Graz University of Technology

Doctoral Thesis

Development of an effective agent to enhance flavor production in strawberries based on a formulation of methylobacteria

Dissertation zur Erlangung des akademischen Grades Doktor der Naturwissenschaften (Dr. techn.) an der Fakultät für Technische Chemie, Verfahrenstechnik und Biotechnologie an der Technischen Universität Graz

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Abstract

With more than 350 different components strawberry flavor is one of the most complex fruit flavors we know. Amongst all these constituents some components are of major importance for the strawberry aroma. These include the substance class of furanones, from which 2,5-dimethyl-4-hydroxy-2H-furanoe (DMHF) is the most important representative in strawberries. One interesting aspect of this compound is, that it is biosynthesized from chemical compounds that are produced by plant-associated bacteria of the genus *Methylobacterium*. Members of this genus can metabolize methanol; this makes them well adapted to a plant-endophytic lifestyle. The influence of *Methylobacterium* species on the DMHF biosynthesis in strawberry has been shown already several years ago on cell cultures of strawberry (*Fragaria* x *ananassa*). In this work it was shown that this effect occurs also on fully developed plants and that it is possible to use it for the improvement of strawberry flavor on a biotechnological way.

The strain *Methylobacterium extorquens* DSM 21961 (Rab1) has been applied on fully developed strawberry plants in the greenhouse and on the field and the colonization behavior of the strain has been investigated. For the quantification of the strain on plants specific oligonucleotides for a TaqMan™ Real Time PCR Assay have been developed and were successfully applied. Results showed that Rab1 after application colonized the strawberry plants in abundances that were stable over a time period of five weeks. The effect of the application on fruit quality was assessed by quantifying and comparing DMHF in fruits of treated and untreated plants. It appeared that DMHF concentration in fruits of treated plants was significantly higher than in fruits of untreated plants. These differences were also of sensoric relevance: a sensory panel was able to distinguish between fruits of treated and fruits of untreated plants and the former were described to be more sweet, ripe and pleasant.

To enable the use of Rab1 in biotechnology fermentation conditions were determined, which allow the production of the strain in an industrial scale. Several media which were already described in literature were tested. The optimal approach was the use of a minimal salt medium supplemented with methanol in a fed batch fermentation. Fermentation conditions were evaluated in a pilot fermenter with a volume of 10 litres. To allow storage and distribution of the bacteria it was necessary to preserve them in a formulation. For that purpose different formulation prodedures were tested and compared. It turned out that for a formulation of the strain in a matrix of xanthan gum, a polysaccharide produced by bacteria, was the best solution. This formulation combined high cell numbers with high shelf lifes even at high storage temperatures.

Zusammenfassung

Das Erdbeeraroma ist mit über 350 verschiedenen Einzelkomponenten eines der komplexesten Fruchtaromen, die wir kennen. Unter diesen vielen Einzelkomponenten existieren einige Verbindungen, die von herausragender Bedeutung für das Aroma der Frucht sind. Zu diesen gehören Vertreter aus der Substanzklasse der Furanone, von welchen 2,5-Dimethyl-4-hydroxy-2H-furanon (DMHF) in Erdbeeren die wichtigste Rolle spielt. Ein interessanter Aspekt in der Biosynthese dieser Verbindung ist, dass Vorstufen davon nicht von der Erdbeerpflanze selbst, sondern von pflanzenassoziierten Mikroorganismen der Gattung *Methylobacterium* produziert werden. Diese Gattung zeichnet sich dadurch aus, dass ihre Vertreter Methanol als Substrat nutzen können, wodurch sie sehr gut an das Leben als Pflanzenepiphyt angepasst sind. Der Eingriff dieser Bakterien in die Furanonbiosynthese von Erdbeeren wurde schon vor einigen Jahren an Zellkulturen von Erdbeeren (*Fragaria* x *ananassa*) gezeigt. In der vorliegenden Arbeit wurde untersucht, inwieweit dieser Effekt auch an voll entwickelten Erdbeerpflanzen auftritt und ob er biotechnologisch zur Verbesserung des Erdbeeraromas nutzbar ist.

Der Stamm *Methylobacterium extorquens* DSM 21961 (Rab1), welcher als Favorit aus einem Screening pflanzen-assoziierter Vertreter der Gattung *Methylobacterium* hervorgegangen ist, wurde im Gewächshaus und im Freiland auf Erdbeerpflanzen (*Fragaria* x *ananassa* cv Elsanta) appliziert und dessen Etablierung auf den Pflanzen wurde untersucht. Für die Quantifizierung des Stammes auf Pflanzen wurden spezifische Oligonukleotide für einen TaqMan™ Real Time PCR Assay entwickelt und erfolgreich angewandt. Es zeigte sich, dass Rab1 Populationsdichten auf den Erdbeerpflanzen ausbildete, welche über einen Zeitraum von fünf Wochen annähernd stabil waren. Der Effekt der Besiedelung der Erdbeerpflanzen mit Rab1 wurde durch gaschromatographische Quantifizierung von DMHF in den Früchten der entsprechenden Pflanzen überprüft. Die Behandlung der Erdbeerpflanzen mit Rab1 führte zu einer statistisch signifikanten Erhöhung der Furaneol-Konzentration in den Früchten. Diese Unterschiede waren auch von sensorischer Relevanz: Ein Panel von geschulten Verkostern war in der Lage, Früchte von behandelten und unbehandelten Pflanzen eindeutig zu unterscheiden, wobei die Früchte von behandelten Pflanzen als süßer, reifer und wohlschmeckender kategorisiert wurden.

Um Rab1 einer biotechnologischen Nutzung zugänglich zu machen, wurden Fermentationsbedingungen ermittelt, welche die Produktion des Stammes in großem Maßstab erlauben. Hierfür wurde auf Medien zurückgegriffen, welche bereits in der Literatur beschrieben worden waren. Als optimaler Ansatz erwies sich die Fed-Batch-Fermentation in einem Minimalmedium mit Methanol als Kohlenstoffquelle. Die benötigten Fermentationsparameter wurden an einem Pilotreaktor mit 10 Liter Fassungsvolumen ermittelt. Zur Erleichterung von Lagerung und Logistik war es nötig, die produzierten Bakterien in einer Formulierung haltbar und lagerfähig zu machen. Hierfür wurden verschiedene Formulierungsstrategien erprobt und miteinander verglichen. Eine Formulierung von Rab1 in einer Matrix aus Xanthan, einem von Bakterien produzierten Polysaccharid, hat sich als beste Variante erwiesen. Sie vereinte hohe Zelldichten mit einer sehr guten Haltbarkeit des Produkts auch bei hohen Temperaturen bis 44 °C.

Abbreviations

(v/v)	volume per volume
(w/v)	weight per volume
BLAST	basic local alignment search tool
CFU	colony forming unit
DMHF	2,5-dimethyl-4-hydroxy-3(2*H*)-furanone
DMMF	2,5-dimethyl-4-methoxy-3(2*H*)-furanone
DNA	deoxyribonucleic acid
DSMZ	Deutsche Stammsammlung für Mikroorganismen & Zellkulturen
EDTA	ethylenediamintetraacetic acid
EMBL	European Molecular Biology Laboratory
FAM	carboxyfluorescein
FAOSTAT	food and agriculture organization statistical databases
fw	fresh weight
GC-MS	gas chromatography – mass spectrometry
GFP	green fluorescente protein
HPLC	high pressure liquid chromatography
NCBI	National Center for Biotechnology Information
PCR	polymerase chain reaction
PHA	polyhydroxyalkanoate
PHB	polyhydroxybutyrate
PPFM	pink pigmented facultative methylotrophic bacteria
RAPD	random amplified polymorphic DNA
RT PCR	real time polymerase chain reaction
SPE	solid phase extraction
SSCP	single strand conformation polymorphism
TAMRA	carboxytetramethylrhodamine

Introduction

The strawberry

The strawberry belongs to the genus *Fragaria* (family Rosaceae). Today in this genus there are known more than 20 species and many cultivars and hybrids. In the Stone Age woodland strawberries (*Fragaria vesca*) already were collected by humans and used as food. Typical habitats of woodland strawberries are young woodland, sparse forest, woodland edges and clearings. Flowering time is from April to June. During summertime the small, red, very aromatic fruits are ripening. Seeds of woodland strawberries are spread by animals which feed the fruits, or by water, which washes away the dry seeds. Additionally, the plants produce a lot of runners for vegetative reproduction.

In Europe cultivation of woodland strawberries by humans has started in medieval times. In the 18th century french settlers imported a new strawberry species from the colonies around the St. Lawrence River in Canada to Europe. First, this new species, *Fragaria virginiana,* was cultivated only in botanical gardens. In 1714 the French botanist Armédée Francois Freziér discovered a new strawberry species with remarkable big fruits in South America, *Fragaria chiloensis*. Around 1750 the garden strawberry as we know it today, *Fragaria x ananassa,* originated by a multiple crossing of *Fragaria virginiana* and *Fragaria chiloensis.* Flowering time of garden strawberries is in spring, harvest time in early summer. Fruits of the garden strawberry combine the pleasant flavor of the fruits of *Fragaria virginiana* with the big fruit size of *Fragaria chiloensis*. *Fragaria* x *ananassa* and many cultivars of this species are still in use for commercial strawberry production today.

From a botanic point of view a strawberry is the swollen base of the flower (receptacle). The one seeded fruits are located on the outer surface (Perkins-Veazie, 1995).

Beside their pleasant flavor strawberries present many specific nutritional characteristics known to provide health benefits. They are particularly rich in vitamin C (>100 mg per 100 g fresh fruits) and contain high amounts of potassium. Beside these essential nutrients, strawberries contain a high content of phenolic and polyphenolic compounds, which are known as a naturally occurring dietary antimutagen and anticarcinogen. Anthocyanins are responsible for the intense color of the fruits. Especially vitamin C and the phenolic compounds contribute to the high antioxidative capacity of strawberries (Klopotek et *al.*, 2005; Scalbert and Williamson, 2000).

Nowadays the strawberry is one of the most important crop plants in the “high value sector” worldwide, besides apples and citrus fruits. In 2008 worldwide 4.068.454 tons of strawberries were produced on an area of 255.366 ha. A large part of the cultivated area is located in Europe (about 70%), followed by Asia and North and Central America. In Austria the annual production in 2008 amounted to 19.363 tons on 1.560 ha of cultivated area (FAOSTAT). The worldwide production continues to increase in most growing regions.

While consumption, demand and acreage continue to expand, the strawberry culture needs to adapt to new regulations and to maintain high quality fruits through harvest and shipping. In strawberry cultivation a large number of pesticides and chemicals are in use. Their residues in the harvested fruits and in the environment are an increasing problem (Abad *et al.*, 1999; Baker *et al.*, 2002). Governments and food-retailers address this critical issue by claiming a reduced use of chemicals from the farmers regarding to strawberry growing. In order to reduce the need of chemical pesticides and to increase fruit yield and fruit quality in the last decades a number of new strawberry varieties have been bred. They show a higher resistance against plant pathogens, higher fruit yields and a better resistance

of the fruits against post-harvest fruit spoilage. Unfortunately the new cultivars often show a decrease of the typical, pleasant strawberry aroma.

The flavor of strawberries is composed of more than 350 different chemical substances and is therefore one of the most complex fruit flavors. According to Schieberle and Hofmann (1997) and Larsen *et al.* (1992) most of them can be grouped into the substance classes of esters, alcohols, carbonylic compounds and organic acids. In addition, some heterocyclic compounds and some terpenes were detected in strawberries. The quantitative composition of the flavor depends on the cultivar, origin and cultivation conditions. Despite the high number of different compounds that are present in strawberry flavor, only a limited number of compounds have a major impact on the flavor. Several butanoates, ethylhexanoate and γ-decalacton are responsible for a fruity impression, different organic acids give an acidic impression and 2,5-dimethyl-4-hydroxy-3(2H)-furanone (furaneol®) is responsible for a sweet, caramel-like odor. Because of its low odor-threshold particular importance is assigned to this compound.

2,5-dimethyl-4-hydroxy-3(2H)-furanone (furaneol®)

The flavor of furaneol® is multifaceted. Its description ranges from fruity in low concentrations to caramel-like or roasty in higher concentrations. Due to its low odor-threshold and its flavor-enhancing effect it is used as a food-flavouring in food technology (Hauck et *al.*, 2003). It is formed as a reaction product in the Maillard-reaction and can be found in many bakery products, roasted meat or roasted coffee. Furthermore, furaneol® can also be synthesized by yeasts and can be found in beverages produced by alcoholic fermentation. Also bacteria, especially species from the genus *Lactobacillus*, have the capability to produce furaneol®. It was detected in different cheese varieties. In plants

furaneol® is the principal component of different fruit flavours. It is dominant in the flavor of pineapples, plays a predominant role in the flavor of strawberries and has been detected in many other fruits like mango, raspberries, lychees or tomatoes (Slaughter, 1999). In literature different biosynthetic pathways of furanones are discussed. On the one hand fructose-1,6-bisphosphate is supposed to be an educt for furanone-biosynthesis in plants, which forms the basic structure of the molecule after glycosylation and dehydratation (Roscher et *al.*, 1998), on the other hand a condensation reaction of smaller molecular subunits is discussed. Zabetakis has shown that a cocultivation of cell cultures of *Fragaria* x *ananassa* with the bacterial strain *Methylobacterium extorquens* leads to an increased biosynthesis rate of furanones in the plant cells (Zabetakis, 1997). He suggested a direct intervention of the bacteria into the secondary metabolism of the plant cells. Bacterial cells make available lactaldehyde for the plant cells by the oxidation of the terminal hydroxy-group of propane-1,2-diol. The lactaldehyde is incorporated by the strawberry cells and introduced into the biosynthetic pathway of furanones (Fig. 1). Additionally, the possibility is discussed that the presence of methylobacteria in high abundances induces an unspecific defence response which leads to the acculumation of furanones, which have antimicrobial effects (Sung et *al.*, 2006).

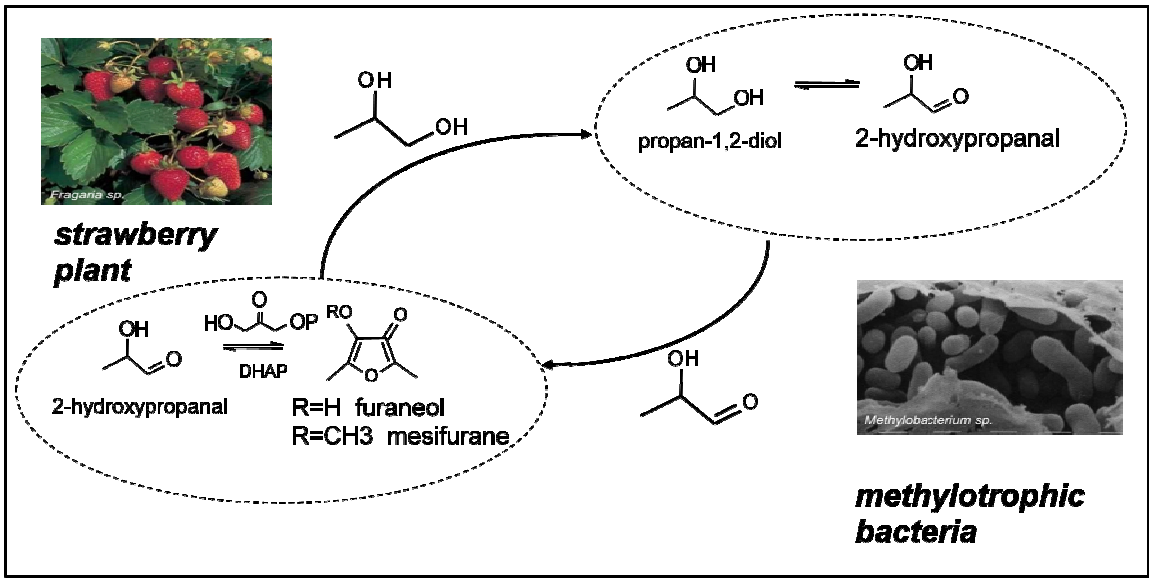

Fig. 1: Interaction between strawberry plants and methylobacteria according to Zabetakis

Methylobacteria

Bacteria of the genus *Methylobacterium* occur ubiquitously and are facultative methylotrophic. Methylobacteria can be found in soils, on plant leaves and in water samples. Even from the human oral cavity *Methylobacterium*-species have been isolated. Although many of the mechanisms of methylobacteria are not fully understood, they are highly studied (Lidstrom and Chistoserdova, 2002).

The first evidence for the existence of methylobacteria was provided by Loew in 1892. He isolated a strain that was able to metabolize methane and methanol as sole carbon source. Even if they occur ubiquitously, in ecological studies they often were not considered on the basis that they didn't appear on isolation agar plates within the usual incubation time if cultivation dependent methods were used. The genus *Methylobacterium* was firstly described by Patt et al in 1976. It belongs to the family of Methylobacteriaceae, which is ranged in the order of Rhizobiales, a subclass of Alphaproteobacteria. Since the 1970ies more interest was focused on the investigation of methylobacteria. Driving power of this

process was the assumption that methylobacteria could have a commercial relevance for the production of heterologous proteins with methanol as carbon source. The genome of *M. extorquens* is completely sequenced and contains open reading frames with significant similarity with genes involved in plant association in rhizobia and in *Agrobacterium (*Lidstrom and Chistoserdova, 2002). *M. extorquens* AM1 is the best-studied methylotroph to date. It has been used to investigate genetic and molecular background of methylotrophy, the serine cycle and formaldehyde oxidation reactions. Methylobacteria are growing by reducing C1-compounds like methanol, methylamine or formaldehyde for energy production. Also other short-chained organic compounds with 2-5 C-atoms can be reduced, but no carbon-carbon-bonds can be cleaved. Their cells are rod-shaped and 3-5 µm long. Due to intracellular deposition of carotenoides they are colored pink, therefore they are also know as Pink Pigmented Facultative Methylotrophs (PPFMs). These carotenoids act as protection against UV radiation. This protection is necessary because methylobacteria are reported to colonize the phyllosphere of many plant species. Especially the surrounding of stomata, where they use methanol and other “waste products” of the plant as carbon source, are occupied by them (Fig. 2).

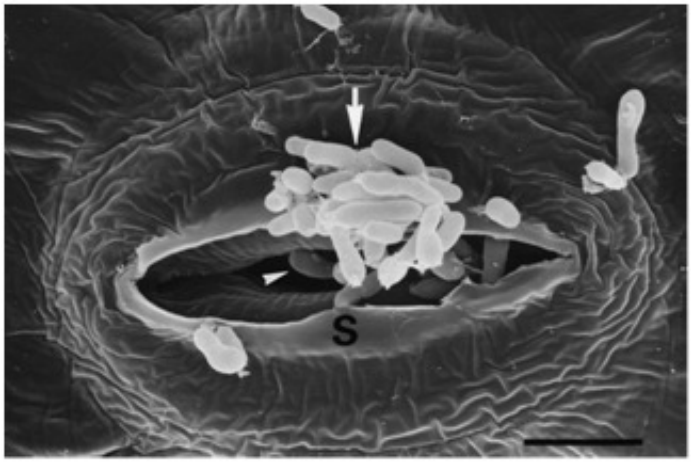

Fig. 2: Methylobacteria surrounding a stomatum of a sunflower plantlet. Bar = 0,5 µm (Kutschera U. (2007) *Plant Signal. Behav.* **2**: 74-78)

Even if they grow rather slowly, this capability to metabolize methanol and their "sun protection" due to incorporated carotenoids gives them a significant advantage over other microbial species on the plant surface. Therefore, methylobacteria can occur on plants in high abundances, especially on young, fast growing plant organs. Their occurrence is proven on more than 70 plant species (Omer et *al*., 2004; Kutschera, 2007) and on some plants they represent more than 80% of the heterotrophic bacterial population (Holland and Polacco, 1994). Also in the rizosphere of plants methylobacteria were detected (Sy et *al.,* 2001). There they interact with plants as rhizobial symbionts that fix atmospheric nitrogen to ammonium. Beside carotenoids they produce a wide range of different secondary metabolites like auxins, cytokinins, vitamins and osmoprotective substances. Therefore, a lot of positive interactions with their host plants like growth or germination promotion are described (Lidstrom and Chistoserdova, 2002; Hornschuh et *al.*, 2002, Abanda-Nkpwatt et *al.,* 2006). Methylobacteria are reported to increase yield in soybean and sugarcane growing (Holland, 2000; Madhaiyan, 2005). There exist several attempts to benefit from these beneficial effects of PPFMs on plants by using them as plant growth promoting inoculants (Holland and Polacco 1996; Joshi and Holland 2001; Holland and Polacco 2006). In 1997 Holland proposed the hypothesis, that methylobacteria are in symbiotic interaction with plants. The bacteria consume toxic methanol and in return they promote growth of their host plant by producing cytokinins. As a consequence the plant grows faster and produces more methanol as substrate for the methylobacteria. The fact that no cytokinin-synthase has been isolated from plants at this time supported this theory. Some years later the hypothetis was corrected because genes for cytokinin-synthase were found in several plants (Haberer and Kieber, 2002).

In biotechnology methylobacteria are in use for the production of vitamins and amino acids (Lidstrom, 2005). There are also attempts to use *M. extorquens* as a heterologous expression system for recombinant proteins with the advantage that this strain can use methanol as carbon source, which is much less expensive than sucrose. Also for the production of polyhoydroxyalkanoates *M. extorquens* is a promising candidate. Like many other bacteria also PPFMs produce polyhydroxyalkanoates (PHAs), if nutrient supply is unbalanced or abiotic conditions deviate from the optimum (Anderson and Dawes 1990). Since PHAs have a chemical structure similar to that of petroleum-derived plastics, they have been investigated as a potential alternative. For *M. extorquens* a high cell density fermentation process for the production of PHB has been developed (Bourque *et al* 1995).

Objective of this doctoral thesis

In previous studies from different plant species and soil samples of different origin isolates of the genus *Methylobacterium* were isolated. Those isolates were subjected to a screening process, which tested their ability to oxidize 1,2-propanediol to 2-hydroxypropanal. Apart from this, several other parameters like growth at 37 °C or growth in liquid culture were assessed. As a result of this screening process one strain has been selected and was used in the present study: *Methylobacterium extorquens* DSM 21961 (Rab1)

The aim of this study was to develop a ready to use product containing one or both of the selected strains being used for the improvement of flavor production in strawberries. For this purpose the colonization of strawberry plants by the two strains and the effect on flavor production has been investigated in detail in a series of field trials and greenhouse experiments. In these experiments the optimum application procedure and the optimum point of time of the application were determined. In a second step a procedure for the

growth of the applied strains in large scale has been developed. Several growth media have been tested in shaking flask cultures and after selection of the most suitable cultivation medium the fermentation was optimized in a 10 L pilot fermenter. Finally, to facilitate application and distribution of the product, a formulation for Rab1 has been developed. Several formulation procedures were tested and shelf life and user-friendliness were estimated.

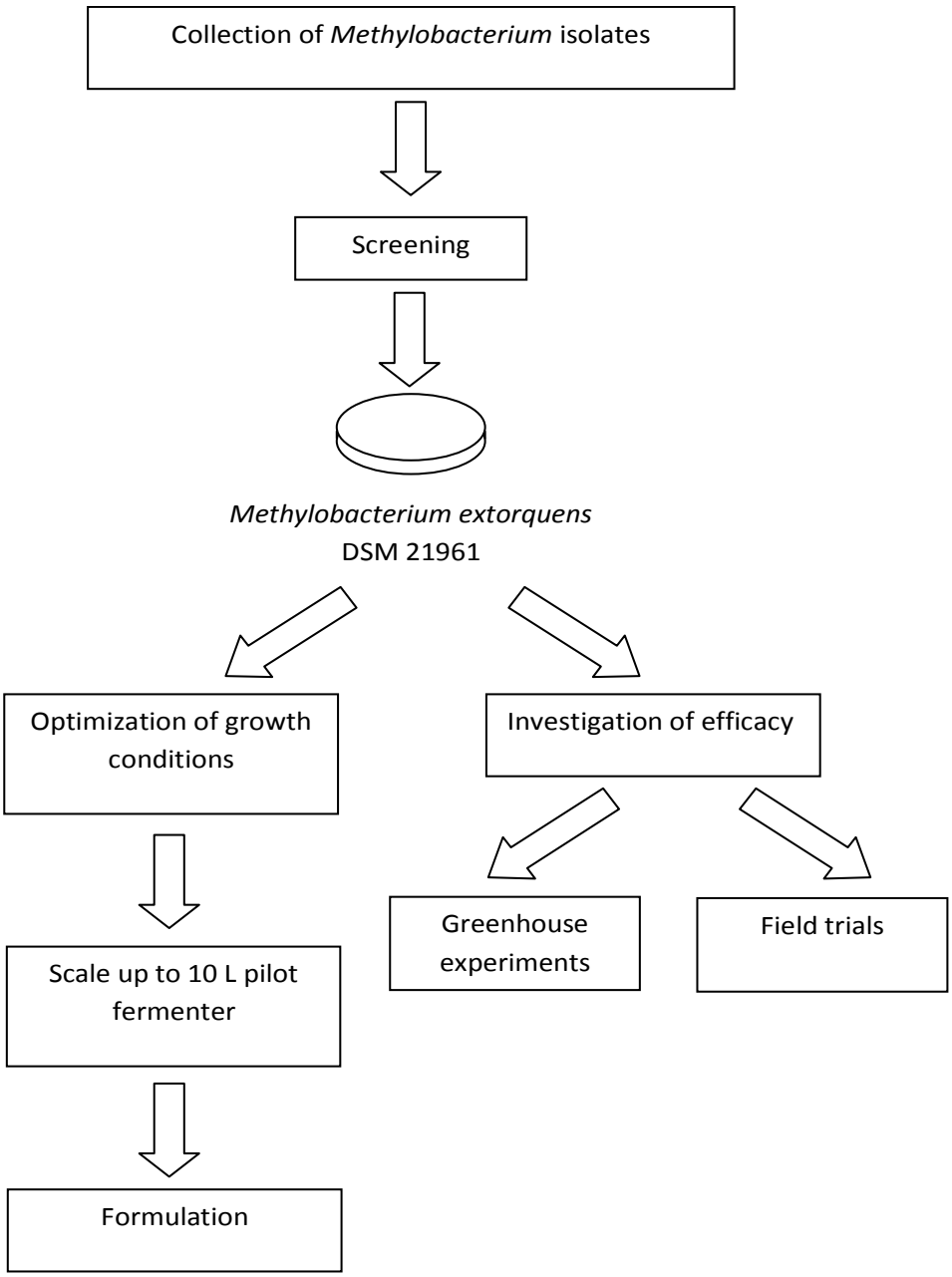

Fig 3: Workflow of the present study

Summary of results

A detailed presentation of the results can be found in the publications and manuscripts located in the appendix of this work. In this section a short summary of the results is given.

