Janne Koskimäki

THE INTERACTION BETWEEN THE INTRACELLULAR ENDOPHYTIC BACTERIUM, METHYLOBACTERIUM EXTORQUENS DSM13060, AND SCOTS PINE (PINUS SYLVESTRIS L.)
JANNE KOSKIMÄKI

THE INTERACTION BETWEEN THE INTRACELLULAR ENDOPHYTIC BACTERIUM, METHYLOBACTERIUM EXTORQUENS DSM13060, AND SCOTS PINE (PINUS SYLVESTRIS L.)

Academic dissertation to be presented with the assent of the Doctoral Training Committee of Technology and Natural Sciences of the University of Oulu for public defence in Auditorium IT116, Linnanmäki, on 27 May 2016, at 12 noon.

UNIVERSITY OF OULU, OULU 2016

University of Oulu Graduate School; University of Oulu, Faculty of Science
University of Oulu, P.O. Box 8000, FI-90014 University of Oulu, Finland

**Abstract**

To date, plant endophytic bacteria have mainly been studied in roots of crop plants. However, shoot-associated endophytes are less diverse than root-associated ones. Hence, endophytic bacteria of plant shoots evolved different traits, than root colonizers, especially with types of host tissues infected and patterns of growth and development. This study found *Methylobacterium extorquens* colonized pine seedlings similarly to stem-colonizing rhizobia of other plants. *M. extorquens* DSM13060 was isolated from meristematic cells in shoot tip cultures of Scots pine (*Pinus sylvestris* L.). *M. extorquens* infected the plant stem through epidermis or stomatal apertures, forming infection pockets in the root and stem epidermis, or cortex. Post-infection, thread-like infection structures passed through the endoderm, invading vascular tissues. This led to systemic colonization of above and below ground-parts, observed in *in vitro* grown Scots pine.

A novel mechanism enabling development of endophyte-host symbiosis is discovered within the *M. extorquens* – Scots pine model. This mechanism involves ability of *M. extorquens* to produce polyhydroxybutyrates (PHB) to protect itself from host-induced oxidative stress during infection. Upon initial colonization on the host surface, *M. extorquens* DSM13060 consumes methanol as a carbon source, using it to biosynthesize PHB. PHB are then degraded, upon host infection, by PHB depolymerases (PhaZ) to yield methyl-esterified 3-hydroxybutyrate oligomers. These oligomers have substantial antioxidant activity towards host-induced oxidative stress, enabling the bacterium to bypass host defenses and colonize further tissues. The bacteria can also store PHBs for future protection. The capacity for PHB production and, thus, protection from oxidative stress, is discovered in a wide taxonomic range of bacteria.

This study also shows meristematic endophytes are important in growth and development of their hosts. Unlike many bacterial root endophytes, *M. extorquens* DSM13060 does not induce plant growth through hormones. However, this bacterium can colonize the interior of living host cells, where it aggregates around the nucleus of the host plant. *M. extorquens* DSM13060 genome encodes nucleomodulins, eukaryotic-like transcription factors, which may intervene in host transcription and metabolism.

**Keywords:** endophyte, intracellular, Methylobacterium, methylotrophy, oxidative stress, PhaZ depolymerase, poly-3-hydroxybutyrate, Scots pine
Koskimäki, Janne, Solunsisäisen Methylobacterium extorquens DSM13060 endofyytiin ja männyn (Pinus sylvestris L.) vuorovaikutussuhde.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Luonnontieteellinen tiedekunta
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä


Asiasanat: endofyytti, Methylobacterium, metylotrofia, männyn, oksidatiivinen stressi, PhaZ depolymeraasi, poly-3-hydroksibutyraatti, solunsisäinen
Tardius, Altius, Fortius
Acknowledgements

The present study was conducted at the Genetics and Physiology Unit (former Department of Biology), University of Oulu. I am grateful for the financial support by the Academy of Finland, the Oulu University Scholarship Foundation, the University Pharmacy Fund, and the Tauno Tönning Foundation enabling me to conduct this study. I especially wish to thank the Niemi Foundation for critical funding when the significance of the outcome of my research was not anticipated.

