-free supernatants for further analysis. Supernatant was freeze-dried and several extraction methods were tested for efficient and cost effective separation of FAAs with preparative HPLC. Purity of the compounds, sufficient for pre-clinical studies, was later determined by LC-MS and NMR.

4. Description of the main results obtained

Based on the preliminary experiments, we were able to produce, derivatize and purify sufficient amounts of the material for the upcoming *in vitro* and *in vivo* studies, stated as the main goal in the STSM workplan. Optimization of the several steps along the procedure facilitates up-scaling and allows production of larger amounts of material for further studies.

5. Foreseen publications/ articles resulting or to result from the STSM

The results obtained in this STSM will be included in joint publication after the *in vitro* and *in vivo* studies are completed successfully.

6. References

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