Optimization of the application procedure and assessment of efficacy of the treatment

In previous experiments two different application procedures for Rab1 have been tested: a root dipping treatment and a leave spraying application. As methylobacteria normally colonize the phyllosphere of plants, the spraying application gave much better results as the root dipping treatment. Therefore for all further experiments bacteria were applied to plants by spraying a suspension of bacterial cells on the plants. For the investigation of the fate of the bacteria after application a species specific quantitative TaqMan™ Real Time PCR assay has been developed and applied. The following oligonucleotides were used: forward primer Ext-f 5'- AGC ATC GCG AGC TCT GGT A -3', reverse primer Ext-r 5'- CGA AAC GTC ACT GAT CGT ATG AG -3' and probe Ext-p 5'- FAM - CTG GAT GCC GGA CTT GGC TCG TC – TAMRA - 3'.

Results showed that Rab 1 was present on the treated plants for more than five weeks after application in stable abundances. These abundances were dependent from the concentration in which Rab1 has been applied. In greenhouse experiments an application of a suspension of $\log_{10}$ 9.0 CFU mL^{-1} resulted in an abundance of $\log_{10}$ 7.5 cells g^{-1} of fresh leaves after five weeks and the application of a suspension of $\log_{10}$ 8.0 CFU ml^{-1} led to an abundance of $\log_{10}$ 6.0 cells g^{-1} of fresh leaves after five weeks. The optimum date of application was determined to be between one and two weeks before fruit harvest. As dosage an application concentration of the bacteria of $\log_{10}$ 8 CFU mL^{-1} was determined. If lower cell numbers were applied, it was not possible to detect Rab1 on the plants by RT PCR.

Higher cell numbers gave no improved effect. Application of $\log_{10}$ 8.0 CFU mL^{-1} yielded strawberries with a DMHF content of 7.8 ± 0.2 mg kg^{-1} while untreated plants yielded fruits with a DMHF content of 2.8 ± 0.1 mg kg^{-1}. For detailed results see publication I and publication II.

Optimization of growth conditions for Rab1

Testing of several liquid media, which were described in literature to be used for growing methylobacteria showed different suitability for our purposes. Finally, for all further experiments a minimal salt medium supplied with 0.5% (v/v) methanol as sole carbon source was chosen. In this medium in batch cultures in shaking flasks after 48 h at 120 rpm and 30 °C a cell density of $\log_{10}$ 9.5 CFU mL^{-1} was reached and no negative effects like biofilm formation, excessive foaming or an unpleasant odor of the fermentation broth were noticed. In the 10 L Rab1 was grown in a fed-batch fermentation over 48 h at 30 °C. Initial methanol concentration was 0.5% (v/v), after 24 h a carbon feed with 3.3 mL h^{-1} of 50% (v/v) methanol was started and maintained by the end of the fermentation. The fermentor was stirred with a Rushton impeller at 300 rpm, pH was automatically adjusted to 7 with 10% (w/v) NH_3OH and an antifoaming agent was added automatically as required. Oxygen saturation in the fermentation medium was kept at 25% by aeration with sterile air. After 48 h a final cell number of $\log_{10}$ 9.9 CFU mL^{-1} was reached. For detailed results see Manuscript I.

Formulation of Rab1

To facilitate application and distribution of Rab1, four formulations have been developed, evaluated and compared: (i) a lyophilisate [LYO], (ii) a formulation in a xanthan matrix [XAN], (iii) an encapsulation in alginate with [ALG] and (iv) without adaptation [ALD]. All formulations consisted of a pink colored powder. Initial cell numbers in $\log_{10}$ CFU g^{-1} were

11.2 for [ALG], 10.1 for [ALD], 12.7 for [LYO], and 10.5 for [XAN]. After storage over 16 weeks at different temperatures ranging from 4 °C to 44 °C clear differences between the formulations became obvious. [XAN] showed the best survival rates over the whole temperature range. Even at a storage temperature of 44 °C only a slight decline of viable cells in the formulation was visible (-0.005 ± 0.012 log N/N_0 $week^{-1}$). Also [ALG] showed good results at high temperatures (-0.040 ± 0.013 log N/N_0 $week^{-1}$ at 44 °C). The survival of bacterial cells in the formulations depended on the temperature. The lower the storage temperature has been, the lower the degradation of bacterial cells occurred and therefore the lower were the differences between the variants. After application of the different variants to strawberry plants no differences in the survival of Rab1 on the plants was detected. For our purpose variant [XAN] was the best solution. This variant combines a good shelf life with low production costs and an easy application. For detailed results see Manuscript I.

References

Abad A., Moreno M.J., Pelegri R., Martínez M.I., Sáez A., Gamón M., Montoya A. (1999) Determination of carbaryl, carbofuran and methiocarb in cucumbers and strawberries by monoclonal enzyme immunoassays and high-performance liquid chromatography with fluorescence detection: An analytical comparison. *J. Chromatogr. A.* 833: 3-12

Abanda-Nkpwatt D., Müsch M., Tschiersch J., Boettner M., Schwab W. (2006) Molecular interaction between *Methylobacterium extorquens* and seedlings: growth promotion, methanol consumption and localization of the methanol emission site. *J. Exp. Bot.* 57: 4025-4032

Baker B.P., Benbrook C.M., Groth III E., Benbrook K.L. (2002) Pesticide residues in conventional, IPM-grown and organic foods: Insights from three U.S. datasets. *Food Addit. Contam.* 19: 427-446

Haberer G., Kieber J.J. /2002) Cytokinins. New insights into a classic phytohormone. *Plant Physiol.* 128: 354-362

Hauck T., Brühlmann F., Schwab W. (2003) 4-Hydroxy-2,5-dimethyl-3(2H)-furanone formation by *Zygosaccharomyces rouxii*: effect of the medium. *J. Agric. Food Chem.* 51: 4753-4756

Holland M.A. (2000) A method for altering the metabolism of plants. *U.S. Patent WO 00/60052* 12. October 2000

Holland M.A. and Polacco J.P. (1994) PPFMs and other covert contaminants: Is there more to plant physiology than just plant? *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 45:197-209.

Hornschuh M., Grotha L., Kutschera U. (2002) Epiphytic bacteria associated with the bryophyte *Funaria hygrometrica*: effects of Methylobacterium strains on protonema development. *Plant Biol.* 4: 682-687

Klopotek Y., Otto K., Böhm V. (2005) Processing strawberries to different products alters content of vitamin C, total phenolics, total anthocyans, and antioxidant capacity. *J. Agric. Food Chem.* 53:5640-5646

Kutschera U. (2007) Plant-associated methylobacteria as co-envolved phytosymbionts. *Plant Signal. Behav.* 2: 74-78

Larsen M., Poll L., Olsen C.E. (1992) Odour thresholds of some important aroma compounds in strawberry. *Z. Lebensm. Wiss. Forsch.* 195: 536-539

Lidstrom M.E., Chistoserdova L. (2002) Plants in the pink: cytokinin production by *Methylobacterium. J. Bacteriol.* 184: 1818

Madhaiyan M., Poonguzhali S., Lee H.S., Hari K., Sundaram S.P., Sa T.M. (2005) Pink-pigmented facultative methylotrophic bacteria accelerate germination, growth and yield of sugarcane clone Co86032 (Saccharum officinarum L.). *Biol. Fertil. Soils* 41: 350-358

Omer Z.S., Tombolini R., Gerhardson P. (2004) Plant colonization by pink-pigmented faculatative methylotrophic bacteria (PPFMs). *FEMS Microbiol. Ecol.* 47: 319-326

Patt T.E., Cole G.C., Hanson R.S. (1967) *Methylobacterium*, a new genus of facultatively methylotrophic bacteria. *Int J Syst Evol Microbiol* 26:226-229

Perkins-Veazie P. (1995) Growth and ripening of strawberry fruits. *Horticult. Rev.* 17: 267-297

Roscher R., Bringmann G., Schreier P, Schwab W. (1998) Radiotracer studies on the formation of 2,5-dimethyl-4-hydroxy-3(2*H)*-furanone in detached ripening strawberry fruits. *J. Agric. Food. Chem.* 1998; 46: 1488-1493

Scalbert A., Williamson G. (2000) Dietary intake and bioavailability of polyphenols. *Br. J. Nutr.* 130: 2073-2085

Schieberle P., Hofman T. (1997) Evaluation of the Character Impact Odorants in Fresh Strawberry Juice by Quantitative Measurements and Sensory Studies on Model Mixtures. *J. Agric. Food Chem.* 45: 227-232

Slaughter C. (1999) The naturally occurring furanones: formation and function from pheromone to food. *Biol. Rev.* 74: 259-276

Sung W.S., Jung H.J., Park K., Kim H.S., Lee I.S., Lee D.G. (2006) 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF); antimicrobial compound with cell cycle arrest in nosocomial pathogens. *Life Sci*. 80: 586-591

Sy A., Giraud E., Jourand P., Garcia N., Willems A., Lajudie P., Prin Y., Neyra M., Gillis M., Boivin-Masson C., and Dreyfus B. (2001) Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *J. Bacteriol.* 183:214-220.

Zabetakis I. (1997) Enhancement of flavor biosynthesis from strawberry (*Fragaria x ananassa)* callus cultures by *Methylobacterium* species, *Plant Cell Tissue Organ Culture* 50: 179-183

Holland M, Polacco JC (2006) Method for altering the metabolism of plants. *US Patent* US 2006/0228797 A1

Joshi J, Holland MA (2001) Method for treating plants. *US Patent* US6,329,320 B1

Holland M, Polacco JC (1996) Seeds, coated or impregnated with a PPFM. *US Patent* 5,512,069

Anderson AJ, Dawes E (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol Rev* 54:450-472

Bourque D, Pomerleau Y, Groleau D (1995) High-cell-density production of poly-β-hydroxybutyrate (PHB) from methanol by *Methylobacterium extorquens*: production of high-molecular-mass PHB. *Appl Microbiol Biotechnol* 44:367-376

Appendix

1. Compendium of Manuscripts

Publication I: Development of an effective agent to enhance flavor production in strawberries based on a bacterial formulation

Publication II: Monitoring the plant epiphyte *Methylobacterium extorquens* DSM 21961 by real time PCR and its influence on strawberry flavor

Manuscript I: High shelf-life formulations for *Methylobacterium extorquens* DSM 21961, a microbial inoculant to enhance strawberry flavor

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2. Curriculum vitae

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Publication I

Development of an effective agent to enhance flavor production in strawberries based on a bacterial formulation[1]

Abstract: Object of this publication is to describe the development of a commercial product based on a formulation of *Methylobacterium extorquens* that is used to enhance flavor production in ripening strawberries. Basis of the project is the interaction of methylobacteria with strawberry plants; the methylobacteria provide lactaldehyde that acts as a chemical precursor for the biosynthesis of furanoic compounds for the strawberry plant. The successive phases of the project are described. At the beginning, strains of the genus *Methylobacterium* were isolated from different plant species and screened regarding their ability to synthesize lactaldehyde. The strain with the highest rate of biosynthesis of lactalehyde was used for the further product development. The production of the bacterium in a pilot bioreactor as well as the application of the selected strain on the plant was optimized and the efficiency of the system was shown in different field studies.

Introduction

Strawberry flavor is extremely popular worldwide as flavoring added in many different foods and of course also as part of the natural fruits. The natural flavor is composed of more than 350 different volatile chemical constituents and is therefore one of the most complex fruit flavors. Amongst this large number of different components, two compounds belonging to

[1] Verginer M., Müller H., Siegmund B., Leitner E., Berg G. Development of an effective agent to enhance flavor production in strawberries based on a bacterial formulation. Biology of Plant-Microbe Interactions, Vol. 7, edited by Antoun H., Avis T., Brisson L., Prévost D., Trepanier M.

the class of furanones, 2,5-dimethyl-4-hydroxy-*2H*-furanone (DMHF, furaneol) and 2,5-dimethyl-4-methoxy-*2H*-furanone (DMMF, mesifurane) play an outstanding role because of their pleasant sensory properties and their low sensory thresholds (Bood and Zabetakis 2002). There is strong evidence that methylotrophic bacteria, which colonize the surface of strawberry leaves, can enhance the biosynthesis rate of these compounds in the plant via molecular interaction (Koutsompogeras et al. 2007). Methylobacteria are ubiquitous, distinct pink pigmented alphaproteobacteria which can use methanol, methylamine as well as C2-, C3- and C4-compounds as carbon source. Their property to metabolize methanol allows them to colonize the phyllosphere of plants, where they utilize methanol that is emitted through the stomata. They have been shown to stimulate seed germination and plant growth by the production of phytohormones (Lidstrom and Chistoserdova 2002, Omer et al 2004). In literature an interaction between methylobacteria and strawberry plant is discussed: a direct intervention of the bacteria into the biosynthetic pathway of furanones in the plant by providing chemical precursors is supposed (Zabetakis 1997). In field trials an increase of the furaneol concentration in ripe strawberries was shown after application of a suspension of *Methylobacterium extorquens* DSM 21961 on the plants (Berg et al. 2009).

Therefore, methylobacteria and their interaction with strawberry plants were a major objective for further investigations with the aim to develop a commercial product based on a formulation of methylobacteria that increases the biosynthesis of furanones in ripening strawberries. This paper outlines the milestones in the ongoing process of product development.

Collection of strains and *in vitro* screening

In the first phase of the project, a collection of 83 different strains of methylobacteria occurring on different plants (cultivars and naturally occurring species of strawberry, bryophytes and others) was created to provide a wide selection from which the favored strain that was used for the product development was chosen. Plants were sampled, and the occurring methylobacteria were enriched on selective agar plates containing ammonium mineral salt medium supplemented with 0.5 % (v/v) methanol as organic carbon source. Sampling was performed by imprinting leaves, roots and sections through inner plant tissues on agar plates. Plates were incubated for 7-10 days at 30 °C; pure cultures were isolated and conserved at -70 °C for further use.

All collected strains were tested for their ability to grow to high cell densities in different liquid media. This property is crucial for a later production in a technical scale. In addition, all strains were grown with 1,2-propanediol as only carbon source and their production of 3-hydroxopropanale was measured via HPLC. The provision of 2-hydroxypropanale to the plant is the central point in the interaction between methylobacteria and strawberry plant, therefore special attention was paid to this property. Regarding to the reached concentrations of 2-hydroxypropanal and cell numbers in liquid cultures, two strains were selected for a first application in field trials (see Table 1).

Tab. 1: Details about the two strains selected for *in vivo* studies

Strain	***Rab1***	***Sab1***
Identification	*Methylobacterium extorquens*	*Methylobacterium mesophilicum*
Deposition number	DSM 21961	DSM 21962
Origin	*Rosa sp.* leave surface	*Galanthus nivalis* leave surface
Production of 2-hydroxypropanal on 1,2-propandiol in batch cultures [ng/10^6 cells]	854	497
cell numbers in batch culture on 1,2-propandiol after 14 d [CFU/mL]	$1.3*10^7$	$3.5*10^7$

In vivo tests of selected strains

In a first field trial, the two strains with the highest rate of 2-hydroxypropanal production were applied on strawberry plants. Two different application procedures, a root dipping before planting and a spraying on the strawberry leaves after planting, were tested. These tests showed that spraying is more efficient in comparison to the root dipping, and resulted in an increased concentration of mesifurane in the harvested fruits compared with fruits of untreated plants. The impact of the application on the autochtonous population of alphaproteobacteria in the strawberry phyllosphere was analyzed with the Single Strand Conformation Polymorphism Analysis (SSCP) using universal and specific primers. No shift in the population of alphaproteobacteria due to the application was observed. To investigate the fate of the applied bacteria in the strawberry phyllosphere, for one strain *Methylobacterium extorquens* DSM 21961 a strain-specific set of PCR-primers and a TaqMan probe were developed and applied in a quantitative Real Time PCR (Verginer, unpublished results). It could be demonstrated that the abundance of the strain on the leaves remained

stable over the total test time period of one month. The optimization of the application of the bacteria regarding cell concentration and point of time is in progress.

Growing of bacteria

For the commercial use of a bacterial strain it is essential to grow it as easy and cost-efficient as possible. Therefore, the growth of *Methylobacterium extorquens* DSM 21961 was optimized in culture flasks and a pilot-bioreactor with a volume of 10 litres. Different liquid media were tested regarding the cells numbers that could be reached. In a complex medium containing peptone and meat extract final cell numbers of $5*10^8$ cells/mL were reached in a batch fermentation after 48 hours, but the cell suspensions had a unpleasant smell and the components of the medium were to expensive for use in technical scale. A change to a mineral salt medium with 0.5% (v/v) methanol as carbon source resulted in almost odourless cell suspension but unfortunately, cells grown in this medium were prone to coagulate if suspensions were not shaken or stirred. Finally, a medium with an increased content of mineral salts optimized by Borque et al. (1995) for the production of polyhydroxybutyrates with *Methylobacterium extorquens* was used. This medium resulted in cell numbers of 10^9 cells/mL in batch cultures and over 10^{10} cells per mL in fed-batch cultures. All constituents of the medium are available for low prices on the market.

Formulation

For *Methylobacterium extorquens* DSM 21961 different formulations were tested. Growing the bacteria in alginate beads with subsequent drying of the beads resulted in a granulate containing about 10^{10} cells per gram with no significant loss of viability after storage at room temperature for four month. Sadly, these beads can be solubilized in water only very slowly. Freeze-drying of cell pellets with different stabilizing additives (e.g. saccharose or milk

powder) resulted in a fine powder with good water solubility and a content of about 10^{10} viable cells per gram with no significant loss of viability after storage for one month at room temperature. Also different liquid formulations were tested with additives that avoided sedimentation and coagulation of the cells. Survival was good, if the cell suspensions were stored at 4 °C. Storage at room temperature resulted in degradation of the cells with a total loss of viable cells. A final choice of a formulation procedure will be made after the evaluation of advantages, costs and effort of all methods.

Summary and outlook

This article describes the milestones and general phases of the development of a formulation of *Methylobacterium extorquens* DSM 21961. In a first project phase, a collection of strains was created from which the strain with the most suitable properties was selected. Special attention was given to a good biotechnological behaviour and a high production rate of 3-hydroxapropanal of the strain. This selection was crucial for the project, because errors in this phase of the project can exceed its scheduling and its financial frame. On the following project phases was worked in parallel. Field trials were performed in compliance with natural vegetation cycles and the availability of suitable plant material. The application procedure was optimized in several subsequent field studies, growth conditions and liquid media were improved in culture flasks and a pilot bioreactor and different formulation procedures were tested and the viability of the formulations at different storage conditions was evaluated.

During the project, the scale of field trials was gradually increased from a harvest of a few kg of strawberries in the first trial to five tons in the last one. In parallel also the growth volume for *M. extorquens* DSM 21961 was increased from 200 mL in culture flasks to 10 L in the pilot bioreactor. In the last field trial a growing volume of 1000 L is planned as well as a

harvest of 50 tons of strawberries. Finally, a formulation strategy has to be chosen, evaluated in larger scales and the shelflife of the product over a longer time period has to be proven.

Acknowledgement

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Literature

Berg, G., Müller, H., Leitner, E., Siegmund B. 2009. Enhancement of strawberry flavor. European Patent 08021731.8-2114.

Bood, K.G., Zabetakis, I. 2002. The Biosynthesis of Strawberry Flavor (II): Biosynthetic and Molecular Biology Studies. Journal of Food Science, 67:2-8

Borque, D, Pomerleau, Y., Groleau, D. 1995. High cell density production poly-β-hydroxybutyrate (PHB) from methanol by *Methylobacterium extorquens*: production of high-molecular-mass PHB. Applied Microbiology and Biotechnology, 44:367-376

Koutsompogeras, P., Kyriacou, A., Zabetakis, I. 2007. The formation of 2,5-dimethyl-4-hydroxy-2H-furan-3-one by cell-free extracts of Methylobacterium extorquens and strawberry (*Fragaria* x *ananassa* cv. Elsanta). Food Chemistry, 104:1654-1661

Lidstrom, M.E., Chistoserdova, L. 2002. Plants in the pink: Cytokinin production by *Methylobacterium*. Journal of Bacteriology, 184:1818

Omer, Z.S., Tombolini, R., Broberg, A., Gerhardson, B. 2004. Indole-3-acetic acid production by pink pigmented facultative methylotrophic bacteria, Plant Growth Regulation. 48:93-96

Zabetakis, I. 1997. Enhancement of flavor biosynthesis from strawberry (*Fragaria* x *ananassa*) callus cultures by *Methylobacterium* species. Plant Cell, Tissue and Organ Culture, 50: 179–183

Publication II

Monitoring the plant epiphyte *Methylobacterium extorquens* DSM 21961 by real time PCR and its influence on strawberry flavor[2]

Abstract

Beside its influence on plant growth and health, plant-associated bacteria show an impact on fruit quality. Methylotrophic bacteria can enhance the biosynthesis of strawberry flavor compounds, especially the two furanoid compounds 2,5-dimethyl-4-hydroxy-*2H*-furanone (DMHF) and 2,5-dimethyl-4-methoxy-*2H*-furanone (DMMF) *in vitro*. Here we report the selection and characterization of *Methylobacterium extorquens* DSM 21961, a strain which was able to enhance the furanone content *ad planta* under greenhouse conditions. For monitoring the colonization of strawberry plants, a strain-specific quantification system for *M. extorquens* DSM 21961 was developed. Specificity, linear range and quantitative limit of the system were shown, and the successful application was demonstrated in a monitoring experiment of *M. extorquens* DSM 21961 on strawberry leaves under greenhouse conditions. Furthermore, quantification of DMHF in strawberry fruits via gas chromatography indicated an increased biosynthesis of this compound in strawberry plants. The colonization behavior analyzed by confocal laser scanning microscopy using GFP-tagged cells revealed high colonization of the upper and lower leave surfaces with a specific

[2] Verginer M, Siegmund B, Cardinale M, Müller H, Choi Y, Miguez C, Leitner E, Berg G (2010) Monitoring the plant epiphyte *Methylobacterium extorquens* DSM 21961 by real time PCR and its influence on strawberry flavor. FEMS Microbiol Ecol 74:136-145

accumulation of bacterial cells on trichomes. The results support a biotechnological application of this promising flavor stimulating agent.

Introduction

Plant-associated bacteria fulfill important functions on their host. Beside their well-studied effect on plant growth and health (rev. in Lugtenberg & Kamilova, 2009; Berg, 2009), they are involved in plant metabolism and can influence fruit quality and flavor. Pink-pigmented facultative methylotrophic bacteria are ubiquitous, plant specific phyllosphere-associated bacteria, which use methanol as a source of carbon and energy (Delmotte *et al.*, 2009). They are known for their ability to interact with strawberry cell cultures: a co-cultivation of the callus cultures with *Methylobacterium extorques* led to an increased biosynthesis rate of furanones (Zabetakis & Gramshaw, 1996; Zabetakis, 1997). Although more than 300 volatile compounds were identified in the flavour of strawberries, only a limited number of compounds is responsible for the formation of the typical and very pleasant flavour. Furanoid compounds, and especially 2,5-dimethyl-4-hydroxy-2H-furanone (DMHF) with a flavour described as ‘caramel’, ‘cotton-candy’ is considered to be one of the most important contributors to strawberry flavour (Larsen *et al.*, 1992; Schieberle & Hofmann, 1997; Bood & Zabetakis, 2002). In addition to its aroma properties, DMHF is a known flavour enhancer; recent studies show, that the presence of DMHF in sucrose solutions increases the perceived sweetness of the solution (Labbe *et al*., 2007). In this context, we suppose that an increased DMHF concentration could improve the flavour of strawberries not only by its aroma properties, but also by its flavour enhancing properties.

Furthermore, it was shown, that the presence of *M. extorquens* activated a defense response of the plant cells and increased the production and accumulation of plant antimicrobial compounds to which also DMHF can be grouped (Sung *et al.*, 2006). Also a

direct intervention of the bacteria in the biosynthesis pathway of furanones by providing lactaldehyde (LA) as a secondary metabolite or by oxidation of endogenous 1,2-propanediol is discussed in literature (Zabetakis, 1997). However, this interaction was only analyzed *in vitro*, and there was no evidence that methylobacteria influence the strawberry flavor under natural conditions.