Conducting a comprehensive experimental setup with a variety of species and new methods has demanded years of work. I wish to express my sincere gratitude to my main supervisor, Doc. Anna Maria Pirttilä and co-supervisor Doc. Sampo Mattila. I have always received guidance and support from you, when needed. Anna Maria is most warmly thanked for introducing me to the topic of plant-microbe interactions and for showing me the importance of viewing research themes in a broader scale. Her kind personality, overly positive attitude towards research, and life in general, has compensated my characteristics and made this journey bearable through the rough patches. Thank you for providing a friendly, relaxed, and most of all innovative working environment. It has been a privilege to be part of your team.

The members of my follow-up group, Prof. Anja Hohtola, Doc. Laura Jaakola, and Doc. Kari Taulavuori, are acknowledged for their valuable comments. I wish to thank Professor Anja Hohtola for her approachable nature and willingness to kindly advice on random ideas regarding any aspects of plant physiology and morphology. Her comments have been of enormous help. In addition, her understanding and prompt measures during rapidly enclosing deadlines has been vital. I would like thank also Doc. Laura Jaakola, the supervisor of my master’s thesis. She has been the person who initiated my interest toward molecular biology and plants. Her kind guidance and expertise has set a high bar for my laboratory practices. I thank Professor Hely Häggman for fostering a warm and welcoming working environment. Her farsighted quest to regularly update the equipment and facilities to meet the requirements of high-quality research has made this study possible. I would like to thank Professor Barry Scott and Professor Stéphane Vuilleumier for reviewing my thesis and for giving valuable comments and constructive criticism. I would also like to thank Marena Kajula, Dr. Juho Hokkanen, Ari Turpeinen, Mirva Pääkkönen and Heidi Hautajärvi (Department of Chemistry, University of Oulu) for collaboration with chemical analyses. Dr. Bruce C. Campell, Dr. Jong H. Kim and the personnel of
the Plant Mycotoxin Research Unit at the United States Department of Agriculture (USDA, WRRC, Albany, CA, USA) are acknowledged for their hospitality and proficient supervision during my research visit in quest to learn yeast mutant methodology for this work. Dr. Bruce C. Campbell is also thanked for kindly revising the language of this thesis, for the authentic California experience, and just for being a great dude. I owe my gratitude also to all the other researchers for their collaboration in the original papers, including Dr. Carolin Frank, Dr. Suvi Sutela, Johanna Pohjanen, Emmi-Leena Ihanhola, Marko Suokas, Elina Hankala, Outi Halonen, Dr. Olga Podolich, and Dr. Natalia Kozyrovska.

I am also grateful for all the support that I have received from the present and past members of Plant-endophyte interactions research group: Dr. Mysore Tejesvi, Jaanika Edesi, Pirjo Riikola, Dr. Pavlo Ardanov, Anna-Kaisa Anttila, Dr. Sayed Baker, and Dr. Constantina Nasopoulou. I have enjoyed our group meetings, solving scientific problems, sharing ideas, and most of all discussing random mundane matters, fueled by coffee. At the plant biology work community, I have had the pleasure to share the lab with great people: Dr. Jaana Vuosku, Terttu Kämäräinen-Karppinen, Dr. Katja Karppinen, Dr. Soile Jokipii-Lukkari, Dr. Laura Zoratti, Dr. Giacomo Cocetta, Emmi Alakärppä, Dr. Jana Krajňáková, and Dr. Kaloian Nickolov, I have really enjoyed your company. The personnel of the Biology Department and the Botanical Gardens at the University of Oulu are acknowledged for their support during this process. Taina Uusitalo, Marja-Liisa Martimo-Halmetoja, Niilo Rankka, Matti Rauman, Soile Alatalo, are thanked for their skillful assistance, with special thanks to Tuulikki Pakonen who many times single handedly made crucial maneuvers to ensure that my experiments could continue without interruptions. The members of pseudo-scientific ‘Prostata’ – society, Jussi, Jarno, Juhani, Jalo and Jouni, with visiting artists Timo and Markku are gratefully acknowledged for their relaxing company with significant rehabilitating effects. These meetings have functioned as a rejuvenating mental base camp between the research and the everyday life. I also want to thank all my friends, including the former biology students of the University of Oulu, with whom I have always felt warmly welcome and truly enjoyed my time.

As far as I can remember, education has been highly valued in our family. This was the case also with my grandparents, who always remembered to emphasize the vast doors of opportunity it opens. I sincerely thank my family, especially my parents Vivi-Ann and Esko, and my brother Ville, for their relentless motivation and support. I have always been able to count on you to
have my back, no matter what I decide to pursue. During these past years this work has occupied the majority of my time and when I haven’t been at the university, Anna, my partner in life, has patiently put up with my absent-mindedness and semi-nocturnal lifestyle. Anna, I cannot thank you enough for holding the fort while I have been able to dwell in my scientific bubble. Thanks for being my sunshine.

April 2016

Janne J. Koskimäki
Abbreviations

2,4-D  2,4-dichlorophenoxyacetic acid
3-HB  3-hydroxybutyrate
ABA  abscisic acid
ACC  1-aminocyclopropane-1-carboxylate
ACCd  1-aminocyclopropane-1-carboxylate deaminase
AO-EB acridine orange–ethidium bromide
CFU colony-forming unit
CMC Carboxymethyl cellulose
CLSM confocal laser scanning microscopy
COGs cluster of orthologous groups of proteins
CTAB Cetyltrimethylammonium bromide
DAMPs damage-associated molecular patterns
EDTA ethylenediaminetetraacetic acid
EPS extracellular polymeric substances
ET ethylene
ETI effector-triggered immunity
GDPs Genome Directed Primers
GFP green fluorescent protein
GST glutathione-S-transferases
HGT horizontal gene transfer
HO· hydroxyl radical
HOSC HO· Scavenging Capacity assay
HPLC High Performance Liquid Chromatography
HR hypersensitive response
IAA indole-3-acetic acid
IMG Integrated Microbial Genomes
ISR Induced Systemic Resistance
JA jasmonic acid
JGI Joint Genome Institute
LPS lipopolysaccharides
M molar
MAMPs microbe-associated molecular patterns
ME-3HB 3-hydroxybutyrate methyl ester
MS Mass Spectrometry
MTI MAMP-triggered immunity
NCBI  National Center for Biotechnology Information
NGS  Next-Generation Sequencing
NMR  Nuclear Magnetic Resonance
PBS  phosphate buffered saline
PCD  programmed cell-death
PHB  polyhydroxybutyrate
PPFM  pink-pigmented facultative methylotrophs
PR  pathogenesis-related
PRRs  pattern-recognition receptors
ROS  reactive oxygen species
RT  room temperature
RT-qPCR  Real-Time quantitative Polymerase Chain Reaction
SAR  systemic acquired resistance
SOD  superoxide dismutase
T3SS  Type-III Secretion System
T4SS  Type-IV Secretion System
TF  Transcription factor
TE  Tris-EDTA
TOF  time-of-flight
UV  ultraviolet
List of original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:


**Major author contributions.**

<table>
<thead>
<tr>
<th>Role</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original idea</td>
<td>JJK</td>
<td>AMP, CF</td>
<td>AMP, SM, BCC</td>
</tr>
<tr>
<td>Study design</td>
<td>JJK</td>
<td>JJK, AMP, CF</td>
<td>JJK, AMP, SM, MK</td>
</tr>
<tr>
<td>Methods development1</td>
<td>JJK</td>
<td>JJK</td>
<td>JJK, AMP</td>
</tr>
<tr>
<td>Molecular methods2</td>
<td>JJK, E-L, JP, SS</td>
<td>JJK, E-L, OH</td>
<td>JJK, JP, E-L</td>
</tr>
<tr>
<td>Chemical analyses3</td>
<td>-</td>
<td>-</td>
<td>MK, JH, AT, SM</td>
</tr>
<tr>
<td>Microscopy</td>
<td>JJK, E-L</td>
<td>JJK, E-L</td>
<td>JJK, E-L</td>
</tr>
<tr>
<td>Bioinformatics4</td>
<td>JJK, SS</td>
<td>JJK, CF, E-L</td>
<td>JJK, AMP</td>
</tr>
<tr>
<td>Data analysis</td>
<td>JJK, AMP, SS, JP</td>
<td>JJK, CF, AMP</td>
<td>JJK, AMP, JP, MS</td>
</tr>
<tr>
<td>Manuscript figures</td>
<td>JJK</td>
<td>JJK, CF</td>
<td>JJK, JP</td>
</tr>
<tr>
<td>Manuscript preparation</td>
<td>JJK, AMP, SS, JP</td>
<td>JJK, AMP, CF</td>
<td>AMP, JJK, BCC</td>
</tr>
</tbody>
</table>