Phyllosphere colonization by bacteria is the key factor for a successful plant-microbe interaction (Whipps *et al*., 2009). Different molecular and microscopic methods are available to study the colonization pattern of plant-associated bacteria. To study colonization at strain level, it is necessary to develop strain-specific primers, e.g. for real time PCR analysis. Many authors used a random amplification of polymorphic DNA (RAPD) (Hadrys *et al*., 1992; Welsh & McClelland, 1990; Williams *et al*., 1990), to provide specific regions in the genome of a target strain for primer design. Most of the strains, for which a specific primer design was described in literature, are used as biocontrol agents in agriculture, e.g. *Pseudomonas fluorescens* EPS62e (Pujol *et al*., 2006), *Aureobasidium pullulans* (Schena *et al*., 2002) and *Trichoderma hamatum* 382 (Abbasi *et al.,* 1999).

The objective of this study was to analyze the *Methylobacterium*-strawberry interaction *ad planta*. Therefore, active *Methylobacterium* strains from different plant species were isolated and characterized. *Methylobacterium extorquens* DSM 21961, the strain for which the highest biosynthesis rate of the precursor LA was measured, was selected to investigate the interaction between bacteria and fully developed strawberry plants. For DSM 21961, a strain-specific primer for qRT PCR was developed. Therefore, we used BOX PCR to provide regions on the genome of the target strain suitable for primer design. The primer used targets the repetitive BOX-elements, which are spread throughout the bacterial chromosome (Koeuth *et al.,* 1995). To complement the results obtained from

qRT PCR, the colonization of strawberry plants by *M. extorquens* was verified by confocal laser scanning microscopy. The influence of *M. extorquens* on flavor production in strawberries was shown by the quantification of DMHF in the strawberry fruits via gas chromatography.

Materials and methods

Bacterial strains and growing media

All strains used in this study are listed in table 1. They were isolated from different host plants by imprinting leaves, roots and sections through inner plant tissues on MIS agar plates, containing in g/L 1.8 $(NH_4)_2SO_4$, 0.2 $MgSO_4*7H_2O$, 1.4 $NaH_2PO_4*2H_2O$, 1.9 K_2HPO_4. After autoclaving, 5 mL/L methanol and 1 mL/L of a trace element stock solution were added. The trace element stock solution contained in mg/L 500.0 EDTA, 200.0 $FeSO_4*7H_2O$, 10.0 $ZnSO_4*7H_2O$, 3.0 $MnCl_2*4H_2O$, 20.0 H_3BO_3, 20.0 $CoCl_2*6H_2O$, 1.0 $CuCl_2*6H_2O$, 2.0 $NiCl_2*6H_2O$ and 3.0 $Na_2MoO_4*2H_2O$. This medium was used both as liquid growing medium and, after addition of 12 g/L of agar agar, as solid medium. Growing temperature of the cultures was 30 °C and incubation time was two days for liquid cultures and five days for agar plates. Long-term storage of bacteria was performed in liquid MIS-medium blended with 20% sterile glycerol at -70 °C.

Table 1: Bacterial isolates used in this study. They were identified by their partial 16S rDNA sequence.

Code	Host plant	Closest database match (EMBL number)	% Homology
Rab1	*Rosa* sp.	*Methylobacterium extorquens* (AB298399.1)	100
Sab1	*Galanthus nivalis*	*Methylobacterium mesophilicium* (AB175636.1	100
EAb1	*Hedera helix.*	*Methylobacterium mesophilicum* (AF408975.1)	99
EAb5	*Hedera helix*	*Methylobacterium mesophilicum* (AM910537.1)	99
EAb8	*Hedera helix*	*Methylobacterium mesophilicum* (AM910537.1)	99
Fab1	*Fragaria x ananassa*	*Methylobacterium extorquens* (AB298401.1)	100
Fab2	*Fragaria x ananassa*	*Methylobacterium extorquens* (AB298401.1)	99
Fab3	*Fragaria x ananassa*	*Methylobacterium extorquens* AB298401.1)	100
Fab5	*Fragaria x ananassa*	*Methylobacterium adhaesivum* (AB302928.1)	99
FmE17	*Fragaria moschata*	*Methylobacterium adhaesivum* (AB302928.1)	98
FmE19	*Fragaria moschata*	*Methylobacterium adhaesivum* (AB302928.1)	98
FmE21	*Fragaria moschata*	*Methylobacterium adhaesivum* (AB302928.1)	98
FmE23	*Fragaria moschata*	*Methylobacterium adhaesivum* (AB302928.1)	98
FmE24	*Fragaria moschata*	*Methylobacterium adhaesivum* (AB302928.1)	98
Hab1	*Hibiscus* sp.	*Methylobacterium extorquens* (AB298399.1)	100
Sab2	*Galanthus nivalis*	*Methylobacterium mesophilicum* (AB175636.1)	100
Sab3	*Galanthus nivalis*	*Methylobacterium mesophilicum* (AM910537.1)	99
Sab7	*Galanthus nivalis*	*Methylobacterium adhaesivum* (AB302928.1)	98
Sab9	*Galanthus nivalis*	*Methylobacterium adhaesivum* (AB302928.1)	98
Sab12	*Galanthus nivalis*	*Methylobacterium mesophilicum* (AM910537.1)	99
Sab13	*Galanthus nivalis*	*Methylobacterium mesophilicum* (AM910537.1)	99
Sab16	*Galanthus nivalis*	*Methylobacterium mesophilicum* (AM910537.1)	99
Sab18	*Galanthus nivalis*	*Methylobacterium mesophilicum* (AM910537.1)	99
Sab 19	*Galanthus nivalis*	*Methylobacterium mesophilicum* (AM910537.1)	99
Sab20	*Galanthus nivalis*	*Methylobacterium adhaesivum* (AB302928.1)	98
Sab21	*Galanthus nivalis*	*Methylobacterium mesophilicum* (AM910537.1)	99

To identify methylobacteria, a single colony on LB-medium was picked with a sterile toothpick into 50 µl de-mineralized water in a 1.5 ml Eppendorf tube. The cell suspension was heated to 96°C for 10 min. The 30 µl reaction mixture contained 6 µl 5xTaq&Go (MP Biomedicals), 1.5 µl of primer pair mix EubI-forward (5'-GAG TTT GAT CCT GGC TCA G-3') and 1492r-reverse (5'- TAC GGY TAC CTT GTT ACG ACT T-3') both in a concentration of 10 pmol μl^{-1} and 20-30 ng template. The PCR products were purified with GeneClean Turbo Kit as recommended by the manufacturer. The fragments were sequenced using the reverse primer 1492r. For identification of related sequences a database alignment using BLAST algorithm was done. One strain *Methylobacterium extorquens* Rab 1 was transfered to the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and got the number DSM 21961. The ability of strains to produce lactaldehyde (LA) *in vitro* was estimated according to Zabetakis (1997).

Isolation of genomic DNA

Bacteria were grown in 2 mL reaction tubes containing 1.5 mL MIS medium at 30 °C for 48 h shaken at 120 rpm. Cells were centrifuged down at 14.000 x *g* for 10 minutes. The supernatant was discharged and cells were resuspended in 1 mL of extraction buffer (2 mM Tris, 200 mM NaCl, 25 mM EDTA, 0.5% SDS). Cell suspensions were transferred into 2 mL reaction tubes with screw caps containing 400 mg sterile glass beads (0.25-0.5 mm, Sigma-Aldrich, Missouri, USA). The tubes were treated with a FastPrep instrument (Qbiogen BIO 101 Systems, Carlsbad, USA) for 30 s at level 5. 150 µL of 3 M sodium acetate were added and the samples were shaken for 2 min by hand. After centrifugation at 14000 x *g* for 5 min the clear supernatant was transferred into a fresh 1.5 mL reaction tube, cleaned by phenol-chloroform extraction and DNA was precipitated at 0 °C for 1 hour adding an equal amount

of ice cold isopropanol. The precipitated DNA was centrifuged down for 15 min at 14000 x *g* and 4 °C and the resulting pellet was washed with 500 µL ice cold 70% ethanol, air-dried, solved in 50 µL TE-buffer (100 mM, pH 8) and stored at -20 °C.

BOX PCR and sequencing of PCR products

Amplification reactions were performed in a final volume of 25 µL, containing 1x Taq-&Go™ Mastermix (MP Biochemicals, Irvine, USA), 2.5 µM of Primer A1R (5´ CTA CGG CAA GGC GAC GCT GAC G 3') (Lloyd-Jones *et al.,* 2005) and 50 ng of template DNA. Thermocycler conditions consisted of an initial denaturation step at 99 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 60 sec, primer binding at 53 °C for 60 sec and elongation at 65 °C for 8 min and ending with an elongation step at 65 °C for 16 min. The success of the amplification was tested in a 1.5 % agarose gel in 0.5x TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8). Electrophoresis was performed at 2.8 V/cm for 4.5 hours. The gel was stained with ethidiumbromide.

The PCR product of the BOX PCR of *M. extorquens* DSM 21961 was purified with the Geneclean Turbo Kit (MP Biomedicals, Irvine, USA) according to the manufacturer's protocol. After purification, DNA concentration was determined photometrically. As sequencing vector the pGEM®-T Vector System with the corresponding cloning kit (Promega, Madison, USA) was applied. Insertion was performed in a final reaction volume of 10 µL, containing 1x ligation buffer, 50 ng pGEM®-T vector, 1 U T4-ligase and 25 ng PCR product. The ligation reaction was incubated over night at 4 °C. Next day, 2 µL of the ligation reaction were used for the transformation of competent cells of *Escherichia coli* JM109 (Promega) according to the protocol published by Sambrook & Russel (2001) Next day, clones were picked and checked on the appropriate vector insert via colony PCR. To provide template DNA, from 20

white colored colonies some cell material was transferred into 1.5 ml reaction tubes with the tip of sterile toothpicks, 100 µL of water were added and the tubes were heated to 100 °C for 5 min in a heating block and afterward frozen for 15 min. PCR was performed in a final volume of 25 µL, containing 1x Taq-&Go™ Mastermix, 4 mM forward primer usp (5´ GTA AAA CGA CGG CCA GT ´3), 4 mM reverse primer rsp (5´ CAG GAA ACA GCT ATG ACC ´3), 1.6 mM additional $MgCl_2$ and 1 µL of template DNA-solution. The thermocycler program consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 20 sec, primer annealing at 54 °C for 15 sec and elongation at 72 °C for 30 sec and ended with a final elongation step at 72 °C for 10 min. The success of the PCR reactions was checked in a 0.8 % agarose gel in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 1mM EDTA pH 8). Amplificons were purified with a Geneclean Turbo Kit (MP Biomedicals) according to the manufacturer's protocol and sequenced with an Applied Biosystems 3130l Genetic Analyser sequencer Data Collection v. 3.0, Sequencing Analysis v. 5.0 (Foster City, USA) at the sequencing core facility ZMF, Medical University of Graz, Austria.

Primer design

Obtained sequences were subjected to a BLAST search according to Altschul *et al*. (1997) against all available DNA sequences in the NCBI database. Sequences that gave any positive result (especially genes for proteins involved in DNA replication were found), were excluded from the further process of primer design. The remaining sequences provided the basis for primer design with the software package Primer Express 5.0 (Applied Biosystems, Carlsbad, USA). As result the software package emitted a list of 200 different sets of primers and probes, from which one set was chosen (forward primer Ext-f, reverse primer Ext-r, probe Ext-p).

DNA-Extraction for quantitative RT PCR

Different methods for DNA extraction and some ready-to-use extraction kits were tested with leave samples containing different bacterial abundances. The FastDNA SPIN for Soil Kit (Qbiogen BIO 101 Systems, Carlsbad, USA) gave the best results regarding reproducibility and linearity over the tested range of bacterial abundances and was used for all further analyses. For DNA extraction strawberry leaves were freeze-dried over night (Labconco Freezedry system/Freezone 4.5, Labconco, Kansas City, USA) and pulverized with mortar and pestle. From the obtained powder 20 mg were used for DNA extraction with the FastDNA SPIN for Soil Kit according the manufacturer's instructions. The obtained DNA extract was cleaned with the Geneclean Turbo Kit according the manufacturer's protocol. DNA concentration in the extracts was determined photometrically and adjusted to 10 ng/µL by diluting the samples with ultrapure water. DNA samples were stored at -20 °C.

Quantitative real-time PCR

qRT PCR was performed in a final reaction volume of 20 µL in 96 well plates (Applied Biosystems, Foster City, USA) in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The reaction mixture contained 1x TaqMan mastermix (Applied Biosystems), 50 nM Primer Ext-f, 50 nM Primer Ext-r and 200 nM Probe Ext-p. To each reaction 10 ng (1µL) of template DNA were added. The thermocycler program consisted of an initial denaturation step of 10 min at 90 °C, followed by 45 cycles composed of a denaturation step of 15 sec at 90 °C followed by an elongation step of 60 sec at 60 °C. Data acquisition was performed during the elongation step. For the evaluation of the obtained data the ABI PRISM detection software was used.

Determination of specificity and limit of quantification and calibration of the system

The specificity of the detection system was tested against pure cultures of methylobacteria, which were isolated from different plants (Table 1). qRT PCR was performed with each 10 ng of DNA extracted from pure cultures and the resulting cycle threshold values were compared.

The limit of quantification of the system was tested with a pure DNA extract from *M. extorquens* DSM 21961, which was diluted to different concentrations and additionally with a DNA extract of strawberry leaves, which were grown under sterile conditions, that was supplemented with different amounts of DNA from *M. extorquens* DSM 21961.

To take into consideration all losses of DNA and other sources of errors during the process of DNA extraction, for the calibration of the system leave samples were supplemented with different cell numbers of *M. extorquens* DSM 21961. From these samples DNA was extracted and qRT PCR was performed as described above. The resulted cycle threshold values were used to calculate a calibration curve for the system.

Quantitative monitoring of M. extorquens DSM 21961 on strawberry plants

Strawberry plants (variety *Elsanta*) were purchased in a local nursery and were planted in pots of a volume of 1.6 liters containing potting soil supplemented with 2.5 g fertilizer (Compo Beerenduenger, Compo, Muenster, Germany) per pot. The plants were grown under artificial light (16 hours illumination per day with metal halide lamps), temperature control (20 °C) and watering as required in a greenhouse. At the stage of flowering the plants were sprayed with suspensions of *M. extorquens* DSM 21961 grown in 1000 mL shaking flasks containing 300 mL medium 4 (Bourque *et al*., 1995) for 48 h at 30 °C and 120 rpm. After enumeration of cell density in a hemocytometer, cell suspensions were diluted with tap

water to concentrations of $\log_{10}$ 6.0, 8.0 and 9.0 cells per mL. As control pure tap water was used. For each concentration, 6 strawberry plants were treated. All leaves that were present at the moment of the treatment were labeled with stripes of adhesive foil to distinguish them from leaves grown after the application of the bacterial suspension. In the following five weeks at intervals of four days samples of treated leaves were taken. From each plant one leave was picked for each sample. The samples were stored at -20 °C until the end of the sampling period, then DNA was extracted from the samples and qRT PCR was performed as described above. Comparing the measured cycle threshold values with the calculated calibration curve the abundance of *M. extorquens* DSM 21961 on the leaves over the time period of sampling was determined.

Confocal laser scanning microscopy

Strawberry plants were grown as described above and at their flowering time they were sprayed with a suspension of *Methylobacterium extorquens* ATCC 55366 GFP5 (Choi *et al*., 2006) with a concentration of $\log_{10}$ 8.0 cells mL^{-1}. After two weeks leave samples were taken and analyzed with a confocal laser scanning microscope Leica TCS SPE (Leica Microsystems, Wetzlar, Germany). Plant tissues were excited with a 635 nm laser beam and the autofluorescence emitted in the range 650-690 nm was recorded. The GFP was excited with a 488 nm laser beam and the detection window was optimized for every field of view, in order to obtain a better discrimination between the signals and the noise. Z-stacks were acquired with a Z-step of 0.4–0.8 µm using the objectives 40X or 63X (Leica). The fluorescent signals from GFP and from plant tissues were acquired sequentially.

Quantification of DMHF in strawberries

In an independent experiment the DMHF concentration in strawberries grown on methylobacterium-treated and on untreated plants was determined and compared. Strawberry plants were grown as described above and also the treatment of the plants with suspensions of *M. extorquens* DSM 21961 was performed with the same cell concentrations as described. For each concentration 16 plants were treated. Ripe strawberry fruits were harvested and fruits of four consecutive days were pooled and frozen at -20 °C. After the harvest of all ripe strawberries a leave sample of each variant was taken and the abundance of *M. extorquens* DSM 21961 was determined as described above.

The quantitative determination of DMHF was performed by gas chromatography-mass spectrometry (GC-MS) after solid phase extraction (SPE). Method development was described elsewhere (Siegmund *et al*., 2010). Gas chromatography was selected as separation technique as with the used polar stationary phase a rapid simultaneous determination of DMHF and DMMF with sufficient sensitivity could be achieved. For the extraction of the analytes, 2 g homogenized strawberries were mixed thoroughly with 25 mL buffer solution (KH_2PO_4 and $Na_2HPO_4.2H_20$, pH 5.0). Maltol (>98%, Sigma-Aldrich, Steinheim, Germany) was added to the system as an internal standard in a concentration of 5mg/kg strawberries. After centrifugation of the slurry, an aliquot of 5 mL was transferred onto the SPE cartridge (Strata X, polymeric reversed phase, particle size 33 µm, 500 mg/3 mL; Phenomenex Ltd., Germany). The SPE cartridge was conditioned with 5 mL methanol (for residue analysis, Promochem, Wesel, Germany) and 5 mL deionized water prior to use. The elution of the analytes was performed with acetone (for residue analysis, Promochem, Wesel, Germany), whereas 1 mL was discarded, 2 mL were collected and used for the GC-MS analysis.

For the GC-MS measurements a Hewlett Packard system (HP G1800A GCD System, electron impact ionisation) was used. The capillary column used was a DB Wax (column dimensions 30 m x 0.25 mm x 0.5 µm, Agilent Technologies Inc., USA). Helium (99.999%, Air Liquide, Austria) was used as carrier gas. The GC conditions were as follows: injector temperature 220 °C, detector temperatur 240 °C; a constant flow method (40 kPa at 60 °C) was used starting at a temperature of 60 °C (hold time 1 min) with a temperature rate of 8°C/min to 245 °C (hold time 5 min). Splitless injection mode was used, the split valve being opened after 1 minute. Electron impact ionization was used (70 eV). Data were acquired in the selected ion mode. Identification of the compound was performed via the calcuation of the linear temperature programmed retention index and comparison with the RI of the database as well as the data obtained from the reference compound. In addition the identity of the compound was cross-check by acquiring data in the scan modus and interpretation of the obtained mass spectra. Quantification of the compounds was performed via the ratio of the response of the internal standard to the analytes' response at four different concentrations levels. Performance of the whole sample preparation procedure was cross-checked by performing various standard addition experiments (i.e. addition of DMHF in four different concentration levels to the pure buffer solution as well as by addition to real strawberry samples, all determinations were performed in true replicate). Recovery for DMHF was determined to be 92 ± 3%. In course of the quantification procedure, possible variations of the recovery were corrected via the internal standard. The quantification procedure was fully validated in a concentration range from 0.25-200 mg DMHF per kg strawberries using an Excel macro especially designed for the validation of analytical procedures (ValiData version 3.02.48). All given numbers are mean values from duplicate extraction of the strawberry samples. Each extract was analysed twice by GC-MS.

Results

Isolation and characterization of Methylobacterium strains

Methylobacterium strains were isolated from different host plants by imprinting leaves and roots on MIS agar plates (see selection of strains in Table 1). All strains were able to grow on 1,2-propanediol. The biosynthesis rate of lactaldehyde differed from strain to strain and ranged from 20 - 855 ng/$\log_{10}$ 6.0 cells. Strain *M. extorquens* DSM 21961, which had the highest production rate, was selected for further investigations.

Design of specific primer for M. extorquens DSM 21961

To find discriminating regions in the genome of *M. extorquens* DSM 21961, BOX PCR fingerprints with the target strain and different reference strains of the genus *Methylobacterium* were performed (Fig. 1).

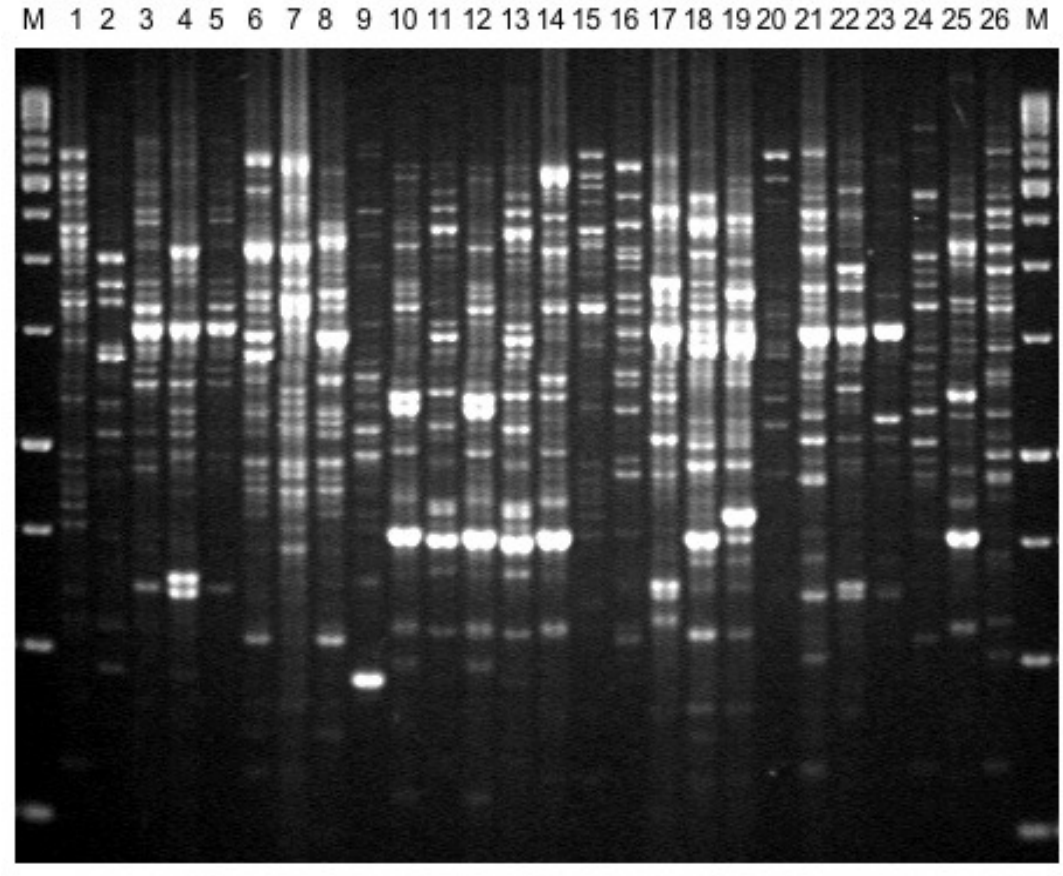

Fig 1: BOX-fingerprints of *Methylobacterium extorquens* DSM 21961 and 25 reference strains. (1) *M. extorquens* DSM 21961, (2) Sab1, (3) EAb1, (4) EAb5, (5) EAb8, (6) Fab1, (7) Fab2, (8) Fab3, (9) Fab5, (10) FmE17, (11) FmE19, (12) FmE21, (13) FmE23, (14) FmE24, (15) Hab1, (16) Sab2, (17) Sab3, (18) Sab7, (19) Sab9, 20 (Sab12), (21) Sab13, (22) Sab16, (23) Sab18, (24) Sab19, (25) Sab20, (26) Sab21, (M) 1 kb DNA Ladder (Fermentas)

For sequencing, PCR products of *M. extorquens* DSM 21961 were cloned into a pGEM®-T vector. Primer design with the obtained sequences was performed with the software package Primer Express 5.0 (Applied Biosystems). From the different primers and probes suggested by the software the following set was chosen: forward primer Ext-f 5'- AGC ATC GCG AGC TCT GGT A -3', reverse primer Ext-r 5'- CGA AAC GTC ACT GAT CGT ATG AG -3' and probe Ext-p 5'- FAM - CTG GAT GCC GGA CTT GGC TCG TC – TAMRA - 3'. These primers target a sequence with a length of 92 bp. The gap between forward primer and probe is 8 bp.

Determination of specificity and limit of quantification and calibration of the system

Specificity of the primer probe system was tested against 25 different isolates of the genus *Methylobacterium*. *M. extorquens* DSM 21961 gave a cycle threshold (ct value) of 20, while all other isolates except Hab1 gave ct values higher than 35 (Fig. 2).

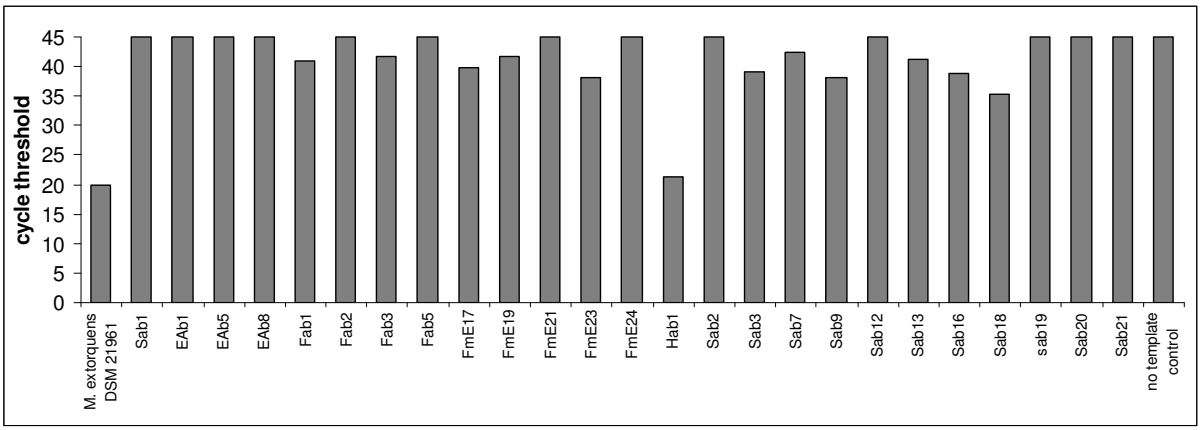

Fig. 2: Specificity of the designed TaqMan system verified against different *Methylobacterium* isolates. *M. extorquens* DSM 21961 and different reference strains (see table 1) were tested, and cycle thresholds were compared.

Comparison of 16S rDNA sequences and BOX-patterns of Hab1 and *M. extorquens* DSM 21961 indicated that both isolates are genetically identical (see Fig. 1). In general, ct values above 35 were considered as negative because also no template controls and even empty PCR vials occasionally gave results in this range. This effect was observed also by other authors (Pujol *et al*., 2005, Lloyd-Jones *et al*., 2005), and has been considered as negligible if the obtained ct values are outside the range of calibration.

The test of the system with pure DNA extract of *M. extorquens* DSM 21961 showed a linear correlation of the ct with the amount of template DNA over a range of more than seven orders of magnitude Fig. 3). The limit of quantification of the system is about 600 fg of

template DNA per PCR reaction. This corresponded to the mass of DNA of approximately 100 bacterial cells, if the size of the genome of *M. extorquens* is assumed to be 5.5 Mbp with a GC-content of 68.2% (cf. GeneBank Accession Number CP000908). Also with leave samples, which were supplemented with bacterial cells, the linear range runs from an abundance of $\log_{10}$ 4.0 cells per gram of fresh leaves to an abundance of more than 10.0 cells per gram. If only ct values less than 35 are considered as reliable, the limit of quantification is $\log_{10}$ 4.6 cells per gram of fresh weight.

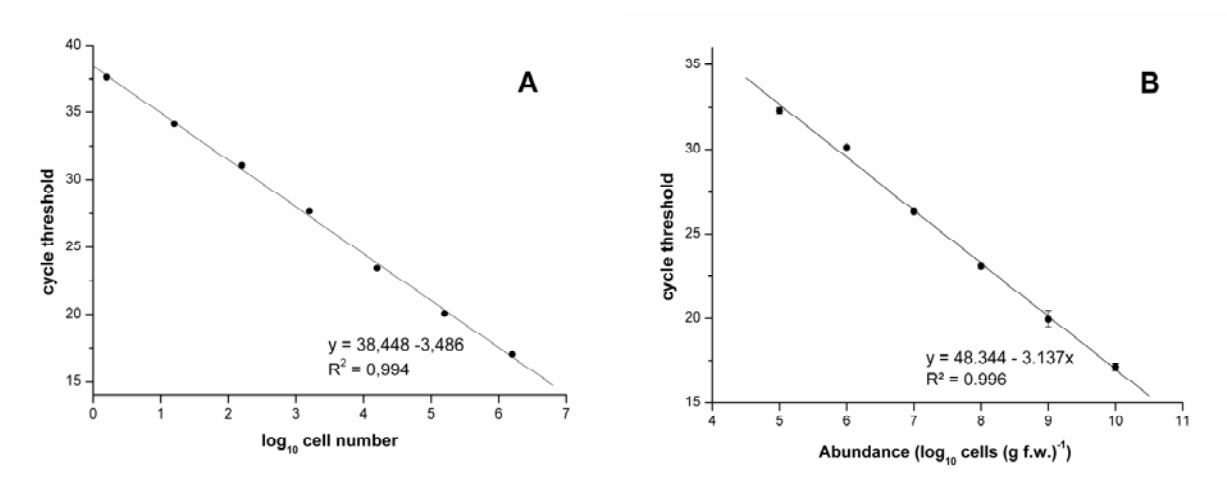

Fig 3: Linearity and detection range of the designed TaqMan system. This was analyzed with (A) pure DNA of *M. extorquens* DSM 21961, and (B) DNA isolated from different cell concentrations *M. extorquens* DSM 21961 from strawberry leaves. Dots represent mean values of three replicates, error bars the corresponding standard deviations; lines represent linear fits of the data. Equation of the calibration functions and correlation coefficient are indicated in the diagrams.

A lowering of the limit of quantification is prevented by the large amount of plant DNA, which is co-extracted during the procedure of sample processing. Optimization experiments have shown that large amounts of non-template DNA can inhibit the PCR reaction (data not shown). Therefore, the total amount of DNA that was applied in one PCR reaction was

limited to 10 ng in total. Additionally, co-extracted plant secondary metabolites may have inhibiting effects on the PCR reaction.

Monitoring of M. extorquens DSM 21961 on strawberry leaves and quantification of DMHF in strawberries

It was possible to monitor the abundance of *M. extorquens* DSM 21961 on strawberry leaves over a time period of 35 days. The variants that were sprayed with cell suspensions with concentrations of $\log_{10}$ 8.0 and 9.0 cells mL^{-1} gave results within the calibration range of the system during the whole monitoring period. Application of the $\log_{10}$ 8.0 variants resulted in abundances between 6.5 cells/g of leaves at the beginning of the monitoring period and 6.0 cells/g of leaves at the end. The $\log_{10}$ 9.0 variants resulted in abundances between 8.7 and 7.3 cells/g of leaves. The control variant that has been treated with tap water gave only ct values below the limit of quantification of the system. The variant that was treated with a suspension with $\log_{10}$ 6.0 cells mL^{-1} resulted in values below or slightly above the limit of quantification. Therefore, the data of this variant are not shown in Fig. 4.

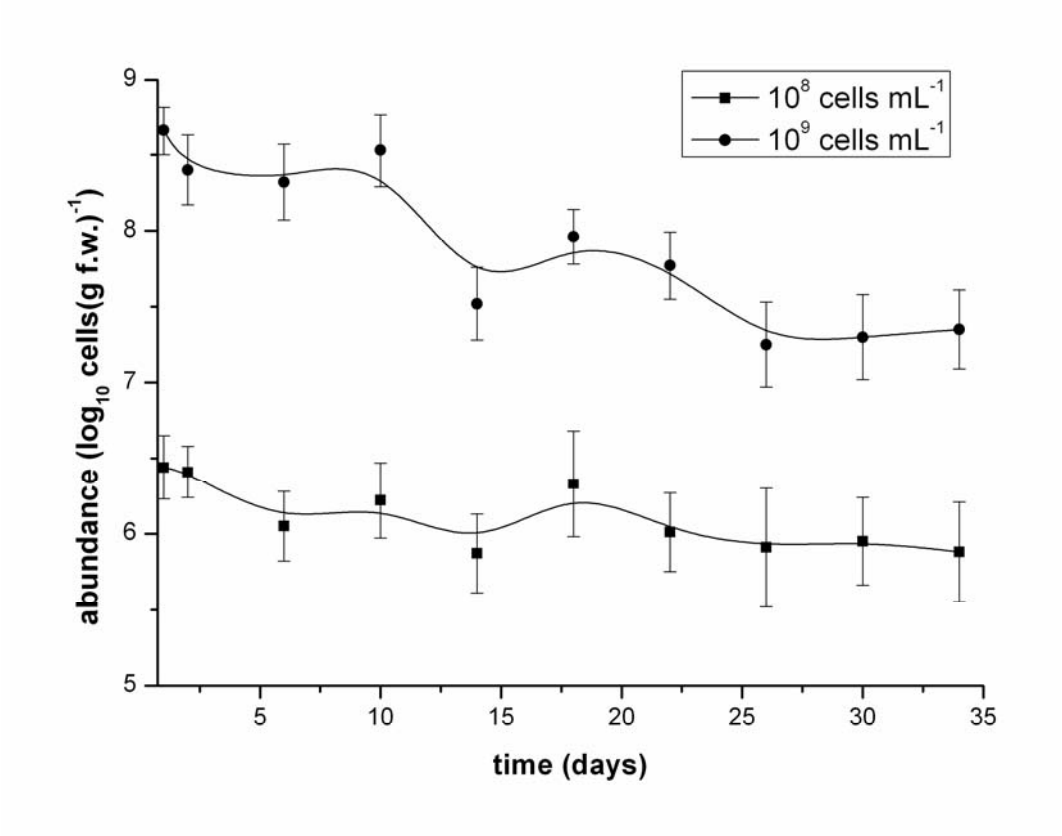

Fig 4: Survival of Methylobacterium extorquens DSM 21961 on strawberry leaves after spraying application with cell suspensions with 108 cells mL-1 (circles) and 109 cells mL-1 (squares) over a time period of 35 days under greenhouse conditions. Datapoints represent mean values of three PCR replications; error bars indicate standard deviations and lines a B-spline interpolation of data points.

In order not to influence fruit quality by the removal of leaves from the plants for the monitoring of the abundance of *M. extorquens* DSM 21961, the concentration of DMHF in strawberries of treated plants was evaluated in an independent experiment. The results of this experiment indicate a significantly increased concentration of DMHF in fruits grown on plants, which were treated with *M. extorquens* DSM 21961 during the stage of flowering (Table 2). The survival of *M. extorquens* DSM 21961 on the plants until the time of harvest was shown by qRT PCR.

Table 2: DMHF concentrations in strawberries and abundance of *Methylobacterium extorquens* DSM 21961 on leaves of the corresponding plants; the given cell numbers correspond to the concentration in the applied suspensions (n.d. means not detectable resp. below the limit of quantification).

	control sample	10^6 cell mL^{-1}	10^8 cells mL^{-1}	10^9 cells mL^{-1}
Abundance [log_{10} (cells / g fresh leaves)]	n.d.	n.d	5.31 ± 0.13	5.73 ± 0.75
DMHF [mg kg^{-1}]	2.8 ± 0.1	7.7 ± 0.4	7.8 ± 0.2	4.9 ± 0.2

Colonization pattern of M. extorquens DSM 21961

Microscopy confirmed the potential of *M. extorquens* to densely colonize strawberry plants. Two weeks after application a high colonization of leaf surfaces was observed (Fig. 5). On the upper leaf surfaces an accumulation of bacterial cells in dents between epidermal cells of the plant was observed (A). However, bacterial cells were not visible in the leaf endosphere (B). On the lower leaf surface especially trichomes were heavily colonized by *M. extorquens* (C).

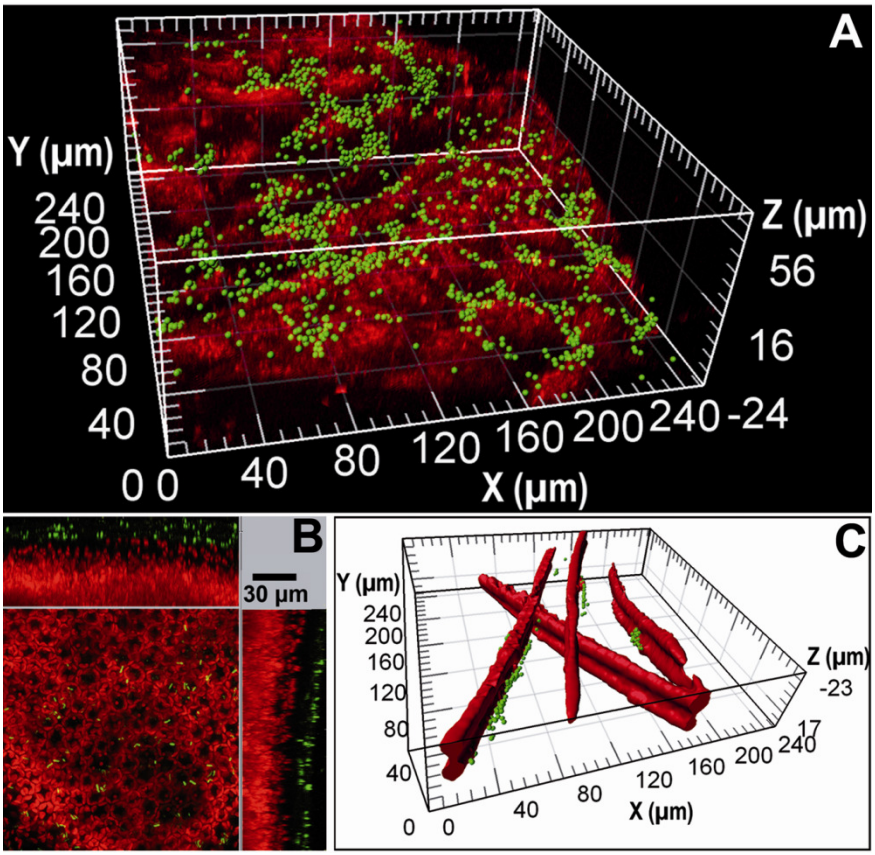

Fig. 5: Colonization of strawberry leaves by GFP-tagged *Methylobacterium extorquens* detected by confocal laser scanning microscopy. A: Volume rendering of a confocal stack showing the three dimensional arrangement of methylobacteria colonies on the leaf surface; small, interconnected bacterial colonies (green spots) occupy the micro-niches formed by the leaf tissues (red). B: XY, XZ and YZ maximum projections of a 60µm thick confocal stack, showing the localization of the bacteria (green) on the surface of the leaf, at a certain distance from the plant parenchimatic cells (red). C: Three dimensional surface-reconstruction of a confocal stack showing methylobacteria colonizing trichomes on the lower leaf surface; green spots: bacteria; red surface: trichomes.

Discussion

Methylobacterium extorquens is a ubiquitous colonizer of the plant phyllosphere and a symbiont for many plants (Delmotte *et al.*, 2009). All *Methylobacterium* strains from different plant sources isolated in this study were able to produce chemical precursor of furanoic compounds *in vitro*. To study the importance of this biosynthesis for the flavor of strawberry fruits, we investigated the interaction between the selected *Methylobacterium*

extorquens DSM 21961 with strawberries *ad planta*. To monitor the colonization, a specific Taqman qRT PCR system was developed. In addition, the function of *M. extorquens* DSM 21961 was evaluated by measuring the concentration of the furanonic compound DMHF in fruits of treated plants. Microscopic analysis by CLSM confirmed the high colonization rate, which is necessary for a beneficial plant-microbe interaction.

In contrast to cultivation dependent methods, qRT PCR offers a number of advantages, e.g. high specificity, low limit of quantification and the possibility of using genetically unmodified wild type strains. To circumvent the time consuming process of optimization of a RAPD PCR reaction for providing suitable target regions for primer design in the target genome, we used an alternative BOX-fingerprinting technique, which does not require any optimization of primers or PCR conditions. The favorable specificity of the system was determined with different isolates of *Methylobacterium,* isolated from the phyllosphere of different plants. The limit of quantification of the system with pure DNA of *M. extorquens* DSM 21961 was determined to be around 100 cells per PCR reaction resulting in a cycle threshold value of 35. This value is higher than values indicated by other authors in literature (Abbasi *et al.,* 1999; Hristova *et al*., 2001). On the other hand, it represents the bottom end of the linear range of the calibration function and not the limit at which a positive sample just can be distinguished from a control sample. This often is not indicated clearly in literature. The limit of quantification with samples of strawberry leaves that were supplied with different cell concentrations is $\log_{10}$ 4.6 cells per gram of fresh leaves. This limit is comparable to the detection limit of other TaqMan systems indicated in literature (Salm & Geider, 2004).

The quantification system was applied in a monitoring experiment of *M. extorquens* DSM 21961 on strawberry plants under greenhouse conditions over a time period of five

weeks. Cell suspensions with three different concentrations were sprayed onto the plants, and the abundance of *M. extorquens* DSM 21961 on the leaves was monitored for five weeks. Applied concentrations of $\log_{10}$ 8.0 and $\log_{10}$ 9.0 cells per mL resulted in a stable population, while untreated control plants gave no signals within the calibration range of the system. A concentration of $\log_{10}$ 6.0 cells per mL resulted in cell concentrations on the leaves around the limit of quantification of the system. In cases where abundances are lower than $\log_{10}$ 4.6 cells per gram of fresh leaves, the method of DNA extraction from leaves will have to be optimized. Optionally, plant DNA should be removed in the extraction procedure or bacterial DNA should be enriched. Simply increasing the amount of template DNA in the PCR reaction is not feasible, because an excess of non-template DNA in the reaction decreases the efficiency of the PCR, mainly due to the co-extraction of PCR-inhibitors.

Confocal Laser Scanning Microscopy confirmed a dense colonization of strawberry leaves by *M. extorquens.* Upper and lower leaf surfaces were occupied by bacterial cells particularly in the dents on the leaves and on trichomes. Bacterial cells were not detected in the leaf endosphere. This was surprising, since methylobacteria are well-known endophytes (Hallmann & Berg, 2006).

The effect of *M. extorquens* DSM 21961 on fruit quality and DMHF content of strawberries was studied using greenhouse-grown plants. Spraying *M. extorquens* DSM 21961 on strawberry plants during the flowering stage resulted in an enhanced concentration of DMHF in the strawberries in comparison to untreated plants. Survival of *M. extorquens* DSM 21961 on the plants, from the time of application until harvest of fruits, was confirmed by qRT PCR. The determined DMHF concentrations underline the hypothesis of an interaction of methylobacteria and strawberry plants which leads to an increased DMHF biosynthesis rate of the strawberry plants. Further investigations are needed to understand

the interaction in detail, especially the phenomenon that the colonization of the phyllosphere by methylobacteria can enhance the flavor of the fruit, and to optimize the *Methylobacterium*-strawberry interaction for biotechnological applications. However, the tools for analysis as developed and presented in this study are suitable for this purpose.

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Literature

Abbasi PA, Miller SA, Meulia T, Hoitink HA & Kim, JM (1999) Precise detection and tracing of *Trichoderma hamatum* 382 in compost-amended potting mixes by using molecular markers. *Appl Environ Microbiol* **65:** 5421-5426.

Altschul SF, Madden TL Schaffer AA, Zhang J, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:** 3389–3402.

Berg G (2009) Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *J Appl Microbiol Biotechnol* **84**: 11-8.

Bood KG & Zabetakis I (2002) The Biosynthesis of strawberry flavor (II): Biosynthetic and molecular biology studies. *J Food Sci* **67:** 2-8.

Bourque D, Pomerlau Y, & Groleau D (1995) High-cell-density production of poly-β-hydroxybutyrate (PHB) from methanol by *Methylobacterium extorquens*: production of high-molecular-mass PHB. *Appl Microbiol Biotechnol* **44:** 367-376.

Choi YJ, Bourques D, Morel L, Groleau D & Miguez CB (2006) Multicopy integration and expression of heterologous genes in *Methylobacterium extorquens* ATCC 55366. *Appl Environ Microbiol* **72**: 753-759.

Delmotte N, Knief C, Chaffron S, Innerebner G, Roschitzki B, Schlapbach R, von Mering C & Vorholt JA (2009) Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proc Natl Acad Sci USA* **22**: 16428-33.

Hadrys H, Ballick M & Schierwater B (1992) Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol Ecol* **1:** 55-63.

Hallmann J & Berg G (2006) Spectrum and population dynamics of root endophytes. In: Microbial root endophytes. Schulz, B., Boyle, C. und T. Sieber (eds.) Springer Verlag Berlin, Heidelberg, New York, 15-32.

Hristova KR, Lutenegger CM & Scow KM (2001) Detection and quantification of methyl tert-butyl ether-degrading strain PM1 by Real-Time TaqMan PCR. *Appl Environ Microbiol* **67:** 5154-5160.

Koeuth T, Versalovic J & Lupski JR (1995) Differential subsequence conservation of interspersed repetitive *Streptococcus pneumoniae* BOX elements in diverse bacteria. *Genome Res* **5:** 408-418.

Labbe D, Rytz A, Morgenegg C, Ali S, Martin N (2007) Subthreshold olfactory stimulation can enhance sweetness. *Chem Senses* **32**: 205-214.

Larsen M, Poll L, Olsen CE (1992) Evaluation of the aroma composition of some strawberry (*Fragaria ananassa* Duch.) cultivars by use of odour threshold values. *ZLUF* **195**: 536-539.

Lloyd-Jones G, Laurie AD, Tizzard AD (2005) Quantification of the *Pseudomonas* population in New Zealand soils by fluorogenic PCR assay and culturing techniques. *J Micriobiol Meth* **60:** 217-224.

Lugtenberg B & Kamilova F (2009) Plant-growth-promoting rhizobacteria. *Ann Rev Microbiol* **63**: 541–556.

Pujol M, Badosa E, Cabrefiga J & Montesinos E (2005) Development of a strain-specific quantitative method for monitoring *Pseudomonas fluorescens* EPS62e, a novel biocontrol agent of fire blight. *FEMS Microbiol Lett* **249:** 343-352.

Pujol M, Badosa E, Manceau C & Montesinos E **(**2006) Assesment of the environmental fate of the biological control agent *Pseudomonas fluorescens* EPS62e on apple by culture and Real-time PCR methods. *Appl Environ Microbiol* **72:** 2421-2427.

Salm H & Geider K (2004) Real-time PCR for detection and quantification of *Erwinia amylovora*, the causal agent of fireblight. *Plant Pathol* **53:** 602-610

Sambrook J & Russel DW (2001) Molecular cloning. 3rd ed. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press.

Schena L, Finetti Sialer M & Gallitelli D **(**2002) Molecular detection of strain L47 of *Aureobasidium pullulans*, a biocontrol agent of postharvest diseases. *Plant Dis* **86:** 54-60.

Schieberle P, Hofmann T (1997) Evaluation of the character impact odorants in fresh strawberry juice by quantitative measurements and sensory studies on model mixtures. *J Agric Food Chem* **45**: 277-232.

Siegmund B, Bagdonaite K, Leitner E (2010) Furaneol® and mesifuran in strawberries – an analytical challenge. In Expression of Multidisciplinary Flavour Science. Blank I, Wüst M,

Yeretzian C (eds) ZHAW Züricher Hochschule für Angewandte Wissenschaften, Winterthur, 537-540.

Sung WS, Jung HJ, Park K, Kim HS, Lee IS & Lee DG (2006) 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF); antimicrobial compound with cell cycle arrest in nosocomial pathogens. *Life Sci* **80:** 586-591.

Welsh J & McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* **18:** 7213-7218.

Williams JGK, Kubelik AR, Kenneth JL, Rafalski JA & Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* **18:** 6531-6535.

Zabetakis I & Gramshaw JW (1996) 1,2-Propanediol in strawberries and its role as a flavour precursor. *Food Chem* **61:** 351-354.

Zabetakis I, Moutevelis-Minakakis P & Gramshaw JW (1999) The role of 2-hydroxypropanal in the biosynthesis of 2,5-dimethyl-4-hydroxy-2H-furan-3-one in strawberry (*Fragaria x ananassa*, cv. Elsanta) callus cultures. *Food Chem* **64:** 311-314.

Zabetakis I (1997) Enhancement of flavour biosynthesis from strawberry (*Fragaria* x *ananassa*) callus cultures by *Methylobacterium* species. *Plant Cell*, *Tissue and Organ Cult* **50:** 179–183.

Whipps JM, Hand P, Pink D & Bending GD (2008) Phyllosphere microbiology with special reference to diversity and plant genotype. *J Appl Microbiol* 105:1744-1755.

Manuscript I

High shelf-life formulations for *Methylobacterium extorquens* DSM 21961, a microbial inoculant to enhance strawberry flavor[3]

Abstract

Methylobacterium extorquens DSM 21961, which belongs to the group of Pink pigmented facultative methylotrophic bacteria, is a promising flavor enhancing agent on strawberries. To use the strain in large scale, a method to produce and formulate the bacterium in high-cell-densities into a product with a long shelf-life is essential. First, we evaluated different fermentation conditions. In a second step, four formulations were tested: (i) a lyophilisate, (ii) a formulation in a xanthan matrix, (iii) an encapsulation in alginate with and (iv) without adaptation of the cells to storage conditions. After storage at six different temperatures and according to the calculated disintegration kinetics, the xanthan formulation as well as the adapted alginate formulation showed the best shelf-life. Strawberry plants were treated with these *Methylobacterium* formulations but no statistically significant differences between abundances were observed: DSM 21961 was established at a level of $\log_{10}$ 6.0 cells g^{-1} leaves monitored by specific real-time PCR. Comparing all criteria (shelf-life, practicability, plant colonization, effect, expenses), the xanthan formulation can be suggested as optimal formulation procedure. For special applications like soil treatments the alginate beads or the

[3] Verginer M, Müller H, Berg G (2010) High shelf-life formulations for *Methylobacterium extorquens* DSM 21961, a microbial inoculants to enhance strawberry flavor. Submitted to Industrial Microbiology and Biotechnology

lyophylisate are appropriate formulations. They open the way for the use of methylobacteria in agriculture.