JJK: Janne J. Koskimäki  
AMP: Anna Maria Pirttilä  
AT: Ari Turpeinen  
BCC: Bruce C. Campbell  
CF: Carolin Frank  
MK: Marena Kajula  
SS: Suvi Sutela  
E-L: Emmi-Leena Ihantola  
JP: Johanna Pohjanen  
MS: Marko Suokas  
OH: Outi Halonen  
BCC: Bruce C. Campbell

1 Optimization and design of the molecular protocols for the species under study  
2 Implementation of molecular methods (nucleic acid extraction, RT-qPCR, promoter-reporter strains, tissue staining, HOSC, plant, bacterial and yeast assays)  
3 Identification, production and analysis of the chemical compounds  
4 Primer design (RT-qPCR, GDPs, constructs), sequence alignments, genomic and phylogenetic analyses
Contents

Abstract
Tiivistelmä
Acknowledgements 9
Abbreviations 13
List of original publications 15
Contents 17

1 Introduction 19
1.1 Endophytic microbes and their interaction with plant hosts .......... 19
1.2 Endophytic traits affecting plant health and growth promotion ...... 21
1.3 Plant colonization by bacterial endophytes .......................... 21
1.4 Bacterial traits required for plant colonization ......................... 23
1.5 Plant defenses against microbial invaders ............................ 25
1.6 Genomics of endophytic bacteria .................................... 27
1.7 Plant association and genomics of the genus Methylobacterium .... 27
1.8 Methanol assimilation and endogenous storage of carbon ......... 29

2 Aims of the study 33

3 Material and methods 35
3.1 Microbial strains and culture conditions (I, II, III) ................. 35
3.2 Plant material and culture conditions (I, II, III) ..................... 35
3.3 Isolation of bacterial genomic DNA and total RNA (II, III) ....... 36
3.4 Genome sequencing, assembly, annotation and analysis (II) .. 37
3.5 Confocal Laser Scanning Microscopy (I, II, III) .................... 37
3.6 Field Emission Scanning Electron Microscopy (FE-SEM) (I) .... 37
3.7 Bacterial promoter-reporter constructs (I, II, III) .................... 37
3.8 Identification of cell viability by AO-EB staining (I, II) ............ 38
3.9 Screening for cellulose hydrolysis and ACC utilization (II) ..... 38
3.10 Bioactive fractions of M. extorquens DSM13060 and viability assays on pine shoot tips (III) .............................................. 39
3.11 Co-cultures of M. extorquens DSM13060 and pine callus (III) .. 39
3.12 Identification of bioactive compounds (III) ......................... 40
3.13 HOSC (HO· Scavenging Capacity) assay (III) ..................... 41
3.14 Hydroxyl-radical induced growth arrest bioassay for yeast (III) 41
3.15 Bacterial gene expression under hydroxyl-radical stress (III) ... 42
3.16 Detection of bacterial PHB under hydroxyl-radical stress (I, III) 43
3.17 Detection of bacterial PHB in pine roots during infection (I, III) 43
3.18 Detection of iron and H$_2$O$_2$ during infection of host plant (III) .............. 44
3.19 Phylogenetic analysis (III) ........................................................................... 44
3.20 Statistical analyses (III) ............................................................................... 45
4 Results
4.1 Endophytic colonization of Scots pine by *M. extorquens*
DSM13060 (I, II) .............................................................................................. 47
4.2 Genome sequence and key characteristics (II) .............................................. 51
4.3 Bioactive compounds of *M. extorquens* DSM13060 (III) ......................... 53
4.4 Expression of genes involved with PHB processing under
hydroxyl-radical stress and host infection (III) .............................................. 55
4.5 Mobilization of bacterial PHB granules under hydroxyl-radical
stress (III) ........................................................................................................ 56
4.6 Detection of bacterial PHB and local accumulation of Fenton’s
reagents in pine tissues during endophytic colonization (I, III) .............. 56
4.7 Analysis for presence of genes involved in PHB biosynthesis
among bacterial taxa (III) ................................................................................. 57
5 Discussion
5.1 Colonization of Scots pine by *M. extorquens* DSM13060 .......................... 59
5.2 Characteristics of the *M. extorquens* DSM13060 genome ......................... 61
5.3 Intracellular host association and eukaryotic-like effectors ....................... 62
5.4 Bacterial methanol utilization and PHB synthesis during pine
colonization ........................................................................................................ 64
5.5 Induction of host defense-response during endophytic
colonization ........................................................................................................ 66
5.6 Identification of bacterial bioactive compounds ......................................... 67
5.7 Hydroxyl-radical scavenging capacity of ME-3HB oligomers ..................... 68
5.8 Intracellular PHB depolymerization in *M. extorquens*
DSM13060 .................................................................................................... 69
5.9 Bacterial PHB degradation in response to hydroxyl-radical stress .......... 70
6 Conclusions and future prospects ........................................................................ 73
References ............................................................................................................. 75
Appendices ........................................................................................................... 95
Original publications ............................................................................................. 101
1 Introduction