Introduction

Pink pigmented facultative methylotrophic bacteria (PPFMs), which are named according to their pigmentation and their ability to grown on one-carbon compounds such as methanol, are typical colonizer of the plant phyllosphere (Holland et al. 2002; Knief et al. 2008, 2010). Different positive effects of PPFMs for their host plants or their environment are described: (i) they act as symbionts of legumes by fixing nitrogen in the nodules (Sy et al. 2001; Madhaiyan et al. 2009), (ii) they stimulate plant growth by the production of phytohormones (Lidstrom and Chistoserdova 2002; Omer et al. 2004; Senthilkumar et al. 2009), and (iii) they can degrade polluting chemicals (Hancock et al. 1998; van Akten and Schnoor 2004). Beside this, plant-associated microorganisms also can influence fruit quality: it is known that microorganisms can produce different flavors *de novo* or they can produce chemical precursors of flavors (Janssens et al. 1992). This has been also shown on strawberry plants: a co-cultivation of strawberry callus cultures (*Fragaria* x *ananassa* cv. Elsanta) with *Methylobacterium extorquens* led to an increased biosynthesis of the compound 2,5-dimethyl-4-hydroxy-2H-furanone (DMHF) (Koutsompogeras et al. 2007; Zabetakis 1997). This substance is a major component of strawberry flavor (Bood and Zabetakis 2002). From a biochemical point of view this compound is remarkable because it has been shown, that PPFMs, which colonize strawberry plants, are involved in its biosynthesis (Zabetakis 1997; Koutsompogeras et al. 2007). It has been shown, that also Methylobacterium extorquens is able to stimulate DMHF biosynthesis in strawberries also *ad planta* (Verginer et al 2010).

Scale-up of fermentation and formulation are often a high hurdle for a successful translation of microbial inoculants into commercial products (Ehlers 2001; Berg 2009). Today literature is abundant with studies on screening for microorganisms with beneficial effects; however, very few of the successful studies were translated into commercial applications. Beneficial microorganisms have been frequently applied in the greenhouse in formulations that are not commercially feasible or without any formulation. For many microorganisms no formulation is available or unexpected difficulties with the formulation or large scale fermentation led to the abandonment of the research (Hynes and Boyetchko 2006). To develop improved fermentation and formulation protocols, especially for gram-negative bacteria, is a promising solution for a sustainable and environmentally friendly world food production (Morrissey et al. 2004; Berg 2009).

The objective of this study was to develop a fermentation yielding high-cell-densities and a formulation method with a high shelf-life for *Methylobacterium extorquens* DSM 21961. We have reported earlier that a DSM 21961 treatment on strawberry plants results in an increased concentration of DMHF in the fruits in greenhouse as well as field studies (Verginer et al. 2010). To take advantage of this useful effect, three fermentation conditions and four different formulations of this strain were compared and evaluated in a storage experiment. Furthermore, the formulated cells were applied *ad planta* to assess advantages and disadvantages of the different formulations. Optimized protocols for fermentation and formulation were provided.

Materials and Methods

Strains and pre-culture conditions

The *Methylobacterium* strain used in this study has been isolated from the phyllosphere of a rose plant (*Rosa* sp.) and has been identified as *Methylobacterium extorquens* DSM 21961 (Verginer et al. 2010). Long-time storage of the strain has been performed in a liquid MIS medium containing in g L^{-1} 1.8 $(NH_4)_2SO_4$, 0.2 $MgSO_4 \cdot 7H_2O$, 1.4 $NaH_2PO_4 \cdot 2H_2O$, 1.9 K_2HPO_4. After autoclaving, 5 mL L^{-1} methanol and 1 mL L^{-1} of a trace element stock solution were added. The trace element stock solution contained in mg L^{-1} 500.0 EDTA, 200.0 $FeSO_4 \cdot 7H_2O$, 10.0 $ZnSO_4 \cdot 7H_2O$, 3.0 $MnCl_2 \cdot 4H_2O$, 20.0 H_3BO_3, 20.0 $CoCl_2 \cdot 6H_2O$, 1.0 $CuCl_2 \cdot 6H_2O$, 2.0 $NiCl_2 \cdot 6H_2O$ and 3.0 $Na_2MoO_4 \cdot 2H_2O$. This medium was blended with 20% sterile glycerol and the strain was stored in it at -70 °C. DSM 21961 was grown on MIS agar medium and after five days at 30 °C one single colony was used to inoculate pre-cultures in 250 mL liquid medium 4 (Bourque et al. 1995) in 1000 mL Erlenmeyer flasks. After two days at 120 rpm and 30 °C these cultures were used to inoculate the main cultures.

Fermentation operations and downstream processes

After testing several liquid media which were described in literature (Bourque et al 1995; Stepnowski et al 2004) we decided to use medium 4 as described by Bourque et al. (1995). The growth curve of the strain was determined in 1000 mL shaking flasks containing 250 mL medium 4 over a time period of 48 hours. Samples were taken every two hours and the OD_{600} was measured photometrically (Eppendorf BioPhotometer, Eppendorf AG, Hamburg, Germany) and CFU numbers were determined by plating serial dilutions on MIS agar plates. Fermentation of *M. extorquens* DSM 21961 was performed in a 10 L Sartorius Biostat B plus pilot fermenter (Sartorius AG, Göttingen, Germany). The fermenter was filled with 10 L of

water in which the appropriate amounts of $(NH_4)_2SO_4$, KH_2PO_4 and Na_2HPO_4 were dissolved. The fermenter was autoclaved for 70 min at 121 °C and cooled down to room temperature. To avoid precipitation or evaporation of medium components the remaining components were added after autoclaving by pumping them into the fermenter through a sterile filter. The medium was stirred with a Rushton impeller at 300 rpm, the temperature was regulated to 30 °C and oxygen saturation was regulated to 25 % by aeration of the medium with sterile air. The pH was automatically adjusted to 7 with 10 % NH_3 in water and 1 M HCl. An antifoam agent (Antifoam SE-15, Sigma, St. Louis, USA) was added automatically as required. The fermenter was inoculated with 50 mL of a two days old pre-culture. After consumption of the methanol added at the beginning of the fermentation after 24 h, 50% methanol was pumped into the fermenter with a flow rate of 3.3 mL h^{-1}. After 48 h the fermentation was stopped and bacterial cells were harvested by pumping the fermentation broth directly into 500 mL centrifuge bottles. The cell suspension was centrifuged in a FiberLite F10-6x500y centrifuge rotor (Piramoon Technologies Inc., Santa Clara, USA) in a Sorval RC 5B Refrigerated Superspeed Centrifuge (Du Pont Instruments, Wilmington, USA) at 5000 rpm for 5 min at room temperature. Supernatants were decanted and cell pellets were resuspended in remainders of the supernatant by shaking the centrifuge flasks vigorously. The concentrated cell suspensions were collected and transferred into 50 mL falcon tubes. The tubes were centrifuged in a FiberLite F14-14x50cy (Piramoon Technologies Inc.) at 5500 rpm for 10 min at 4 °C. Supernatants were discharged and cell pellets were resuspended in PBS buffer to a final volume of 25 mL in each falcon tube. These cell suspensions were pooled and cell number was determined by a cell count of an appropriate dilution in a

counting chamber (only motile cells were considered) and used for the different formulations.

Preparation of the formulations

(i) Encapsulation in alginate with (ALG) and without adaptation (ALD)

200 mL of a 1.5 % (w/v) solution of alginate (Fluka, St. Louis, USA) in autoclaved, deionized water were prepared and stirred over night. Next day the solution was filtrated through a filter membrane with a pore diameter of 45 µm. Log_{10} 11.9 cells of *M. extorquens* DSM 21961 were added (resulting in a final cell number of log_{10} 9.6 cells mL^{-1} and alginate beads were prepared by dropping the alginate solution with compressed air through a syringe needle into a stirred 1000 mL glass beaker filled with 300 mL of 0.1 M $CaCl_2$. 15 min later alginate beads were sieved out and washed with deionized and autoclaved water. The alginate beads were directly air-dried at room temperature and ground to a fine powder in a coffee grinder.

An additional approach involves a process to adapt entrapped cells to the following desiccation step. Alginate beads containing *M. extorquens* DSM 21961 cells were prepared as described above. Prior drying at room temperature, alginate capsules were subjected to a proprietary adaptation treatment developed by a seed company in germany. Finally, dry beads were ground in a coffee grinder to a fine powder, which was used for storage experiments.

(ii) Lyophilisation (LYO)

30 mL of the cell suspension of *M. extorquens* DSM 21961 were transferred into a 50 mL Falcon tube, 3 g of sucrose were added and dissolved, and the suspension was frozen over

night and dried in a freeze-drier (Freeze Dry System/Freezone 4.5, Kansas City, USA) for 48 h. The lyophilisate was ground to a fine powder in a coffee grinder.

(iii) Xanthan matrix (XAN)

100 mL of a solution of 5 % (w/v) xanthan (VWR, Darmstadt, Germany) in autoclaved, deionized water were prepared. Log_{10} 12.3 cells of *M. extorquens* DSM 21961 were added and the suspension was mixed thoroughly. Then the mixture was spread on Petri dishes and air dried at 30 °C for 12 h. The dry formulation was ground in a coffee grinder to a fine powder.

Storage experiments

Small amounts of all formulation variants were filled in 1.5 mL reaction tubes and were stored at 4, 10, 20, 30, 37 and 44 °C, respectively. For each temperature and each formulation at intervals of four weeks, 5-10 mg were re-hydrated in water (lyophilisates and xanthan formulation) or disintegrated in 0.1 M sodium citrate (alginate formulations). Sequential dilutions were prepared and the numbers of colony forming units were determined on MIS agar plates. Sequential dilutions and plating were performed in duplicates each.

Application of the formulations ad planta

Strawberry plants (*Fragaria* x *ananassa* variety *Elsanta*) were grown in pots in a greenhouse under artificial light (16 hours illumination per day with metal halide lamps), temperature control (20 °C) and customized watering. At the stage of flowering the different formulations were applied to the plants. The appropriate quantity of each formulation to give 100 mL of a suspension of *M. extorquens* DSM 21961 with log_{10} 8.0 CFU mL^{-1} was dissolved in tap water

after a storage period of 16 weeks at 20 °C. Cell numbers of the suspensions were determined in a counting chamber (only motile cells were considered) and the cell concentration was adapted, if necessary, with tap water. Then, the suspensions were sprayed on the phyllosphere of the strawberry plants until the plants were completely wetted by the suspension. For each variant three plants were treated. At three sampling times, 1, 7 and 14 days after the treatment, leave samples were taken from the plants. Cell numbers of *M. extorquens* DSM 21961 in the samples were determined by real time PCR as described by Verginer et al. (2010). Briefly, after freeze-drying of the leaf-samples total DNA was extracted with the FastDNA SPIN for Soil Kit (Qbiogen BIO 101 Systems, Carlsbad, USA). Amounts of bacterial DNA in the extracts were determined in a quantitative RT PCR assay in a Rotor-Gene 6000 real time analyzer (Corbett Life Science, Hilden, Germany) using the following oligonucleotides: forward primer Ext-f 5'- AGC ATC GCG AGC TCT GGT A -3', reverse primer Ext-r 5'- CGA AAC GTC ACT GAT CGT ATG AG -3' and probe Ext-p 5'- FAM - CTG GAT GCC GGA CTT GGC TCG TC – TAMRA - 3'. Due to the fast decrease of cell number in the formulation ALD, this variant was excluded from this experiment. On the one hand, too little material has been available for application, on the other hand, this variant has only a minimal potential for a further use.

Results

Fermentation conditions for Methylobacterium extorquens DSM 21961

Testing of several liquid media, which were described in literature to be used for growing methylobacteria (Bourque et al 1995; Stepnowski et al 2004), were differently suitable for our purposes. The most important criteria, which were decisive for the selection of one medium, are summarized in Table 1.

Table 1 Comparison of three different cultivation media for *Methylobacterium* species

	Complex medium	Medium 784	Medium 4
Log_{10} cell number after 24 h	8.5 ± 0.2	8.8 ± 0.3	8.8 ± 0.1
Log_{10} cell number after 48 h	7.6 ± 0.3	9.5 ± 0.1	9.6 0.2
Odor	strong, unpleasant	weak, unobtrusive	weak, unobtrusive
Price*	1.2 € L^{-1}	0.2 € L^{-1}	0.3 € L^{-1}
Biofilms formation	coagulation of cells to clumps in not continuously shaked cell suspensions	formation of biofilms on surfaces of shaking flasks, coagulation of cells to clumps	no formation of biofilms or coagulation of cells

* calculated from the price list of a local chemical supplier for order quantities in laboratory scale

A complex medium containing meat extract and soy peptone as nutritional source exhibited a very unpleasant flavor at the end of the fermentation process. The final cell density of M. extorquens DSM 21961 in this medium after 48 h at 30 °C was low (log10 7.6 CFU mL-1). Minimal salt media supplemented with methanol as carbon source, which are also used for the selective enrichment of methylobacteria from environmental samples, showed a better bacterial growth (log10 9.5 after 48 h at 30 °C), and the fermentation broth was nearly odorless at the end of the fermentation process. As example, medium 784, recommended by the American Type Culture Collection for cultivation of methylotrophic bacteria, is mentioned. However, in these media M. extorquens DSM 21961 tended to form biofilms on the surface of the shaking flasks, and the centrifugation pellets were difficult to re-suspend. These problems could be avoided using a minimal salt medium which has been optimized for the production of poly-β-hydroxybutyrate from methylobacteria (Bourque et al. 1995).

In this medium, in batch-fermentations in 1000 mL shaking flasks and a growth time of about 48 hours, a final cell number of $\log_{10}$ 9.6 CFU mL^{-1} was reached. In a 10 L pilot fermenter, cell growth was significantly faster; after 24 hours the methanol added in the beginning was consumed. Afterwards, for another 24 hours methanol was fed continuously. In fed batch fermentations after 48 h of growth an average cell number of $\log_{10}$ 9.9 cells mL^{-1} was reached. The progress of the most important parameters of the fed batch fermentation is shown in Fig. 1. To avoid excessive foaming of the fermentation broth, the feed rate for methanol was limited to 3.3 mL h^{-1} of 50 % methanol.

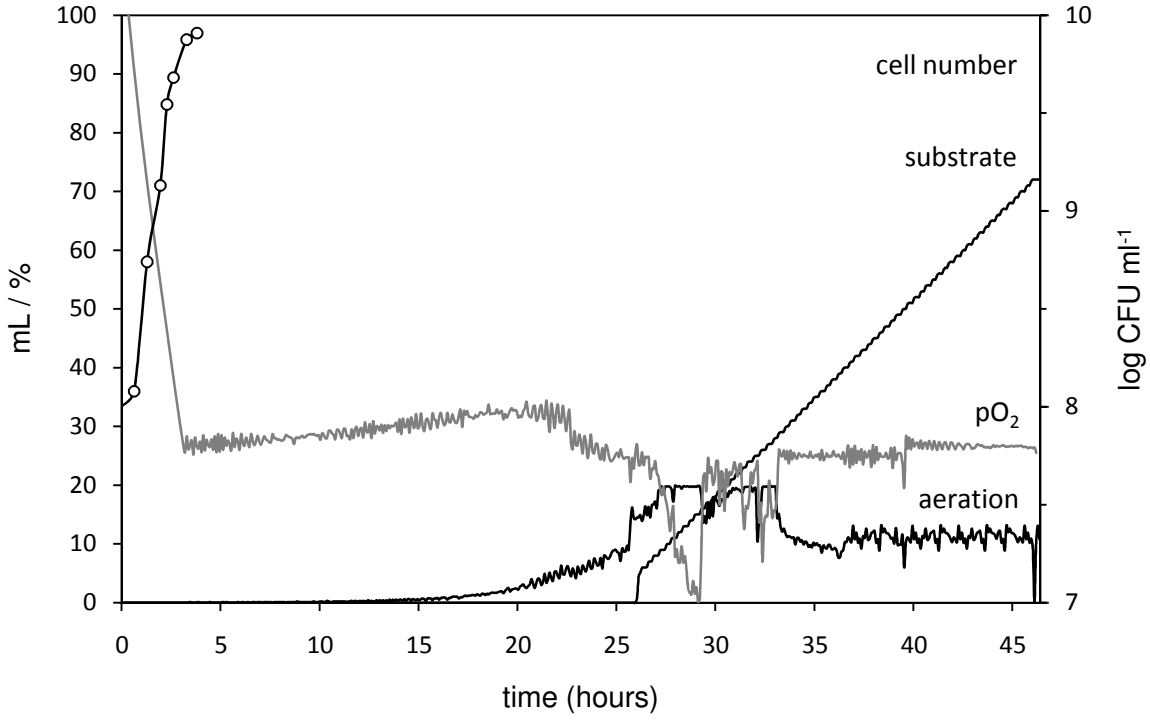


Fig 1 Progress of a fermentation of *M. extorquens* DSM 21961 in medium 4. The diagram shows the cell number in log CFU mL^{-1}, methanol feed in mL, oxygen saturation of the medium in % and the aeration rate of the fermenter in L min^{-1}

Development and evaluation of different formulations

Four different formulations of *M. extorquens* DSM 21961 were prepared: (i) a lyophilisate [LYO], (ii) a formulation in a xanthan matrix [XAN], (iii) an encapsulation in alginate with [ALG] and (iv) without adaptation [ALD] of the cells to storage conditions. At the end, all formulations consisted of a pink-colored powder. Alginate formulations yielded a coarse powder with relatively hard and dense particles with sharp break edges (see Fig. 2). The powders had a good pourability and didn't change their physical properties during storage. Solubilization of the powders in 0.1 M sodium citrate took 30 min of shaking and partially clumps were formed. Initial cell number in the formulation ALG was $\log_{10}$ 11.2 CFU g^{-1}. For the variant ALD, alginate beads were prepared in the same way than for the variant ALG, but they were dried and ground immediately after production. Initial cell number in the fresh formulation was $\log_{10}$ 10.1 CFU g^{-1}. For the variant LYO a bacterial suspension was freeze-dried after addition of 10% sucrose (w/v). Initial cell number was determined to be $\log_{10}$ 12.7 CFU g^{-1}. The lyophilisate was a fine, dusty and light powder. Under the microscope, a mixture of crystals with a fine powder was visible (Fig. 2). It was well pourable after production but during storage it tended to agglutinate, especially at higher temperatures. Solubilization of the product in water occurred immediately. For the variant XAN, xanthan was used as matrix. After incorporation and air-drying of the bacteria the product was ground to a fine powder with a cell content of $\log_{10}$ 10.5 CFU g^{-1}. The xanthan formulation consisted of fine thin light platelets (Fig. 2). The formulation didn't change its physical properties during storage. Solubilization in water took some time of shaking but was faster than that of alginate formulations.

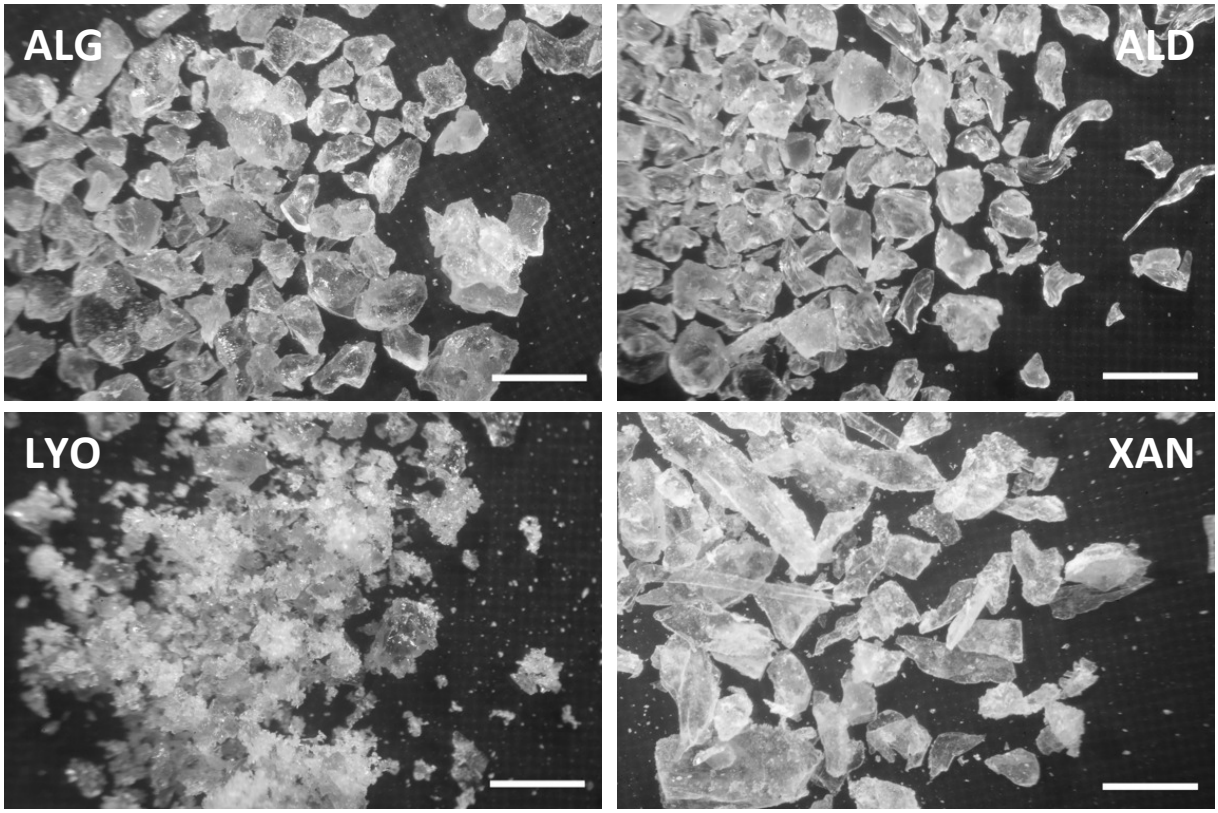
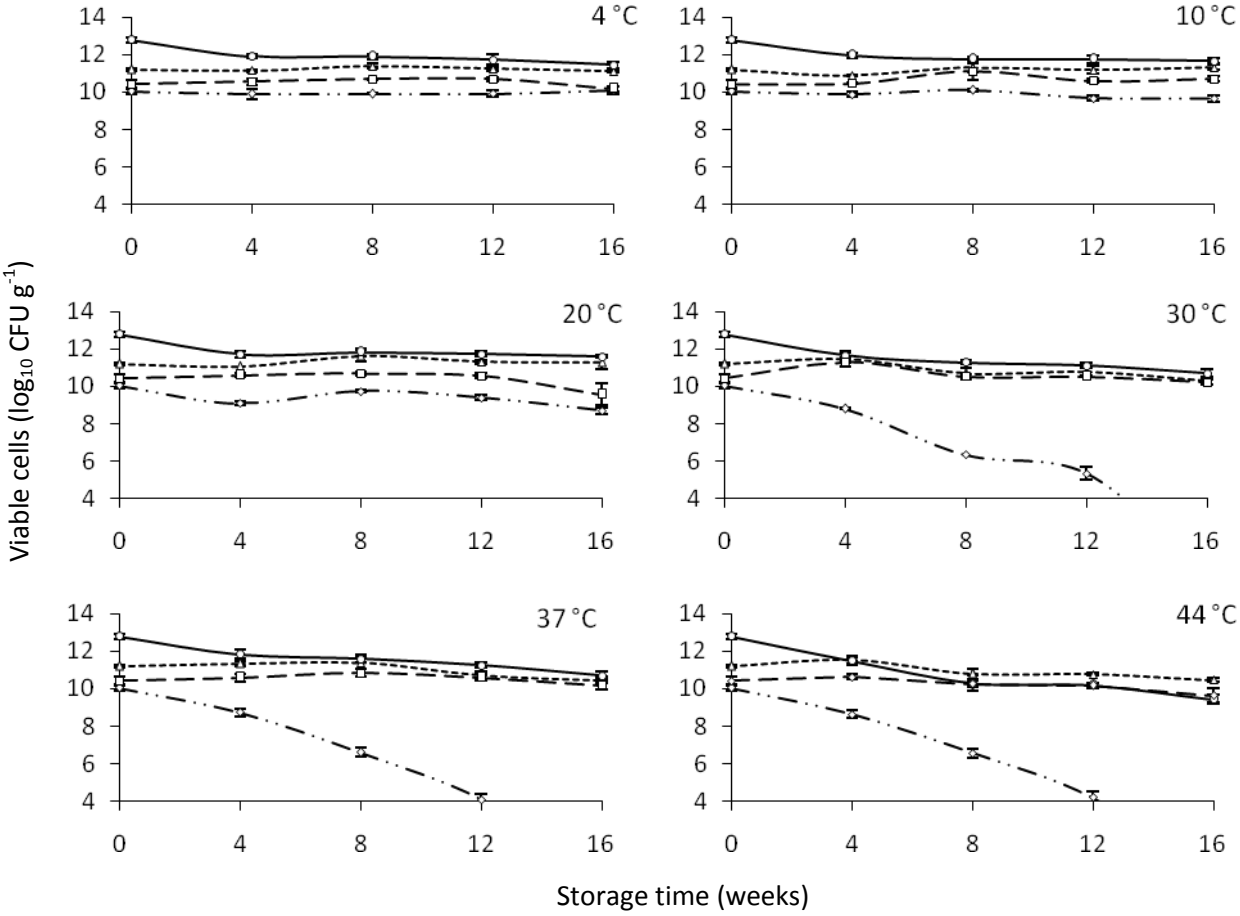


Fig 2 Microscopic images of the different formulations at 40x magnification. Bar = 100 µm. ALG: encapsulation in alginate with adaptation; ALD: encapsulation in alginate without adaptation; LYO: lyophilisate; XAN: formulation in xanthan matrix

Storage experiments

Survival rates of the four formulations at different temperatures are summarized graphically in Fig. 3. Initial cell numbers in $\log_{10}$ CFU g^{-1} were 11.2 for ALG, 10.1 for ALD, 12.7 for LYO, and 10.5 for XAN. In general, all four formulations showed good survival of cells at temperatures up to 20 °C during 16 weeks. No drastic decrease of cell numbers in the formulations was observed. At 4 °C and 10 °C, the variants ALG, ALD and XAN showed constant cell numbers for the whole storage period, ALD showed a decrease to $\log_{10}$ 9.9 CFU g^{-1} in the first four weeks. In contrast, at 20 °C first differences between the variants

appeared. While cell numbers in the variant XAN remained constant also at this



temperature, in the other variants a decline of cell numbers is visible. At 30 °C, clear

Fig 3 Survival of *M. extorquens* DSM 21961 in different formulations stored ad different temperatures. Data points represent mean values of two independent determinations in duplicates, lines represent a B-spline interpolation of data points and error bars represent standard deviations; --Δ—ALG: encapsulation in alginate with adaptation; —□–– XAN: formulation in xanthan matrix; —○— LYO: lyophilisate; - .. -◊- ..- ALD: encapsulation in alginate without adaptation

differences in the survival rates of the different formulations are noticeable. While the decline of cell numbers is relatively weak for ALG and XAN with cell numbers of $\log_{10}$ CFU g^{-1} 10.1 and 10.2 after 16 weeks ALD decreases fast and falls below the detection limit of the method ($\log_{10}$ 5.0 CFU g^{-1}) after this time. Cell numbers in LYO decrease faster than in ALG

and XAN but much slower than in ALD (log_{10} 10.6 CFU g^{-1} after 1 week). At 37 °C the situation is similar as at 30 °C. ALG, LYO and XAN show no differences, ALD decreases faster than at 30 °C and reaches a value of one thousandth of the initial value after 12 weeks (log_{10} 4.2 CFU g^{-1}). Finally, at 44 °C also the variants ALG and XAN show a slight decrease (log_{10} 10.4 and 10.0 CFU g^{-1} after 16 weeks). Also the variant LYO declines faster to a value of log_{10} 9.4 CFU g^{-1} after 16 weeks.