1.1 Endophytic microbes and their interaction with plant hosts

There has been an interaction between plants and microbes since emergence of terrestrial plants, more than 400 million years ago (Krings et al., 2007). Thus, the co-evolution of plants exposed to microbes has resulted in a typical retainment, by plants, of a vast microbiome consisting of bacteria, yeasts and fungi, in aerial and below ground tissues (Berg et al., 2015; Partida-Martínez and Heil, 2011). Root surfaces together with the surrounding soil, the rhizosphere, house the richest microbial community in terms of diversity and cell mass. Moreover, aerial plant surfaces, the phyllosphere, are colonized by epiphytic communities, competing for plant-derived exudates (Delmotte et al., 2009; Lindow and Brandl, 2003; Vorholt, 2012).

Structural and chemical barriers, such as rigid, cellulose-based cell walls, antimicrobial secondary metabolites, and reactive oxygen species (ROS), released against putative pathogens, greatly limit microbial access to the plant interior. As opposed to epiphytes, internal plant-associated microbes, endophytes (‘endo’, inside; ‘phyte’, plant), are bacteria or fungi living inside plant tissue, without eliciting symptoms of disease (Chanway, 1996; Hallmann et al., 1997; Wilson, 1995). Many endophytic species are facultative, capable of surviving as free-living microbes in environments such as soil, without having association with host plant (Hardoim et al., 2008). Obligate endophytes are a group of tightly host-bound symbionts, such as the extensively studied nitrogen-fixing Rhizobacterium species, and mycorrhizae-forming fungi that are the extreme cases of the definition. Interactions in these symbioses involve specific recognition and coordinated differentiation of both bacterial and host cells into organelle-like structures, nodular symbiosomes and arbuscules (Ivanov et al., 2012).

Endophyte, as a term, primarily defines microbes based on their presence in asymptomatic plant tissues, without characterizing the association in further detail (Wilson, 1995). In some cases, an endophytic association can transform to a pathogenic association, depending on growth stage of the microbe, host species, its stress level and overall viability (Schardl et al., 2004). In addition, single-gene mutations have been shown to result in transformation of harmless endophytes to pathogens and vice versa, indicating the potential fragility of perpetuating certain benign or mutually beneficial associations (Freeman and Rodriguez, 1993;
Redman et al., 1999; Scott et al., 2007; Tanaka et al., 2006). Thus, in theory, mutualistic and commensalistic microbes living in the plant interior, and even pathogens in a latent state, can be considered endophytes (Porras-Alfaro and Bayman, 2011). Over the past decades, several reports have described how important endophytes are to plant host viability and that microbes are present in virtually all plant species, occupying all tissues (Hardoim et al., 2015). The knowledge on endophytic lifestyle is rapidly expanding as more diverse set of plants and tissues are being studied. Endophytes provide their host plants a capacity to inhabit a wider range of environments, to endure stress, and often are absolutely essential for plant growth, defense, and development. Co-evolution between host and microbe has succeeded as selective pressures mutually benefited the counterparts to subsist, adapt, and eventually refine their interaction into a balanced state.

Current knowledge on endophytic interactions is predominated by studies performed on root-associated endophytes (Gaiero et al., 2013) in agricultural crops, or model plant species (Rosenblueth and Martínez-Romero, 2006). Alternatively, few studies have focused on characterizing the relationship between bacterial endophytes and shoot tissues of woody plant species (Germaine et al., 2004; Pirtilä et al., 2000; Pohjanen et al., 2014; Prieto et al., 2011; Rincón et al., 2005; Weyens et al., 2012). Shoot-associated endophyte communities significantly differ from their counterparts in root-associated communities by lesser diversity (Pirttilä et al., 2005), indicating that colonization of plant shoots may require different traits for endophytes and their association with the host for promotion of growth and development (Pirttilä et al., 2005; Pohjanen et al., 2014). Endophytes of shoot tips or buds are rarely studied, apart from their occurrence in tissue cultures (Carrell and Frank, 2014; Pirttilä et al., 2000; Pirttilä et al., 2005).

Research on agricultural crops and, to a lesser extent, on forest trees, supports that symbiosis between trees and bacterial endophytes is wide-spread and important to plant health and survival (Carrell and Frank, 2014; Hardoim et al., 2015). Coniferous species are the dominant gymnosperms in the boreal ecosystems of North Europe, North America and Siberia. Scots pine (Pinus sylvestris L.) is the only native pine species in Northern Europe having great ecological and economic value to the northern forest ecosystems (Matías and Jump, 2012). Characterization of plant-microbe interactions in these ecosystems has potential to provide a vast amount of new information, unobtainable in the monotonous agricultural environment, on endophytic transmission routes, host
specific adaptations, gene sets, and bioactive compounds that are the foundation for successful symbiosis.

1.2 Endophytic traits affecting plant health and growth promotion

Colonization of a host plant by beneficial microbes can prevent pathogen invasion and allow plants to direct limited resources to growth (Holland and Polacco, 1994). Many plant-associated microbes are capable of synthesizing phytohormones that affect growth and development of their host. Biosynthesis of phytohormones, together with fixation of atmospheric nitrogen (N₂), is often regarded as the main endophytic trait associated with growth promotion of the plant host (Fig. 1) (James et al., 1997; Reinhold-Hurek and Hurek, 1998b; Rosenblueth and Martínez-Romero, 2006). Production of phytohormones, auxin, gibberellins and cytokinins, by endophytic bacteria and fungi can increase root growth, aerial biomass, and affect plant morphology (Taghavi et al. 2009, Kuklinsky-Sobral et al. 2004, Tsavkelova et al. 2007, Dias et al. 2009). Also, regulation of ethylene (ET) levels via degradation of the ET precursor, 1-aminocyclopropane-1-carboxylate (ACC), by endophyte-encoded ACC deaminase (ACCd) has been shown to result in indirect positive growth effects in plant hosts during abiotic stress. Bacteria utilize the end products of ACC metabolism as carbon and nitrogen sources, which in simultaneously decreases ET levels in colonized plant tissues (Glick, 2005). The meristematic endophyte M. extorquens DSM13060 lacks the capacity for phytohormone production, but is capable of biosynthesis of plant growth-inducing adenine derivatives (Pirttilä et al., 2004). This endophyte was shown to increase lateral root formation, root length, and aboveground biomass, *in vitro*, to the same extent as ectomycorrhizal fungi (Pohjanen et al., 2014). The mechanisms behind successful plant association resulting in mutual benefits are distinctive to each plant-endophyte interaction and potentially a combination of several endophytic traits.

1.3 Plant colonization by bacterial endophytes

Many bacterial endophytes were first discovered living intercellularly in roots, where their nitrogen fixation was studied (Rosenblueth and Martínez-Romero, 2006). After germination, the root is the first plant organ exposed to soil microbiota, long before emergence of the seedling. The general view on endophyte transmission suggests bacterial endophytes initially enter the plant
from the soil through the root and later colonize intercellular spaces (Hardoim et al., 2008). This has been proposed to result in an advantage for systemic colonization and occupation of aerial plant tissues by the rhizospheric bacteria, compared to phyllospheric species (Hallmann, 2001). Another method for endophyte colonization of new plants is through vertical transmission, where seeds from infected parental plants result in infected emerging seedlings (López-López et al., 2010; Truyens et al., 2015).

Early colonization events often involve bacterial chemotaxis towards plant exudates followed by eventual recognition by the host (Bais et al., 2006; Lugtenberg and Kamilova, 2009). Developing, undifferentiated root surfaces, with wounds, cracks of lateral root emergence, and root hairs provide abundant sites for bacterial colonization (James et al., 2002; Prieto et al., 2011). Conversely, stems, leaves, and needles, with thick-walled epidermal cells and waxy cuticles, limit access of bacteria to plant exudates, exposing them to starvation, desiccation, and harmful UV-radiation (Hallmann, 2001). Entry from the phyllosphere requires several adaptive traits for bacteria in order to protect them from desiccation, nutrient deprivation and chemical plant defenses. Before entering the plant, the first line of defense, the epidermal cell wall, must be passed. This usually involves a combination of passive and active mechanisms, as bacteria can migrate via stomatal openings, hydathodes, or directly through epidermis, by secretion of cell-wall degrading enzymes (Hallmann, 2001).