To express the degradation behavior of the different formulations numerically, a numerical evaluation of the data shown in Fig. 2 was used. If it is assumed, that the degradation of the bacterial cells in the formulations is a first-order reaction (Xiong et al. 1999; Achour et al. 2001), cell degradation can be described by the equation

$$N = N_0 \cdot e^{-kt}$$

where

N cell number at time t

N_0 initial cell number

k degradation rate constant

t time

If log (N/N_0) is plotted against time, the slopes of the linear regressions represent the degradation rate constants at the prevalent temperature. Using this method, the rate constants of all formulations stored at different temperatures were determined and listed in Table 2. A dependence of degradation rate constants of the way of formulation of the strain is visible. On the other hand also higher storage temperatures lead to a faster dying of the cells. This temperature dependence is not the same for all formulations. While XAN and ALG

show only a weak temperature dependence, survival rates in LYO and ALD decrease clearly with increasing storage temperature.

Table 2 Degradation rate constants of the different formulations at different temperatures determined by linear regression of experimental data (week^{-1}) ALG: encapsulation in alginate with adaptation; ALD: encapsulation in alginate without adaptation; LYO: lyophilisate; XAN: formulation in xanthan matrix

Formulation	Degradation rate at different storage temperatures					
	4 °C	10 °C	20 °C	30 °C	37 °C	44 °C
ALG	0.000 ± 0.002	0.000 ± 0.003	-0.014 ± 0.011	-0.049 ± 0.018	-0.033 ± 0.016	-0.040 ± 0.013
ALD	0.000 ± 0.005	-0.015 ± 0.006	-0.080 ± 0.019	-0.403 ± 0.024	-0.460 ± 0.029	-0.456 ± 0.022
LYO	-0.089 ± 0.011	-0.077 ± 0.011	-0.081 ± 0.014	-0.135 ± 0.012	-0.129 ± 0.007	-0.215 ± 0.014
XAN	0.000 ± 0.009	0.000 ± 0.015	-0.004 ± 0.011	0.000 ± 0.021	-0.005 ± 0.012	0.000 ±0.007

Application ad planta

All three variants were able to establish *M. extorquens* DSM 21961 on strawberry plants. The determined abundances of the strain on the treated plants after several days are summarized in Table 3. All applied formulations resulted in abundances of *M. extorquens* DSM 21961 on the plants about $\log_{10}$ 6.0 cells g^{-1} immediately after the treatment. No serious differences between the variants could be observed. Two weeks after application, the abundances decreased slightly for all three variants; they show still comparable abundances. These results indicate that the way of formulations of *M. extorquens* DSM 21961 doesn't significantly influence the vitality of the bacterial cells on the plant after application.

Table 3 Abundances of *M. extorquens* DSM 21961 on strawberry plants after application of different formulations determined by RT PCR (log (cells g^{-1})). Indicated values are means of three PCR replications ALG: encapsulation in alginate with adaptation; LYO: lyophilisate; XAN: formulation in xanthan matrix

Formulation	Abundances DSM 21961		
	1 day	7 days	14 days
ALG	5.72 ± 0.15	5.66 ± 0.05	5.58 ± 0.09
LYO	6.02 ± 0.12	5.58 ± 0.14	5.42 ± 0.05
XAN	6.05 ± 0.09	5.94 ± 0.10	5.81 ± 0.11

Discussion

Evaluation of different fermentation conditions for M. extorquens DSM 21961

In our study, we evaluated different fermentation media, which showed different suitability for the growth of *M. extorquens* DSM 21961 in large scale. In detail: the complex medium containing meat extract and soy peptone was excluded from our selection, because the fermentation broth exhibited a very unpleasant flavor at the end of the fermentation. This is disadvantageous if the final product should be applied to crop plants. Furthermore, the components of the medium are too expensive for use in large scale fermentations. Therefore, minimal salt media supplemented with methanol as carbon source were tested. These media are much cheaper than most of the complex media. With medium 784 containing low amounts of different trace elements an intense biofilms formation of bacteria on surfaces of shaking flasks and fermentor components was observed. The peculiarity of *Methylobacterium* species of forming biofilms or of being part of bacterial communities in heterogeneous biofilms is well known and described in literature (Rickard et al 2002*;* Simões et al. 2007). With the use of medium 4 that was developed for production of biopolymers

with methylobacteria and contains higher concentrations of trace elements, this problem was overcome and this medium was used for all further fermentations in our pilot fermenter. For the growth of *M. extorquens* DSM 21961 in the pilot fermenter experience has shown that in the feed-phase cell growth under substrate limitation is preferable, because if cell growth was oxygen-limited, foaming of the fermentation broth increased drastically and high amounts of antifoam agent had to be used. The change of metabolic pathways of a cultured microorganism under oxygen limitation is frequently described in literature (Clark and Bushell 1995; Büchs 2001). In contrast, under substrate limitation foaming of the fermentation broth was low.

Evaluation of different formulations for M. extorquens DSM 21961

In a second step, four different formulations of *M. extorquens* DSM 21961: (i) a lyophilisate [LYO], (ii) a formulation in a xanthan matrix [XAN], (iii) an encapsulation in alginate with [ALG] and (iv) without adaptation [ALD] of the cells to storage conditions were evaluated with a special focus on shelf-life. The initial cell numbers of the alginate formulations were at the same level or higher than those obtained with other bacteria in alginate formulations (Bashan 1986; Russo et al. 2001; Bashan et al. 2002). Also the lyophilisates showed higher initial cell rates than lyophilisates of other bacterial species presented in literature (Heckly et al. 1967; Owen et al. 1989) as well as the xanthan formulation yielded markedly higher cell numbers than other xanthan-based formulations reported earlier (Kloepper and Schroth 1981; Caesar and Burr 1991). The comparison of the degradation rate constants of the formulations (see Table 2) shows, that the rate of degradation of bacterial cells is influenced by the way of formulation as well as by the storage temperature. For all formulations a dependence of the shelf-life on the storage temperature was observed. Lower storage

temperatures achieve a slower degradation of bacterial cells, independent of the method of formulation. This fact is well known and described in literature (Achour et al. 2001). On the other hand also the method of formulation has a strong impact on the survival rates of the bacteria. While at low temperatures all variants showed good survival rates, at higher storage temperature clear differences between the degradation rate constants of the different formulations appear. Xanthan as formulation matrix (XAN) gave the best survival rates over the whole temperature range that was tested. Also alginate is a well suitable formulation matrix, if cells have the possibility to adapt to storage conditions (ALG). In this case, good survival rates were obtained, which are only slightly lower than those of the xanthan formulation. Also LYO and ALD show acceptable results at low temperatures up to 20 °C. Above, survival of cells decreases rapidly with rising temperature. At temperatures of 30 °C or above in ALD after 16 weeks no more viable cells could be detected (detection limit $\log_{10}$ 5.0 CFU g^{-1}). At temperatures of 20 °C or below this variant was comparable with the lyophilisate. In the variant LYO the highest initial cell number in the formulation was reached. These data show that different formulations or formulation procedures result in absolutely different properties of the corresponding products. While the alginate formulation ALD provides absolutely unsatisfactory results, the same formulation procedure with xanthan XAN gave excellent results with good survival rates over 16 weeks. On the other hand also the performance of alginate as formulation matrix could be enhanced drastically, if to the encapsulated cells was given the opportunity to adapt to the new environment before drying. It is known, that methylobacteria produce exopolysaccharides which are involved in biofilm formation (Omer et al. 2004). These substances are known to act as osmoprotectives and enable survival of bacterial cells after drying (Singh and Fett

1995). The big advantage of the lyophilisate LYO is the fast solubilisation in water. This makes the formulation well-suited for spraying applications of methyolobacteria, where homogeneous suspensions of bacteria are needed for the application. Also the xanthan formulation XAN is well suitable for this purpose, even if this variant needs more time to solubilise. On the other hand the xanthan acts as a thickener in the resulting bacterial suspension (Katzbauer 1997), which will lead to a better adhesion of the suspension on plant surfaces. Alginate formulations are suitable for applications, where a slow release of the formulated bacteria into the environment is desired (Bashan 1986). Taking into account all these observations, the formulation in a xanthan matrix is suggested as optimal formulation procedure for our purpose. Formulation operations for this variant are simple and do not require special equipment. This is important if the product should be produced in large scale, where costs for machinery and energy significantly contribute to the final product costs. Furthermore survival rates over a large temperature range were the best in the XAN formulation. The insensitivity to higher temperatures is also of major importance for a large scale production, because the expensive need for cooling of the product during storage and distribution is avoided.

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Literature

Achour M, Mtimet N, Cornelius C, Zgouli S, Mahjoub A, Thonart P, Hamdi M (2001) Application of the accelerated shelf life testing method (ASLT) to study the survival rates of freeze-dried *Lactococcus* starter cultures. J Chem Technol Biotechnol 76:624-628

Bashan Y (1986) Alginate beads as synthetic inoculants carrier for slow release of bacteria that affect plant growth. Appl Environ Microbiol 51:1089-1098

Bashan Y, Hernandez JP, Leyva LA, Bacilio M (2002) Alginate microbeads as inoculants carriers for plant-growth promoting bacteria. Biol Fertil Soils 35:359-368

Berg G (2009) Plant-microbe interactions promoting plant growth and health: perspective for controlled use of microorganisms in agriculture. Appl Microbiol Biotechnol 84:11-18

Bood KG, Zabetakis I (2002) The Biosynthesis of Strawberry Flavor (II): Biosynthetic and Molecular Biology Studies. J Food Sci 67:2-8

Bourque D, Pomerlau Y, Groleau D (1995) High-cell-density production of poly-β-hydroxybutyrate (PHB) from methanol by *Methylobacterium extorquens*: production of high-molecular-mass PHB. Appl Microbiol Biotechnol 44:367-376

Büchs J (2001) Introduction to advantages and problems of shaken cultures. Biochem Eng J 7:91-98

Caesar AJ, Burr TJ (1991) Effect of conditioning, betaine, and sucrose on survival of rhizobacteria in powder formulations. Appl Environ Microbiol 57:168-172

Clark GJ, Bushell ME (1995) Oxygen limitation can induce microbial secondary metabolite formation: investigations with miniature electrodes in shaker and bioreactor cultures. Microbiology 141:663-669

Ehlers RU (2001) Mass production of entomopathogenic nematodes for plant protection. Appl Microbiol Biotechnol 56):623-33

Hancock TLC, Costello AM, Lidstrom ME Oremland RS (1998) Strain IMB-1, a novel bacterium for the removal of methyl bromide in fumigated agricultural soils. Appl Environ Microbiol 64:2899-2905

Heckly RJ, Dimmick RL, Guard N (1967) Studies on survival of bacteria: rhythmic response of microorganisms to freeze-drying additives. Appl Microbiol 15:1235-1239

Holland MA, Long RLG, Polacco JC. (2002). *Methylobacterium* spp.: Phylloplane bacteria involved in cross-talk with the plant host. In: Lindow SE, Hecht-Poinar EI, Elliott VJ (ed) Phyllosphere Microbiology. APS Press, St Paul, Minnesota, pp 125–135.

Hynes RK, Boyetchko SM (2006) Research initiatives in the art an science of biopesticide formulation. Soil Biol Biochem 38:845-849

Janssens L, De Pooter HL, Schamp NM, Vandamme EJ (1992) Production of flavours by microorganisms. Process Biochem 27:195-215

Katzbauer B (1997) Properties and applications of xanthan gum. Polym Degrad Stabil 59:81-84

Kloepper JW, Schroth MN (1981) Development of a powder formulation of rhizobacteria for inoculation of potato seed pieces. Phytopatol 71:590-592

Knief C, Frances L, Cantet F, Vorholt JA (2008). Cultivation-independent characterization of Methylobacterium populations in the plant phyllosphere by automated ribosomal intergenic spacer analysis. Appl Environ Microbiol 74: 2218–2228.

Knief C, Ramette A, Frances L, Alonso-Blanco C, Vorholt JA (2010) Site and plant species are important determinants of the *Methylobacterium* communities composition in the plant phyllosphere. ISME J 4:719-728

Koutsompogeras P, Kyriacou A, Zabetakis I (2007) The formation of 2,5-dimethyl-4-hydroxy-2H-furan-3-one by cell-free extracts of *Methylobacterium extorquens* and strawberry (*Fragaria* x *ananassa* cv. Elsanta). Food Chem 104:1654-1661

Lidstrom ME, Chistoserdova L (2002) Plants in the pink: Cytokinin production by *Methylobacterium*. J Bacteriol 184:1818

Madhaiyan M, Pooguzhali S, Senthilkumar M, Sundaram SP, Sa T (2009) Nodulation and plant-growth promotion by methylotrophic bacteria isolated from tropical legumes. Microbiol Res 164:114-120

Morrissey JP, Dow JM, Mark L, O'Gara F (2004) Are microbes at the root of a solution to world food production? EMBO rep 5:922-926

Omer ZS, Tombolini R, Broberg A, Gerhardson B (2004) Indole-3-acetic acid production by pink pigmented facultative methylotrophic bacteria, Plant Growth Regul 48:93-96

Omer ZS, Tombolini R, Gerhardson B (2004) Plant colonization by pink-pigmented facultative methylotrophic bacteria (PPFMs). FEMS Microbiol Ecol 47:319-326

Owen RJ, On SLW, Costas M (1989) The effect of cooling rate, freeze-drying suspending fluid and culture age on the preservation of *Campylobacter pylori*. J Appl Bacteriol 66:331-337

Rickard AH, Leach SA, Hall LS, Buswell CM, High NJ, Handley PS (2002) Phylogenetic relationships and coaggregation ability of freshwater biofilms bacteria. Appl Environ Microbiol 68:3644-3650

Russo A, Basaglia M, Tola E, Casella S (2001) Survival, root colonization and biocontrol capacities of *Pseudomonas fluorescens* F113 LacZY in dry alginate microbeads. J Ind Microbiol Biotechnol 27:337-342

Senthilkumar M, Madhaiyan M, Sundaram SP, Kannaiyan S (2009) Intercellular colonization and growth-promoting effect of *Methylobacterium* sp. with plant-growth regulators on rice (*Oryza sativa* L. cv. CO-43). Microbiol Res 164:92-104

Simões LC, Simões M, Vieira MJ (2007) Biofilm interactions between distinct bacterial genera isolated from drinking water. Appl Environ Microbiol 73:6192-6200

Singh S, Fett WF (1995) Stimulation of exopolysaccharide production by fluorescent pseudomonads in sucrose media due to dehydratation and increased osmolarity. FEMS Microbiol Lett 130:301-306

Stepnowski P, Blotevogel KH, Jastorff B (2004) Extraction of carotenoids produced during methanol waste biodegradation. Int Biodeterioration Biodegrad 53:127-132

Sy A, Giraud E, Jourand P, Garcia N, Willems A, de Lajudie P, Prin Y, Neyra M, Gillis M, Boivin-Masson C, Dreyfus B (2001) Methylotrohpic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J Bacteriol 183:214-220

Van Akten B, Yoon JM, Schnoor JL (2004) Biodegradation of nitro-substituted explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine by a phytosymbiotic *Methylobacterium* sp. associated with poplar tissues (*Populus deltoides nigra* DN34). J Appl Environ Microbiol 70:508-517

Verginer M, Siegmund B, Cardinale M, Müller H, Choi Y, Miguez C, Leitner E, Berg G (2010) Monitoring the plant epiphyte *Methylobacterium extorquens* DSM 21961 by real time PCR and its influence on strawberry flavor. FEMS Microbiol Ecol, online

Xiong R, Xie G, Edmontson AE, Sherad MA (1999) A mathematical model for bacterial inactivation. Int J Food Microbiol 46:45-55

Zabetakis I (1997) Enhancement of flavor biosynthesis from strawberry (*Fragaria* x *ananassa*) callus cultures by *Methylobacterium* species. Plant Cell Tissue and Organ Cult 50:179-183.

Manuscript II

From Agriculture to Biotechnology: Applications of Pink Pigmented Facultative Methylotrophic Bacteria[4]

Abstract

Since more than 40 years pink pigmented facultative methylotrophic bacteria of the genus *Methylobacterium* are at the center of interest of microbiologists and biotechnologists. Due to several metabolic and physiological peculiarities members of this genus are a comprehensive resource for strains suitable for the application in agriculture and biotechnology. As plant symbionts, methylobacteria increase growth, yield and fitness of crop plants. In a close association with their host plants they stimulate plant growth by producing phytohormones, fixing nitrogen or by suppressing disease symptoms. As cell factories, methylobacteria are used for the production of biopolymers, a sustainable alternative to plastics produced from petroleum. Additionally, they can be used for the conversion of methanol - an alternative and cheap carbon source – to high value products. In bioremediation, methylobacteria are useful for the decomposition of different chemical pollutants that are released by humans. All these applications open up new ways in agriculture and biotechnology. This manuscript summarizes the different applications of pink pigmented facultative methylotrophs and the current state of research on the development of biotechnological processes using these bacteria is reported.

[4] Verginer M and Berg G (2010) From Agriculture to Biotechnology: Applications of Pink Pigmented Facultative Methylotrophic Bacteria. In preparation

Introduction

First evidence for the existence of methylotrophic bacteria was given by Loew already in 1892. He isolated a bacterium that was able to grow on methane or methanol as sole carbon and energy source and named it *Bacillus methylicus*. Almost 70 years later, Peel and Quayle (1961) isolated a strain with similar physiological properties and assigned it as *Pseudomonas* AM1. Due to their ability to metabolize C_1-compounds more attention was paid to methylotrophic bacteria since the 1960s. In 1970 Whittenbury and coworkers published different methods for the isolation of methylotrophic bacteria. The genus *Methylobacterium* was firstly described by Patt et al. in 1976. It belongs to the family of Methylobacteriaceae, which is ranged in the order of Rhizobiales, a subclass of Alphaproteobacteria. The genus description was based on one single type strain that was able to use methane as carbon source (*M. organophilicum*). Because the type strain has lost its ability to grow on methane, in 1983 Green and Bousfield emended the genus description of Patt et al. and transferred several facultative methylotrophic species into the new genus (Green and Bousfield 1983). Many of the strains which today are comprised in the genus *Methylobacterium* have a checkered taxonomic history and were previously assigned to other genera. In the following decades many new metabolic pathways such as the serine cycle and the ribulose monophosphate cycle were discovered and investigated in *Methylobacterium* strains (Anthony 1982). Driving force in the investigation of the metabolism of methylobacteria was the intention to use them for biotechnological applications, e.g. as expression system for heterologous proteins (Bélanger et al. 2004). Methane or methanol are much cheaper carbon sources as sugars that are needed for the growth of many of the standard expression hosts for proteins in biotechnology, e.g. *Escherichia coli*. In addition, methylobacteria were

found out to accumulate poly-β-hydroxybutyrate, the base material for biodegradable plastic (Babel 1992). Since the 1990s, the focus was put progressively on the investigation of the association of methylobacteria and plants, where close plant-microbe interactions were discovered. Plant-associated methylobacteria consume methanol, which is emitted from the plant as a "waste"-product in lignin biosynthesis (Nemecek-Marshall et al. 1995). Kutschera (2007) developed a theory, that plant-associated methylobacteria actively enhance methanol production of the plants by producing cytokinins, which stimulate the growth of the host plant.

In 2005 the Bergey's Manual of Systematic Bacteriology (Garrity et al. 2005) described 12 species belonging to the genus *Methylobacterium*, but due to the frequent description of new species this number is growing rapidly (Gallego et al. 2006; Kang et al. 2007). Today, the genus comprises 33 reported species (NCBI taxonomy database, http://www.ncbi.nlm.nih.gov/Taxonomy; August 2010) (see Fig 1) and from eight strains the genomes are sequenced (NCBI genome database http://www.ncbi.nlm.nih.gov/sites/genome; August 2010).

Methylobacteria are rod-shaped cells with a size of 0.8-1.2 x 1.0-8.0 µm. They stain Gram negative and are motile by means of a single flagellum. Most strains contain carotenoid pigments that lead to a pink to orange-red coloring of the cells. Their metabolism is strictly aerobic, mesophilic, chemoorganotrophic and facultatively methylotrophic. They are capable to grow on a variety of C_1 compounds like formaldehyde, methanol, formate and methylated amines, but also short-chained organic acids, ethylated amines and sugars can be used as carbon source. Due to their metabolism and their pigmentation methylobacteria were referred to as Pink Pigmented Facultative Methylotrophs (PPFMs) (Garrity et al. 2005).

PPFMs occur ubiquitously and can be found in different habitats. Mostly they were isolated from air, soil, water and from plants (Knief et al. 2008). To plants they are closely related (Holland 1997; Kutschera 2007); they are capable to use the methanol which is emitted through plant stomata (Nemecek-Marshall et al. 1995) as sole source of carbon and energy. Methylobacteria already have been isolated from many plant species and probably they can be found on plants of all taxa (Corpe and Rheem 1989; Holland 1997). Partially they were also reported to occur as endophytes of plants, e.g. as nitrogen-fixing symbionts in legumes (Sy et al. 2001; Jaftha et al. 2002) or in the endosphere of *Pinus sylvestris* (Pirttilä et al. 2000).

Their metabolic peculiarities make PPFMs to suitable tools to make accessible new technologies and strategies. In the following chapters the suitability of methylobacteria for different purposes in agriculture and biotechnology is evaluated, results of scientific research are summarized and compared and existing technological applications of PPFMs are described.

Interaction of PPFMs with plants

PPFMs are frequently reported to be part of the heterotrophic microbial flora on the surface of many plant species and a very close association of methylobacteria with their host plants is assumed. Highest cell numbers of PPFMs are present on actively growing tissues with 10^4-10^6 cells per leaflet (Madhaiyan et al. 2005). Therefore, a molecular interaction of the bacteria with their host plants is obvious. In 1994 Holland presented the hypothesis that cytokinins - a group of phytohormones that stimulate cell division and growth in plants - are not produced by plants themselves but by microbial symbionts. He based his theory on the

fact that a reduction of PPFMs in plant seeds resulted in a decreased germination rate and insufficient growth of the seedlings. This effect could be reversed by treating the seeds with PPFMs or alternatively with cytokinins (Holland and Polacco 1994). Holland speculated that plants and PPFMs live in a tight symbiosis: the bacteria consume methanol - a waste product of lignin biosynthesis in plants (Nemecek-Marshall et al. 1995) - and stimulate plant growth by producing cytokinins. He designated methylobacteria as coenvolved participants in plant physiology and remarked the biotechnological potential of PPFMs due to their close connection with plant metabolism (Holland 1997). Indeed, in the following years many experimental data which confirmed his theory and the biotechnological use of methylobacteria were reported in literature: it has been shown *in vitro* that *Methylobacterium* isolates are able to produce cytokinins. In the supernatants of liquid cultures of *Methylobacterium* isolates indole-3-acetic acid (IAA) has been detected. IAA concentrations in the supernatants were between 6 and 13.3 mg L^{-1} in the presence of L-tryptophan (Koenig et al. 2002; Omer et al. 2004). Hornschuh et al. (2002) provided first information and experimental data about the interaction of methylobacteria with their host plants. He showed that methylobacteria preferably colonize growing plant parts and stimulate protonema development in the bryophyte *Funaria hygrometrica*. In the following years the growth-promoting effect of methylobacteria on different plant species was investigated extensively. For several plant species an increased germination, growth and yield due to a treatment with PPFMs has been reported (see table 1). Also an application of methanol on the phyllosphere of sugarcane to stimulate the activity of PPFMs was tested. The application showed similar results as a direct treatment of the plants with PPFMs. The PPFM abundance on methanol-treated plants was significantly higher than on untreated

control plants. The author assumed that the foliar application of methanol may mimic the production of methanol by the plant and stimulate cytokinin production by PPFMs which enhances plant growth. This leads to enhanced methanol emission by the plant which then again stimulates PPFMs (Madhaiyan et al. 2006c). The foliar application of methanol was also tested on soybean. Here a yield increase due to the methanol treatment was reported, when the plants suffered water stress. Under non-stressed condition the application showed no effect (Joshi and Holland 2001). All the examples listed in table 1 indicate a close association of PPFMs with plants. The bacteria interact actively with their hosts by producing cytokinins that stimulate plant metabolism. Examples have shown that this effect can be used to increase growth, yield and health of crop plants. There exist already several attempts to commercialize PPFMs in biotechnological products. Numerous patents concerning the use of *Methylobacterium* strains in agriculture have been filed since 1996 (Holland and Polacco 1996; Joshi and Holland 2001; Holland and Polacco 2006). The company CST growth, LLC markets the product TrophoMax™, a liquid formulation of *Methylobacterium mesophilicum*. According to the manufacturer, the product increases several parameters of plant growth, e.g. the vigor of germination, yield, and resistance against pathogens and abiotic stress factors as drought or heat. In addition, Holland isolated different 'elite strains' that overproduced specific compounds as vitamin B_{12} or methionine. These strains are used to enrich food crops with those substances to raise their nutritional value and can be used for the biological production of functional foods (Holland and Polacco 2006).