Depending on the endophytic strain and host species, various routes and mechanisms for colonization have been suggested (Hardoim et al., 2015). After the initial penetration of the epidermal barrier, endophytes can remain in proximity to the entry site (Timmusk et al., 2005), or continue to invade spaces between host cells, and to establish intercellular colonies in the cortex (Gasser et al., 2011; James et al., 1994; Reinhold-Hurek and Hurek, 1998b). In vascular plants, the boundary between cortex, vascular tissues and endoderm regulates translocation of water, ions, and hormones into and out of the vascular system. The thick-walled suberized cells of the endoderm function as the final obstacle for microbial entry to vascular tissues. Traversing the endodermal barrier typically takes place through unsuberized cells at the apical or basal root zones. It is here where emerging lateral roots interrupt the continuity of the Casparian strip. Only few endophytic species have been reported to gain access to the vascular system (Compant et al., 2005; Gasser et al., 2011; Hurek et al., 1994; James et al., 1994), responsible for long-distance translocation of water, ions and low-molecular weight organic compounds, such as sugars, organic and amino acids.
Transpiration streams of xylem vessels have been proposed to be the main route to aerial tissues for competent endophytes (Compant et al., 2005; Hurek et al., 1994; James et al., 2002). The plant interior sets limitations to population densities of endophytic bacteria, generally lower than those of rhizospheric bacteria. Densities of endophytic populations are highly species-dependent and affected by a variety of host-related factors, such as genotype, developmental stage, and environmental conditions (Andreote et al., 2010; Ardanov et al., 2012; Hardoim et al., 2008).

During the early stages of rhizobium-legume symbiosis, bacteria form a specialized structure, the infection thread located inside the root-hair cell, to enter the host (Callaham and Torrey, 1981; Gage, 2002). At later stages, the infection threads reach cortex cells where bacteria differentiate into bacteroids and, together with the plant host, form specialized nitrogen-fixing nodules (Gage, 2004; Goormachtig et al., 2004). Endophytic bacteria rarely colonize plant cells intracellularly and form specific structures, similar to nodule-forming rhizobacteria. Intracellular endophytes are mainly only observed in dead and hollow plant cells, such as xylem vessels and root border cells (Compant et al., 2005; Germaine et al., 2004; Reinhold-Hurek and Hurek, 1998a). Reports of intracellular colonization of living cells by endophytic bacteria are rare. One such rare case includes native M. extorquens DSM13060, detected in Scots pine (Pinus sylvestris L.) shoot tissue using in situ hybridization (Pirttilä et al., 2000). One other possible example includes bacteria in axenic peach palm (Bactris gasipaes) (Vierira de Almeida et al., 2009).

### 1.4 Bacterial traits required for plant colonization

The majority of bacterial endophytes colonizing host plants originate from the rhizosphere or phyllosphere, thus sharing many traits with rhizospheric and epiphytic bacteria (Dong et al., 2003; Gaiero et al., 2013; Whipps, 2001). Most endophytes possess metabolic versatility allowing them to utilize various carbon compounds, such as, sugars, amino acids, and one-carbon compounds, such as methanol, to gain an advantage over other species (Fig. 1) (Chistoserdova and Lidstrom, 2013; Sy et al., 2005). In fact, plant-produced methanol provides a carbon source for numerous methylotrophic bacteria and yeasts in the phyllosphere (Chistoserdova and Lidstrom, 2013; Doty, 2013; Pirttilä et al., 2005). Inactivation of bacterial methylotrophy reduces the competitive fitness of altered, compared to wild-type, bacterial strains in epiphytic populations (Sy et
and negatively affects host-plant growth (Jourand et al. 2005). Plant emissions have been estimated to be the major source of atmospheric methanol (Fall, 1996; MacDonald and Fall, 1993). Most of the methanol is created as a toxic by-product in enzymatic modification of plant cell walls (Gout et al., 2000; Micheli, 2001). Previous reports have hypothesized that internal plant spaces could offer an even more abundant source of methanol (Nemecek-Marshall et al., 1995; Sy et al., 2005).