Apart from phytohormone production another characteristic of some *Methylobacterium* species leads to a close association to and a beneficial effect for their host

plants. Some species are reported to act as nitrogen fixers associated with legumes. Plants of the *Leguminosae* family are of major ecological and agricultural importance since they are responsible for the majority of nitrogen fixation on land. Nitrogen fixation occurs in specialized root organs, called nodules, where symbiotic bacteria referred to as rhizobia reduce atmospheric nitrogen to ammonium (Peoples and Craswell 1992). Rhizobia are no homogenous group of bacteria but they belong to different genera within the α-subclass of proteobacteria. In 2001, the first rhizobial species belonging to the genus *Methylobacterium* was described: *Methylobacterium nodulans*. Phylogenetic analysis of the *NodA*-gene (coding for a signaling factor which induces nodule formation in the host plant) of the strain showed that the *M. nodulans NodA* is closely related to *Bradyrhizobium NodA,* suggesting that this gene was acquired by horizontal gene transfer. At this time, no other *Methylobacterium* species was known to harbor this gene. Physiological properties and the *mxaF* gene showed the affiliation of the strain to the genus *Methylobacterium*, even if *M. nodulans* does not show the typical pigmentation of PPFMs. This may be an adaptation to the subterrestrial lifestyle of this species. The strain is highly specific to *Crotalaria* ssp. Some *Crotalaria* species are of major interest since they are used as green manure to increase the nitrogen level in agricultural soils (Sy et al. 2001). Jourand and coworkers demonstrated the importance of methylotrophy during the symbiosis between *M. nodulans* and *Crotalaria* sp. If non-methylotrophic mutants were applied to the plants, nodule number and nitrogen fixation capacity of the plant decreased dramatically (Jourand et al. 2005). From *Lotononis bainesii*, a pasture plant, several rhizobia were isolated and identified to be closely related to *M. nodulans* (Jaftha et al. 2002). Raja et al. (2006) isolated nodulating and non-nodulating *Methylobacterium* sp. with high nitrogenase activity from legumes. Similarly, Madhaiyan et

al. (2006) isolated several nodulating *Methylobacterium* isolates with high nitrogenase activity from tropical legumes such as field beans, cowpea, black gram and soybean. In 2009 the plant-growth stimulating effect of nodulating and nitrogen fixing *Methylobacterium* species was shown in greenhouse experiments. *Crotalaria juncea* and *Macroptilium atropurpureum* showed significantly increased shoot and root length as well as increased dry weight and nitrogen content if the seedlings were inoculated with rhizobial *Methylobacterium* isolates (Madhaiyan et al. 2009).

Madhaiyan and coworkers reported about *Methylobacterium* isolates that showed 1-aminocyclopropan-1-carboxylate (ACC) deaminase activity and therefore were able to use ACC as nitrogen source (Madhaiyan et al. 2004). ACC in plants acts as a precursor of ethylene, a phytohormone that inhibits plant growth and induces senescence (Pierik et al. 2006). After infection with a pathogen an increased ethylene level in plants leads to the induction of defense mechanisms in the early stage of infection and to the formation of disease symptoms in the later stage of infection (Ecker and Davis 1987). Therefore, bacteria that degrade ACC inhibit senescence and protect the plant from disease symptoms. Indeed, after treatment with PPFMs, plant showed a reduced disease severity compared with untreated control plants (for examples see table 1).

In addition to influencing plant growth by secreting phytohormones or by supplying the plant with nitrogen methylobacteria also can influence fruit development and flavor production in crop plants. It is known that microorganisms are able to produce different flavors *de novo* or they can produce chemical precursors of flavors (Janssens et al. 1992). This has been shown on strawberry plants: a co-cultivation of strawberry callus cultures (*Fragaria* x *ananassa* cv. Elsanta) with *Methylobacterium extorquens* led to an increased

biosynthesis of the compound 2,5-dimethyl-4-hydroxy-2H-furanone (DMHF) (Zabetakis 1997; Koutsompogeras et al. 2007). This substance is a component of strawberry flavor with major importance (Bood and Zabetakis 2002). The stimulation of DMHF biosynthesis by *Methylobacterium extorquens* has also been shown *ad planta* (Verginer et al. 2010).

Methylobacteria as cell factories: PPFMs in industrial processes

Due to rising oil prices caused by increasing demand and the industrial development in many countries of the world the interest for alternative raw materials increased rapidly during the last decades. Additionally, the new demand for sustainable products speeded up the search for new feedstocks for the chemical industry. Methanol is a promising candidate as an alternative raw material for biotechnological fermentation processes. As by-product from industrial processes it is worldwide available in large amounts for low prices. In contrast to other feedstocks of agricultural origin it is independent from seasonal variations and weather conditions, and – if demanded – it can be also being produced from renewable raw materials on commercial scale (Schrader et al. 2008). Because traditional biotechnology is mainly based on sugars and carbohydrates as substrates, for the use of methanol new microorganisms and fermentation processes had to be found. Due to their methylotrophic metabolism, PPFMs are well suited for this purpose and in the last decades the potential of methylobacteria to be used in industrial biotechnology has been elucidated intensively.

The most investigated application is the production of biopolymers with methylobacteria. Like many other bacteria also PPFMs produce polyhydroxyalkanoates (PHAs), if nutrient supply is unbalanced or abiotic conditions deviate from the optimum (Anderson and Dawes 1990). Since PHAs have a chemical structure similar to that of petroleum-derived polypropylene, they have been investigated as a potential alternative.

Over 100 different hydroxyacid monomers have been detected in PHAs, but poly-3-hydroxybutyrate (PHB) is the most promising candidate since it is biodegradable and has thermoplastic properties. (Yezza et al. 2006). It shows a similar physical and chemical behavior as isotactic polypropylene (Ghatnekar et al. 2002). Despite of intense research and development efforts, production costs of PHB are much higher than those of conventional plastic. The price of the substrate constitutes a significant proportion of the total production costs of the polymer. For PHAs substrate costs contribute with 28-50% to the final price of the product (Solaiman et al. 2006). Therefore, industry is in search of cheap substrates to decrease the production costs of PHAs. PHB producer of the genus *Methylobacterium* offer a way out of this problem, because with their help methanol, one of the cheapest substrates available on the market in large volumes (Bourque et al. 1995) can be used for PHB production. Also the conversion of waste- or side products of agriculture of food industry as cheese whey or glycerol to PHAs can be performed with bacteria of the genus *Methylobacterium* (Solaiman et al. .2006).

From environmental samples different *Methylobacterium* strains that were able to accumulate PHB have been isolated (Anderson et al. 1992; Ackermann and Babel 1997; Yellore et al. 1999; Yezza et al. 2006). Quantity and quality of the accumulated PHB in these strains is highly dependent on the quality and concentration of the carbon source. On succinate *M. extorquens* produces PHB with a higher molecular weight (1700 kDA) than on methanol (600 kDA). With increasing methanol concentrations the molecular weight of the product and product concentration decrease (Anderson et al. 1992). On fructose *M. rhodesianum* accumulates up to 30% (w/v) PHBs under growth association without nitrogen limitation. However, on methanol nitrogen limitation is necessary to induce PHB

accumulation. Ackermann and Babel (1997) speculated that growth-associated PHB accumulation is caused by a metabolic bottleneck in the tricarboxylic acid cycle of *Methylobacterium* strains. Its capacity is not sufficient to convert all the carbon of high molecular substrates. By storing exceeding carbon in PHAs, this bottleneck is circumvented. However, in literature several examples for growth-associated PHB-production by methylobacteria are described (Yellore et al. 1999; Yezza et al. 2006) Yellore et al. (1999) reported that addition of low concentrations (16.7 mM) of formate to the fermentation medium increased the biomass production rate of *Methylobacterium* ZP24 and therefore also the yield of PHB produced in growth-association increased. An explanation for that is the high NADH and ATP yield of formate oxidation, which overcomes the bottleneck in the TCA cycle. In 1992, Bourque and coworkers isolated over 100 putative methylotrophic bacteria from environmental samples. The most promising candidate regarding growth and PHB accumulation was obtained from a soil polluted with oil products and identified as *M. extorquens.* With this strain, biomass concentrations of 9-10 g/L with a PHB content of 30-33% (w/v) were reached under non-optimized fermentation conditions in a minimal salt medium supplemented with methanol. Average molecular weight of the produced PHB was 500 kDA (Bourque et al. 1992). At this time it has been generally recognized that methylotrophic bacteria cannot produce high quality PHB, i.e. high molecular mass PHB above 400 kDA. By optimizing the fermentation conditions for this strain the authors were able to refute this theory. The strain, designated as *M. extorquens* ATCC 55366, was successfully cultivated in fed-batch fermentations with methanol as sole carbon source. Cell biomass levels above 100 g L^{-1} (dry weight) were reached after 5 days of cultivation and cells contained between 40 and 46% (w/v) PHB. Important and essential nutrients were

determined and the mineral composition of a minimal salt medium was optimized to reach these high biomass concentrations. Oxygen limitation was avoided by reducing the methanol feed when the oxygen transfer limit of the reactor has been reached. Methanol concentration was regulated on a very low level (below 0.01 g L^{-1}) to increase the molecular weight of the obtained PHB to 900-1800 kDA, a remarkably high value. Low methanol concentration in the cultivation medium promoted the formation of long-chained PHB. The authors suggested a prevented oxygen limitation due to the low methanol concentration as reason for the increased quality of the produced PHB. By use of multivariable process control strategies they were able to increase yield and quality of the produced PHB. PHB yield of the overall process was between 0.09 and 0.12 g g^{-1} methanol. (Bourque et al. 1995). Ghatnekar et al. (2002) described an efficient strategy to extract accumulated PHB from bacterial cells. PHB extraction in a soxhlet extractor has yielded the highest quality (regarding the molecular weight) of the end product, but with an extraction time of 48 h the method was uneconomical. In a chloroform extraction the yield of the extraction was considerably lower (78%). If a sodium hypochlorite-chloroform dispersion was used for the extraction (90 min contact time) the recovery rate raised to 95% with a purity of 97%. Cell permeabilization was identified as the rate-limiting step in the extraction. To shorten the extraction time a mechanical cell disruption in a high-pressure homogenizer was carried out. In a two cycle process a yield of 98% and a purity of 95% were reached (Ghatnekar et al. 2002). Beside the improvement of fermentation conditions the exploitation of alternative cheap and easily available substrates for the production of biopolymers was evaluated to reduce the production costs of PHB. A *Methylobacterium rhodesianum* strain was reported to convert glycerol to PHB. Glycerol is available in large amounts as by-product in the biofuel

production. A cell biomass of over 20 g L^{-1} was reached and the strain accumulated an average of 50% PHB after 50 h of fermentation (Bormann and Roth 1999). Also cheese whey has been shown to be a suitable substrate for the production of PHB with a *Methylobacterium* strain (Yellore et al. 1998). Cheese whey is produced in large amounts worldwide and most of it is discharged because it is of limited use for other biotechnological applications.

Beside the lowering of the production costs and the increase of the production efficiency also the improvement of the quality of the final product has been a major challenge in the last years: because PHB is highly crystalline, the resulting plastic is hard and britt. Additionally its melting point is close to its temperature of thermal decomposition which complicates the processing of the bioplastic (Yamane et al. 1993). Copolymers containing 3-hydroxybutyrate and other hydroxyacidic monomers have more useful physical properties. The softness of the final product increases and its melting point is lowered, if 3-hydroxybutyrate is copolymerized with other hydroxyacid monomers, e.g. hydroxyvalerate (Bourque et al. 1992; Yamane et al. 1993). With *M. organophilicum* the copolymer could be obtained when valeric acid was supplemented as an additional carbon source beside methanol. With a ratio of valeric acid/methanol of 1/10 best results were obtained with a copolymer content of 41% and a hydroxyvalerate fraction of 11 mol% in the copolymer. Valeric acid inhibited polymer biosynthesis, therefore feeding ratio and starting point of the feeding have to be strictly regulated (Kim et al. 1999). Also with the addition of *n*-amyl alcohol to the fermentation medium as secondary carbon source the accumulation of polyhydroxybutyrate-polyhydroxyvalerate-copolymers could be induced (Ueda et al. 1992; Yamane et al. 1993) Yezza and coworkers isolated a *Methylobacterium* strain that produced

biopolymer granules with a copolymer content of 30% and a hydroxyvalerate content of 66% in the copolymer fraction. This is the highest content of hydroxyvalerate in the heteropolymer reported until now (Yezza et al. 2006).

Despite the 80 years of research since the occurrence of PHAs from bacteria was described the first time, PHAs seem to be still far away from large scale production. Only one attempt to commercialize PHAs has been made during the major oil crisis in the 1970s, when due to the rising price of crude oil bioplastics became an economically interesting alternative to conventional plastics. In the 1980s the company Imperial Chemical Industries has developed a process for PHB production from bacteria to pilot plant stage but it became clear, that the product costs were too high to compete with conventional polypropylene (Anderson and Dawes 1990). The lack of commercialization of PHAs has been attributed to the high investments for fermentation, product recovery and substrates (Lenz and Marchessault 2005). With the discovery of methylobacteria as PHB producer at least the last hurdle could be cleared. They provide access to substrates that are much cheaper than conventional substrates used so far. Perhaps also the biodegradability of PHAs gains in importance in future years, so that biopolymers are preferred over conventional plastics. Another field of application for PHAs are medical applications like surgical sutures, implants or wound dressings. Due to the high biocompatibility – PHBs produce only very mild foreign body responses – PHB materials are well suitable for such applications and here the price plays only a minor role (Holmes 1985). In any case, science already has provided a selection of *Methylobacterium* strains that can be used for the production of PHB in large scale and also fermentation and downstream processes have been optimized, so that they are ready for an application in industrial scale.

Another attempt to take advantage of the metabolic peculiarities of methylobacteria in industrial processes was the production of proteins from methanol. The development of a biotechnological process to grow *Methylobacterium extorquens* to high cell densities on methanol (Bourque et al. 1995) and genetic informations about the species obtained from the sequencing of *M. extorquens* (Vuilleumier et al. 2009) makes this species to an interesting expression system for recombinant proteins and for the production of bulk chemicals. With GFP as a model-protein a plasmid-based expression system for heterologous proteins has been developed several years ago. Promoters usually used in *E. coli* showed only low protein expression levels in *M. extorquens*; therefore alternative, strong promoters were needed. *mxaF* from the methanol dehydrogenase gen of *M. extorquens* has been identified to be a valuable promoter for protein expression in *M. extorquens* (Figueira et al. 2000; Marx and Lidstrom 2001; Bélanger et al. 2004). The effectiveness of this expression system has been shown in a high level expression of a haloalkane dehalogenase in *M. extorquens*. Under optimal conditions an expression rate of 10% of total soluble proteins was achieved. This is significantly lower than expression levels that are reported for *E. coli*; nevertheless for special applications *M. extorquens* can represent an appropriate expression system. The production of isotopically labeled proteins, a prerequisite for NMR-based structure determination, is one possible application. Isotopically labeled methanol that is needed for the introduction of the isotopes into the proteins is much cheaper than labeled glucose, which is needed for protein expression in *E. coli* (FitzGerald and Lidstrom 2003). Also the expression of toxic proteins like enterocins in *M. extorquens* has been approved. The obtained protein yield was higher than in *E. coli* because in contrast to *E. coli*, *M. extorquens* remained unaffected by the enterocin. Also other proteins like the *Lactobacillus*

casei esterase, the *Bifidobacterium infantis* β-galactosidase or the *Bacillus thuringiensis* Cry1aA toxin have been expressed in *M. extorquens* yielding product concentrations from some milligrams to grams per liter (Gutiérrez et al. 2005). For several proteins also the integration of the expression constructs directly into the genome of *M. extorquens* has been tested. This allows the stable maintenance of the expression construct in the host without any selective pressure, e. g. antibiotic. By a multi-copy integration of the target gene the expression has been 20fold higher than the level exhibited by a single-copy integration (Choi et al. 2006).

The potential of *M. extorquens* as a cell factory is further enhanced by its ability to produce metabolites of high importance for humans, e.g. vitamin B_{12}, coenzyme Q_{10} or L-lysine (Holland and Polacco 2006; Schrader et al. 2008)

Use of PPFMs in bioremediation

Pollution due to chemicals which are released by humans is a rising problem in our time. To protect the environment from anthropogenic pollution it is necessary to clean wastewater and residues from industrial processes before they are released into the environment. For some chemicals well established degradation processes are available, for others the removal is laborious and time consuming. In such cases, biotechnological processes can provide a promising solution. Methylobacteria are described to be highly resistant against different pollutants e.g. halogenated organic compounds, aromatic compounds and metal cations and are often isolated from extreme habitats. (De Marco et al. 2004) Therefore, different *Methylobacterium* strains are well suitable for the biodegradation of chemicals. Due to their ability to metabolize C1-compounds, they are optimal for the removal of methanol, formamide and methylated amines (Stepnowski et al. 2004). Beside this, methylobacteria

often are reported to metabolize different halogenated organic compounds (Vanelli et al. 1998; Connell Hancock et al. 1998). Metabolism of halogenated organic compounds in methylobacteria is closely related to the metabolism of the corresponding unhalogenated compounds. In a first step a dehalogenation occurs and the resulting intermediate is introduced into the metabolic pathway of the corresponding unhalogenated compound (Vanelli et al. 1998). Chloromethane is made responsible for 15% of the destruction of the ozone layer of the earth. Sources of the compound are biological and nonbiological processes that occur in nature. In 1998, Vanelli and coworkers described the *Methylobacterium* strain CM4 that is able to utilize chloromethane, bromomethan and iodomethan up to a concentration of 2% in the headspace above the cultivation medium. Growth rates are the same than on methanol (Vanelli et al. 1998; Leisinger and Braus-Stromeyer 1995)). Connell Hancock et al. (1996) assessed the application of the *Methylobacterium* strain IMB-1 to agricultural areas, that were treated with methylobromide. In contrast to other pesticides it doesn't leave high molecular toxic residues in the soil, but on the other hand methylbromide that is released into the atmosphere, is highly harmful for the ozone layer. Therefore, a degradation of the applied methylbromide in the soil is desirable. A pretreatment of methylbromide fumigated soils with strain IMB-1 clearly increased its potential to consume the fumigant and the emission of methylbromide to the atmosphere was reduced significantly. Beside halogenated organic compounds also nitrated aromatics and nitroamines are mineralized by methylobacteria. From the phyllosphere of a poplar three (*Populus deltoides*) the species *Methylobacterium populi* has been isolated. This species was able to degrade the explosives TNT (2,4,6-trinitrotoluene), RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) and HMX (octahydro-1,3,5,7-

tetranitro-1,3,5,7-tetrazocine). These explosives have been found to be toxic to most classes of organisms, including humans and bacteria. Due to their widespread use in military ammunition they led to large-scale contaminations of soil and groundwater on military areas. Therefore there is a considerable interest in developing ecological methods to decontaminate polluted areas. While RDX and HMX are mineralized under release of CO_2, TNT is only reduced to less toxic compounds that may be bound to soil particles or be conjugated to organic molecules, resulting in a reduced bioavailability of the compounds (Van Akten et al. 2004). Beside the application of bioremediation strains directly in the contaminated soil also a more controlled implementation in a bioreactor is possible. One example for such an application has been presented by Campos et al. He used *Fursarium oxysporum* and an unidentified *Methylobacterium strain* to decontaminate industrial wastewater from cyanide. Due to its extreme inhibitory effects towards cell respiration cyanide is highly toxic. The cyanide was converted in a two-step-reaction to formiate. In the first step the cyanide was hydrolyzed by *Fursarium oxysporum* and the resulting formamide was converted to formiate and ammonium by the *Methylobacterium* isolate. The reaction occurred in reaction columns where the microorganisms were immobilized in alginate hydrogel beads. It was possible to reduce cyanide concentrations in the wastewater below the analytical detection limit. (Campos et al. 2006). Beside the use of pure cultures of single strains also a combination of bacterial communities was tested in several examples. Dimethyl isophtalate is decomposed in a cooperation of *Klebsiella oxytoca* and *Methylobacterium mesophilicum* (Li and Gu 2007); phosphonates are degraded by a consortium of 7 bacterial strains, including *Methylobacterium radiotolerans* (Fry et al. 2006). Also in a bioreactor used for the removal of MtBE – a widely used gasoline oxygenate

additive – from ground water *Methylobacterium* species were part of the naturally established microbial flora (Zein et al. 2004).

Risk assessment for PPFMs

Even if PPFMs can be found ubiquitously and occur also in anthropogenic environments e.g. water supply systems or hospitals, only few reports about infections of humans by methylobacteria exist (Kaye et al. 1992; Engler and Norton 2001). These reports refer mainly to immunocompromised people, who were infected during their therapy or stay in hospital. To date, no infection of healthy people with *Methylobacterium* species in environment have been reported. Therefore, it may be assumed, that there is no risk for humans, if they get in closer contact with methylobacteria, e.g. during the application of formulations of the bacteria in agriculture or during the operation of biotechnological processes with methylobacteria. Nevertheless the pathogenic potential of each strain has to be assessed before it is released into the environment. For this, several molecular and biological tests were developed, e.g. the *Caenorhabditis elegans* assay (Zachow et al., 2009). Only if these tests give negative results a release of large quantities of PPFMs into the environment is acceptable.

Summary and future prospectives

Due to changes in industry and agriculture the importance of biotechnology is rising from year to year. Scarcity of resources and an increasing request for sustainable production processes leads to the replacement of chemistry by biotechnology. This development leads to a new demand for microbial strains featuring physiological properties that can be used by humans. PPFMs are a comprehensive resource for strains with useful peculiarities. As plant symbionts they show a close interaction with their hosts leading to an increased growth,

yield and fitness of the plants. Beside the increased yield, also fruit quality can be enhanced by PPFMs. Due to the rising quality awareness of consumers this aspect is of increasing importance in the future. An ongoing problem is the lack of appropriate formulations for Gram negative bacteria in general that allow an easy distribution and application of PPFM strains. The development of stable formulations with a high shelf life is essential for the commercialization of PPFMs in agriculture. The challenge will be to transfer the accumulated knowledge from science to commercial products. Beside this it is necessary to study the fate of PPFMs after their application on the field, especially their influence on the endemic microbial flora and on eukaryotes. For that, new standardized protocols for risk evaluation have to be developed to facilitate an appropriate risk management.

As cell factories, methylobacteria are used for the sustainable production of biopolymers. The future challenge will be to improve the existing processes. At the moment they cannot compete with traditional production methods financially. Further investigations are needed to enhance efficiency and product quality. Scientific results from the last decades show an upward trend regarding product yield and product quality. If this trend is continued in the next years it is a matter of time until PHB can compete with conventional plastics. One possibility that has not been applied yet is the use of molecular techniques to manipulate PHB producing strains genetically to improve their performance. Here the fast progress in molecular biotechnology and the improvement of molecular techniques can open new ways in the next years.

In bioremediation methylobacteria are useful for the decomposition of different chemical pollutants that are released by humans. Increasing demands on the quality of wastewater and waste treatment open a broad market for bioremediation technologies. As

the mentioned examples have shown, PPFMs can play an important role in this area of application. As in the case of agriculture also for bioremediation a risk assessment for the used strains is necessary. For on-site bioremediation, formulations for PPFMs are necessary. Here, formulations that were developed originally for agricultural applications can be used.

In conclusion it can be said that PPFMs possess a range of properties that make them to useful tools in agriculture and biotechnology. Their use has been shown in a large number of scientific papers and some attempts of commercialization have been made. Nevertheless so far no product or process associated with PPFMs was marketed in large scale. The future success of PPFMs in biotechnology will depend on a successful transfer of scientific knowledge to product development and marketing. If that has been done PPFMs will contribute to the success of biotechnology in the twenty-first century.

Literature

Ackermann JU, Babel W (1997) Growth-associated synthesis of poly(hydroxybutyric acid) in *Methylobacterium rhodesianum* as an expression of an internal bottleneck. Appl Microbiol Biotechnol 47:144-149

Anderson A, Williams DR, Taidi B, Dawes EA, Ewing DF (1992) Studies on copolyester synthesis by *Rhodococcus ruber* and factors influencing the molecular mass of polyhydroxybutyrate accumulated by *Methylobacterium extorquens* and *Alcaligenes eutrophis*. FEMS Microbiol Rev 103:93-102

Anderson AJ, Dawes E (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 54:450-472

Anthony C. (1982) The biochemistry of methylotrophs. Academic Press, London

Babel W. (1992) Pecularities of methylotrophs concerning overflow metabolism, especially the synthesis of polyhydroxyalkanoates. FEMS Microbiol Rev 103:141-148

Bélanger L, Figueira MM, Bourque D, Morel L, Béland M, Laramée L, Groleau D, Míguez CB (2004) Production of heterologous protein by *Methylobacterium extorquens* in high cell density fermentation. FEMS Microbiol Lett 231:197-204

Bood KG, Zabetakis I (2002) The Biosynthesis of Strawberry Flavor (II): Biosynthetic and Molecular Biology Studies. J Food Sci 67:2-8

Bormann EJ, Roth M (1999) The production of polyhydroxybutyrate by *Methylobacterium rhodesianum* and *Ralstonia eutropha* in media containing glycerol and casein hydrolysates. Biotechnol Lett 21:1059-1063

Bourque D, Oulette B, André G, Groleau D (1992) Production of poly-β-hydroxybutyrate from methanol: characterization of a new isolate of *Methylobacterium extorquens*. Appl Microbiol Biotechnol 37:7-12

Bourque D, Pomerleau Y, Groleau D (1995) High-cell-density production of poly-β-hydroxybutyrate (PHB) from methanol by *Methylobacterium extorquens*: production of high-molecular-mass PHB. Appl Microbiol Biotechnol 44:367-376

Campos MC, Pereira P, Roseiro JC (2006) Packed-bed reactor for the integrated biodegradation of cyanide and formamide by immobilized *Fusarium osysporum* CCMI 876 and *Methylobacterium* sp. RXM CCMI 908. Enzyme Microb Tech 38:848-854

Cervantes-Martinez J, López-Díaz S, Rodríguez-Garay B (2004) Detection of the effects of *Methylobacterium* in *Agave tequilana* Weber var. Azul by laser-induced fluorescence. Plant Sci 166:889-892

Choi YJ, Bourque D, Morel L, Groleau D, Míguez CB (2006) Multicopy integration and expression of heterologous genes in *Methylobacterium extorquens* ATCC 55366. Appl Environ Microbiol 72:753-759

Connell Hancock TL, Costello AM, Lidstrom ME, Oremland RS (1998) Strain IMB-1, a novel bacterium for the removal of methyl bromide in fumigated agricultural soils. Appl Environ Microbiol 64:2988-2950

Corpe WA, Rheem S (1989) Ecology of the methylotrophic bacteria on living leaf surfaces. FEMS Microbiol Lett 62: 243-249

De Marco P, Pacheco CC, Figueiredo AR, Moradas-Ferreira P (2004) Novel pollutant-resistant methylotrophic bacteria for use in bioremediation. FEMS Microbiol Letters 234:75-80

Ecker JR, Davis RW (1987) Plant defense genes are regulates by ethylene. Proc Natl Acad Sci USA 84:5202-5206

Engler C, Norton R (2001) Recurrent *Methylobacterium mesophilicum* sepsis associated with haemodialysis. Pathology 33:536-537

Figueira MM, Laramée L, Murrell JC, Groleau D, Miguez CB (2000) Production of green fluorescent protein by the methylotrophic bacterium *Methylobacterium extorquens*. FEMS Microbiol Lett 193:195-200

FitzGerald KA, Lidstrom ME (2003) Overexpression of a heterologous protein, haloalkane dehalogenase, in a poly-β-hydroxybutyrate-deficient strain of the facultative methylotroph *Methylobacterium extorquens* AM1. Biotechnol Bioeng 81:263-268

Fry IJ, DeFrank JJ, Earley JP (2006) Microbial biodegradation of phosphonates. US Patent 7,001,758 B1

Gallego ., García MT, Ventosa A (2006) *Methylobacterium adhaesivum* sp. nov., a methylotrophic bacterium isolated from drinking water. Int J Syst Evol Microbiol 56:339-342

Garrity GM, Brenner DJ, Krieg NR, Staley JR (2005) Bergey's Manual of Systematic Bacteriology, Volume Two: The Proteobacteria, Part C., 2nd edition, Springer-Verlag, Berlin

Ghatnekar MS, Pai JS, Ganesh M (2002) Production and recovery of poly-3-hydroxybutyrate from *Methylobacterium* sp. V49. J Chem Technol Biotechnol 77:444-448

Green PN, Bousfield IJ (1983) Emendation of *Methylobacterium* Patt, Cole, and Hanson 1976: *Methylobacterium rhodinum* (Heumann 1962) comb. nov. corrig.; *Methylobacterium radiotolerans* (Ito and Iizuka 1971) comb. nov. corrig.; and *Methylobacterium mesophilicum* (Austin and Goodfellow 1979) comb. nov.. Int J Syst Bacteriol 33:875-877

Gutiérrez J, Bourque D, Criado R, Choi YJ, Cintas LM, Hernández PE, Míguez CB (2005) Heterologous extracellular production of enterocin P from *Enterococcus faecium* P13 in the methylotrophic bacterium *Methylobacterium extorquens*. FEMS Microbiol Lett 248:125-131

Holland M, Polacco JC (2006) Method for altering the metabolism of plants. US Patent US 2006/0228797 A1

Holland MA (1997) Are cytokinins produced by plants? Plant Physiol 115:865-868

Holland MA, Polacco JC (1994) PPFMs and other covert contaminants: is there more to plant physiology than just plants? Annu Rev Plant Physiol Plant Mol Biol 45:197-209

Holland MA, Polacco JC (1996) Seeds, coated or impregnated with a PPFM. US Patent 5,512,069

Holmes PA (1985) Applications of PHB – a microbially produced biodegradable thermoplastic. Phys Technol 16:32-36

Hornschuh M, Grotha R, Kutschera U (2002) Epiphytic bacteria associated with the bryophyte *Funaria hygrometrica*: effects of *Methylobacterium* strains on protonema development. Plant Biol. 4:682-687

Indiragandhi P, Anandham R, Kim K, Yim W, Madhaiyan M, Sa T (2007) Induction of defense responses in tomato against *Pseudomonas syringae* pv. *Tomato* by regulating the stress ethylene level with *Methylobacterium oryzae* CBMB20 containing 1-aminocyclopropane-1-carboxylate deaminase. World J Microbiol Biotechnol 24:1037-1045

Jaftha JB, Strijdom BW, Steyn PL (2002) Characterisation of pigmented methylotrophic bacteria which nodulate *Lotononsis bainesii.* System Appl Microbiol 25:440-449

Janssens L, De Pooter HL, Schamp NM, Vandamme EJ (1992) Production of flavours by microorganisms. Process Biochem 27:195-215

Joshi J, Holland MA (2001) Method for treating plants. US Patent US6,329,320 B1

Jourand P, Renier A, Rapior S, De Faria SM, Prin Y, Galiana A, Giraud E, Dreyfus B (2005) Role of methylotrophy during symbiosis between *Methylobacterium nodulans* and *Crotalaria podocarpa*. Mol Plant Microbe Interact 18:1061-1068

Kang Y.S., Kim J., Shin H.D., Nam Y.D., Bae J.W., Jeon C.O., Park W. (2007) *Methylobacterium platani* sp. nov., isolated from a leaf of the tree *Platanus orientalis*. Int J Syst Evol Microbiol 57:2849-2853

Kaye KM, Macone A, Kazanjian PH (1992) Catheter infection caused by *Methylobacterium* in immunocompromised hosts: report of three cases and review of the literature. Clin Infect Dis 14:1010-1014

Kim SW, Kim P, Kim JH (1999) Production of poly(3hydroxybutyrate-co-3-hydroxyvalerate) from *Methylobacterium organophilicum* by potassium-limited fed-batch culture. Enzyme Microb Tech 24:555-560

Knief C, Frances L, Cantet F, Vorhold JA (2008) Cultivation-independent characterization of *Methylobacterium* populations in the plant phyllosphere by automated ribosomal intergenic spacer analysis. Appl Environ Microbiol 74:2218-2228

Koenig RL, Morris RO, Polacco JC (2002) tRNA is the source of low-level *trans*-Zeatin production in *Methylobacterium* spp. J Bacteriol 184:1832-1842

Koutsompogeras P, Kyriacou A, Zabetakis I (2007) The formation of 2,5-dimethyl-4-hydroxy-2H-furan-3-one by cell-free extracts of *Methylobacterium extorquens* and strawberry (*Fragaria* x *ananassa* cv. Elsanta). Food Chem 104:1654-1661

Kutschera U. (2007) Plant-associated methylobacteria as co-envolved phytosymbionts. Plant Signal Behav 2: 74-78

Lee HS, Madhaiyan M, Kim CW, Choi SJ, Chung KY, Sa TM (2006) Physiological enhancement of early growth rice seedlings (*Oryza sativa* L.) by production of phytohormone of N_2-fixing methylotrophic isolates. Biol Fertil Soils 42:402-408

Leisinger T, Braus-Stromeyer SA (1995) Bacterial growth with chlorinated methanes. Environ Health Perspect 103:33-36

Lenz RW, Marchesault RH (2005) Bacterial polyesters: biosynthesis, biodegradable plastics and biotechnology. Biomacromolecules 6:1-8

Li J, Gu JD (2007) Complete degradation of dimethyl isophtalate requires the biochemical cooperation between *Klebsiella oxytoca* Sc and *Methylobacterium mesophilicum* Sr isolated from wetland sediments. Sci Total Environ 380:181-187

Madhaiyan M, Pooguzhali S, Lee HS, Hari K, Sudaram SP, Sa TM (2005) Pink-pigmented facultative methylotrophic bacteria accelerate germination, growth and yield of sugarcane clone Co86032 (*Saccharum officinarum* L.) Biol Fertil Soils: 41:350-358

Madhaiyan M, Poonguzhali S, Ryu R, Sa T (2006a) Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocyclopropan-1-carboxylate deaminase-containing *Methylobacterium fujisawaense*. Planta 224:268-278

Madhaiyan M, Poonguzhali S, Senthilkumar M, Seshadri S, Chung H, Yang J, Sundaram S, Sa T (2004) Growth promotion and induction of systemic resistance in rice cultivar Cu-47 (*Oryza sativa* L.) by *Methylobacterium* spp. Bot Bull Acad Sin 54:315-324

Madhaiyan M, Poonguzhali S, Senthilkumar M, Sundaram SP, Sa T (2009) Nodulation and plant-growth promotion by methylotrophic bacteria isolates from tropical legumes. Microbiol Res 164:114-120

Madhaiyan M, Poonguzhali S, Senthilkumar M, Sundaram SP, Sa T (2006b) Nodulation and plant growth promotion by methylotrophic bacteria isolated from tropical legumes. Microbiol. Res. 164:114-120

Madhaiyan M, Poonguzhali S, Sundaram SP, Sa T (2006c) A new insight into foliar applied methanol influencing phylloplane methylotrophic dynamics and growth promotion of cotton (*Gossypium hirsutum* L.) and sugarcane (*Saccharum officinarum* L.). Environ Exp Bot 57:168-176

Maliti CM, Basile D, Corpe W (2005) Effects of *Methylobacterium* ssp. strains on rice *Oryza sativa* L. callus induction, plantlet regeneration, and seedling growth *in vitro*. J Torrey Bot Soc 132:355-367

Marx CJ, Lidstrom ME (2001) Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative bacteria. Microbiology 147:2065-2075

Nemecek-Marshall M, MacDonald RC, Franzen JJ, Wojciechowski CL, Fall R (1995) Methanol emission from leaves: enzymatic detection of gas-phase methanol and relation of methanol fluxes to stomatal conductance and leaf development. Plant Physiol 108:1359-1368

Omer ZS, Tombolini R, Broberg A, Gerhardson B (2004) Indole-3-acetic acid production by pink-pigmented facultative methylotrophic bacteria. J Plant Growth Regul 43:93-96

Patt T.E., Cole G.C., Hanson R.S. (1976) *Methylobacterium*, a new genus of facultatively methylotrophic bacteria. Int J Syst Evol Microbiol 26:226-229

Peel D., Quayle J.R. (1961) Microbial growth on C1 compounds. 1. Isolation and characterization of *Pseudomonas* AM1. Biochem J 81:465-469

Peoples MB, Craswell ET (1992) Biological nitrogen fixation: investments, speculations and actual contribution to agriculture. Plant Soil 141:13-39

Pierik R, Tholen D, Poorter H, Visser EJW, Voesenek LACJ (2006) The Janus factor of ethylene: growth inhibition and stimulation. Trends Plant Sci 11:176–183

Pirttilä AM, Laukkanen H, Pospiech H, Myllylä R, Hohtola A (2000) Detection of intracellular bacteria in the buds of Scotch pine (*Pinus sylvestris* L.) by in situ hybridization. Appl Environ Microbiol 66: 3073-3077

Raja P, Uma S, Sundaram S (2006) Non-nodulating pink-pigmented facultative *Methylobacterium* sp. with a functional *nifH* gene. World J Microbiol Biotechnol. 22:1381-1384

Schrader J, Schilling M, Holtmann D, Sell D, Filho MV, Marx A, Vorholt J (2008) Methanol-based industrial biotechnology: current status and future perspectives of methylotrophic bacteria. Trend Biotechnol 27:107-115

Solaiman DKY, Ashby RD, Foglia TA, Marmer WN (2006) Conversion of agricultural feedstock and coproducts into poly(hydroxyalkanoates). Appl Microbiol Biotechnol 71:783-789

Stepnowski P, Blotevogel KH, Jastorff B (2004) Extraction of carotenoids produced during methanol waste biodegradation. Int Biodeterioration Biodegrad 53:127-132

Sy A, Giraud E, Jourand P, Garcia N, Willems A, de Lajudie P, Prin Y, Neyra M, Gillis M, Boivin-Masson C, Dreyfus B (2001) Methylotrophic *Methylobacterium* bacteria nodulate and fix Nitrogen in symbiosis with legumes. J Bacteriol 183:214-220

Ueda S, Matsumoto S, Takagi A, Yamane T (1992) Synthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from methanol and *n*-amyl alcohol by the methylotrophic bacteria *Paracoccus denitrificans* and *Methylobacterium extorquens*. Appl Environ Microbiol 58:3574-3579

Van Akten B, Peres CM, Doty SL, Yoon JM, Schnoor JL (2004) *Methylobacterium populi* sp. nov., a novel aerobic, pink pigmented, facultatively methylotrophic, methane-utilizing bacterium isolated from poplar trees (*Populus deltoides* x *nigra* DN34). Int J Syst Evol Microbiol 54:1191-1196

Vanelli T, Studer A, Kertesz M, Leisinger T (1998) Chloromethane metabolism by *Methylobacterium* sp. Strain CM4. Appl Environ Microbiol 64:1933-1936

Verginer M, Siegmund B, Cardinale M, Müller H, Choi Y, Miguez C, Leitner E, Berg G (2010) Monitoring the plant epiphyte *Methylobacterium extorquens* DSM 21961 by real time PCR and its influence on strawberry flavor. FEMS Microbiol Ecol 74:136-145

Vuilleumier S, Chistoserdova L, Lee M-C, Bringel F, Lajus A, et al. (2009) *Methylobacterium* genome sequences: a reference blueprint to investigate microbial metabolism of C1 compounds from industrial and natural sources. PLoS ONE 4:e5584

Whittenbury R, Phillips KC, Wilkinson JF (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. J Gen Microbiol 61:205-218

Yamane T, Ueda S, Imagawa S, Tahara T, Tokunaga Y, Iesaka H, Urakami T (1993) Process for preparation of copolymer. US Patent 5,200,332

Yellore V, Desai A (1998) Production of poly-3-hydroxybutyrate from lactose and whey by *Methylobacterium* sp. ZP24. Lett Appl Microbiol 26:391-394

Yellore VS, Thakur NB, Desai AJ (1999) Enhancement of growth and poly-3-hydroxybutyrate production from *Methylobacterium* sp. ZP24 by formate and other organic acids. Lett Appl Microbiol 29:171-175

Yezza A, Fournier D, Halasz A, Hawari J (2006) Production of polyhydroxyalkanoates from methanol by a new methylotrophic bacterium *Methylobacterium* sp. GW2. Appl Microbiol Biotechnol 73:211-218

Zabetakis I (1997) Enhancement of flavor biosynthesis from strawberry (*Fragaria* x *ananassa*) callus cultures by *Methylobacterium* species. Plant Cell Tissue and Organ Cult 50:179-183.

Zachow C, Pirker H, Westendorf C, Tilcher R, Berg G (2009) The *Caenorhabditis elegans* assay: a tool to evaluate the pathogenic potential of bacterial biocontrol agents. Eur J Plant Pathol 125:367-376

Zein MM, Suidan MT, Venosa AD (2004) MtBE Biodegradation in a gravity flow, high-biomass retaining bioreactor. Environ Sci Technol 38:3449-3456

Tab 1 Beneficial effects of PPFMs for crop plants

Mode of action	Host plant	Effect	Literature
Production of phytohormones	*Agave tequilana*	increased number of stomata, increased photosynthetic activity	Cervantes-Martinez et al 2004
	Oryza sativa	enhanced germination vigor, biomass production and grain yield	Maliti et al 2005; Lee et al. 2006; Madhaiyan et al 2004
	Saccharum officinarum	enhanced germination, enhanced sugar yield and sugar quality	Madhaiyan et al. 2005
	Glycine max	increased grain yield	Joshi and Holland 2001
Nitrogen fixation	*Crotalaria* sp.	growth promotion	Jourand et al 2005
	Macroptilium atropurpureum	growth promotion	Madhaiyan 2009
1-aminocyclopropan - 1-carboxylate deaminase activity	Oryza sativa	reduced disease symptoms	Madhaiyan et al. 2006b
	Solanum lycopersicum	reduced disease symptoms	Indiragandhi et al. 2008
	Brassica campestris	extended root growth	Madhaiyan et al. 2006a
alcohol dehydrogenase activity	*Fragaria* x *ananassa*	stimulation of flavor production	Verginer et al. 2010

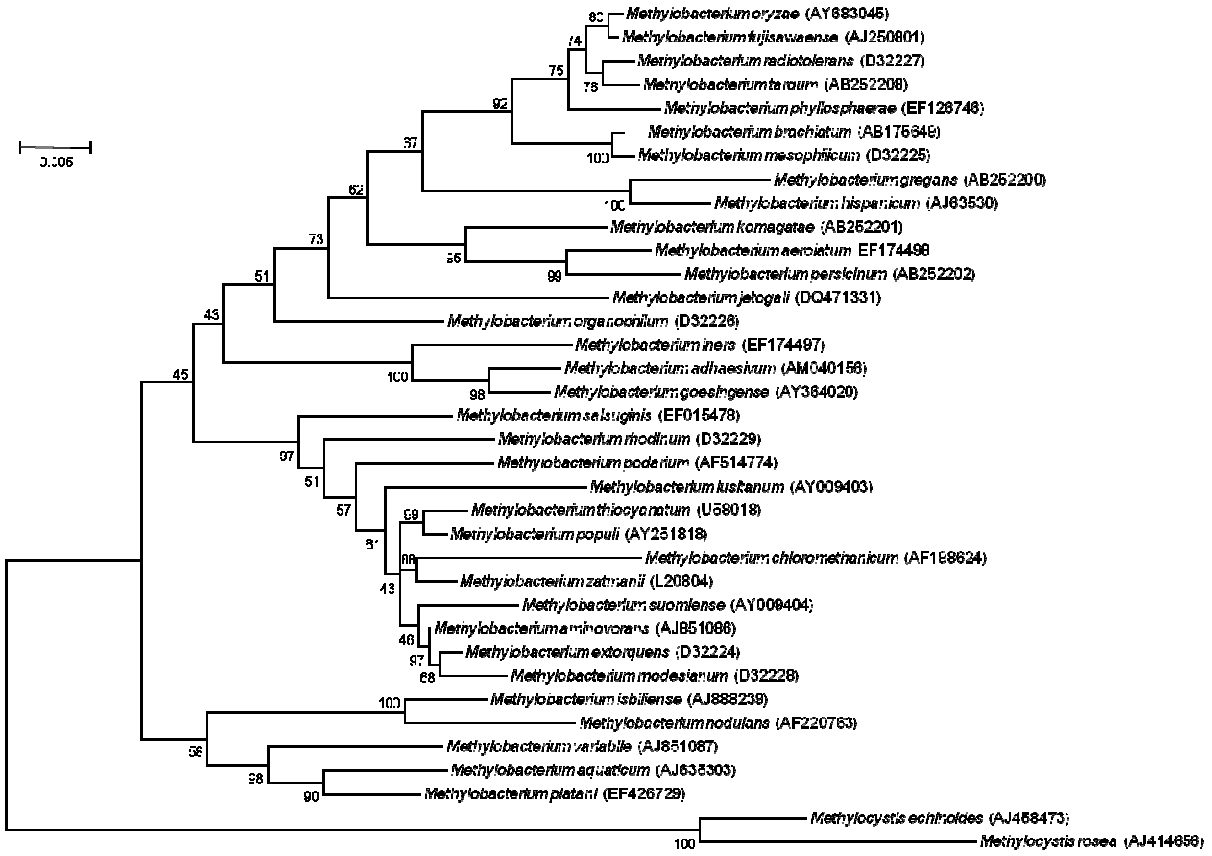

Fig 1 Phylogenetic tree showing the relationship of all species belonging to the genus *Methylobacterium* described until 2010. Data base were 16S rDNA sequences of type strains obtained from GenBank (Accession numbers are listed in the diagram). The tree was constructed using the neighbor joining method. Bootstrap values are indicated at branch points. Bar = 0.5% sequence divergence.

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Publication List

Publications

2010 Verginer M., Siegmund B., Cardinale M., Müller H., Migúez C.B., Leitner E., Berg G. Monitoring the plant epiphyte *Methylobacterium extorquens* DSM 21961 by real time PCR and its influence on strawberry flavor. Journal of FEMS Microbiology Ecology 74:136-145

Verginer M., Leitner E., Berg G. Production of volatile metabolites by grape-associated microorganisms. Journal of Agricultural and Food Chemistry 58:8344-8350

2009 Verginer M., Müller H., Siegmund B., Leitner E., Berg G. Development of an effective agent to enhance flavor production in strawberries based on a bacterial formulation. Biology of Plant-Microbe Interactions, Vol. 7, edited by Antoun H., Avis T., Brisson L., Prévost D., Trepanier M.

Oral Presentations

2010 Verginer M., Siegmund B., Müller H., Leitner E., Berg G. Die Rolle von Methylobakterien bei der Aromaproduktion in Erdbeeren. 65. ALVA Jahrestagung, Wels, Austria

2009 Verginer M., Müller H., Zachow C., Berg G. Development of effective agents enhancing plant quality and health based on ecological backgrounds and molecular mode of actions. XIV International Congress on Plant-Microbe Interactions, Quebec, Canada

2008 Verginer M., Leitner E., Berg G. Mikroorganismen an Wein: Analyse der Diversität und Aromaproduktion. Arbeitskreistagung „Biologische Bekämpfung“, Darmstadt, Germany

Schmid F., Verginer M., Leitner E., Berg G. Grape-associated microorgansisms: their antagonistic potential towards plant-pathogens and impact on wine aroma. Xth meeting of the working group “Biological control of bacterial and fungal plant pathogens. Interlaken, Switzerland

2007 Schmid F., Verginer M., Leitner E., Berg G., Wein-assoziierte Mikroorganismen: Analyse der strukturellen Diversität und Funktion. 25. Internat. Weinwiss., Graz, Austria

Poster Presentations

2009 Berg G., Verginer M., Siegmund B., Leitner E. Impact of associated bacteria on flavor of grapes and strawberry. Bageco 10 bacterial genetics and ecology – coexisting on a changing planet, Uppsala, Sweden

Verginer M., Müller H., Siegmund B., Leitner E., Berg G. Development of an effective agent to enhance flavor production in strawberries based on a bacterial formulation. XIV Congress on Plant-Microbe Interaction, Quebec, Canada

Verginer M., Müller H., Siegmund B., Pöllinger-Zierler B., Bagdonaite K., Leitner E., Berg G. Enhancement of flavor production in strawberries by microbial inoculants. XIV Congress on Plant-Microbe Interaction, Quebec, Canada

Müller H., Verginer M., Zachow C., Berg G. Development of effective agents enhancing plant quality and health based on ecological backgrounds and molecular modes of action. 8th International PGPR workshow. Portland, USA

Berg G., Verginer M., Siegmund B., Leitner E. Plant-associated bacteria and their impact on fruit flavor. 8th International PGPR workshow. Portland, USA

Siegmund B., Pöllinger-Zierler B., Verginer M. , Müller H., Berg G. Leitner E. The flavour of strawberries – can we enhance it on a natural way? 8th Pangborn Sensory Science Symposium, Florence, Italy

2008 Verginer M., Siegmund B., Pöllinger-Zierler B., Bagdonaite K., Leitner E., Berg G. Improving the flavour of strawberries on a natural way. Life Sciences, Graz, Austria

Verginer M., Leitner E., Berg G. Wein-assoziierte Mikroorganismen und deren Aromastoffproduktion. Österreichische Lebensmittelchemikertage, Eisenstadt, Austria

Siegmund B., Pöllinger-Zierler B., Bagdonaite K. Verginer M., Müller H., Berg G., Leitner E. Improving the flavour of strawberries on a natural way – Sensory and instrumental-analytical investigantions. XII Weurman Flavour Research Symposium, Interlaken, Switzerland

2007 Verginer M., Leitner E., Moser G., Berg G. Microbial communities living in and on grapes and their impact on wine aroma. In Vino Scientia Analytica, Melbourne, Australia

Statutory Declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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