Although aerial plant surfaces sustain diverse microbial communities, this habitat is often harsh having broad fluctuations of abiotic conditions, lacking adequate nutrients and being a highly competitive substrate. Successful colonization of this surface requires numerous, complex traits. As a protection against desiccation, surfaces, bacteria form large aggregates (biofilms) on plant surfaces and produce extracellular polymeric substances (EPS) that maintain a hydrated layer around bacterial cells (Fig. 1) (Danhorn and Fuqua, 2007; Ramey et al., 2004). Additionally, surface polysaccharides are important in plant-microbe interactions in several systems (Breedveld et al., 1993; Cheng and Walker, 1998; Jones et al., 2008). For example, in rhizobia, bacterial surface polysaccharides are crucial for establishment of symbiosis, acting as signals to the plant host (Jones et al., 2008; Kawaharada et al., 2015). Endophytes potentially use similar strategies internally, in plant hosts. Motility and the ability to recognize plant-associated chemotactic cues, is fundamental for successful colonization by bacteria. These two factors contribute to overall bacterial competence and survival in the plant environment (Balsanelli et al., 2015; Bulgarelli et al., 2013; Buschart et al., 2012). Bacteria can detect root exudates through sensory complexes involving transmembrane chemoreceptors. These receptors direct flagella-mediated motility and colony establishment onto the root surface (Fig. 1) (Hazelbauer et al., 2008). Attachment and formation of microcolonies on root surfaces involve bacterial exopolysaccharides, adhesins, pili and secretion of effectors via several secretion systems (namely Type I-IV) (Fig. 1). Endophytes actively colonizing a host secrete enzymes (endoglucanases, exoglucanases and endopolygalacturonases) that hydrolyze plant cell-wall polymer components (Reinhold-Hurek et al., 1993). The domain of endophytes can be more accommodating to survival than the phyllosphere in that there is less competition and greater stability. However, the internal plant zone can result in endophytes to greater exposure to a wide array of natural chemical host defenses.
1.5 Plant defenses against microbial invaders

Plants do not possess a circulatory system that transports cells involved in immunogenic activity. Alternatively, they defend themselves against pathogenic microbes by relying on an innate defense of each cell, and on systemic signals like jasmonic acid (JA) and salicylic acid (SA) released by infection sites (Boller and Felix, 2009; Jones and Dangl, 2006). Nonetheless, plants are capable of launching defense responses that are highly specific with restricted self-reactivity, and that often generate a long-lasting 'memory' of the encountered pathogens. Once the plant interior has been breached, microbes encounter extracellular pattern-recognition receptors (PRRs) in the host plasma membrane that recognize microbe-associated molecular patterns (MAMPs), such as bacterial outer membrane lipopolysaccharides (LPS) or flagellin (Felix et al., 1999; Meyer et al., 2001; Millet et al., 2010). Damage-associated molecular patterns (DAMPs) are endogenous molecules (e.g. plant cell-wall fragments) generated at the infection site, and are capable of inducing defense reactions, in a similar fashion to the MAMPs (Krol et al., 2010; Zipfel and Robatzek, 2010). Signals arising from MAMPs and DAMPs activate a signaling cascade which eventually initiates the basal plant defense known as MAMP-triggered immunity (MTI). However, in cases where pathogenic and beneficial microbes have co-evolved with a host plant, the microbes developed a multitude of effector molecules. These molecules are delivered inside host cells to disrupt MTI and constrain basal defenses (Block et al., 2008; Deakin and Broughton, 2009; Kambara et al., 2009).

Beneficial microbes usually promote systemic responses called induced systemic resistance (ISR) having SAR-like characteristics (Kloepper et al., 2004; Zamioudis and Pieterse, 2012). Similar to SAR, ISR provides distal protection against a broad spectrum of pathogens. SAR and ISR provide enhanced basal plant resistance, resulting in accumulation of reactive oxygen species (ROS, also referred to as oxidative burst), cell-wall reinforcements, and induction of biosynthesis of antimicrobial compounds and pathogenesis-related (PR) proteins (Fu and Dong, 2013). The main regulator of SAR is the plant defense hormone salicylic acid. Whereas, jasmonic acid and ethylene (ET) play key roles in regulating ISR (Ahn et al., 2007; Feys and Parker, 2000). Hormone-mediated cross communication can occur between SAR and ISR, depending on the microbial species involved (Pieterse et al., 2014; Ton et al., 2002).

Production of ROS can also occur as a local response to infection by symbiotic bacteria, consistent with the concept all microbes are initially perceived
as potential pathogens by the plant-host (D'Haeze et al., 2003; Pieterse et al., 2014). Endophytic bacteria can even promote stronger and faster defense responses than pathogens, characterized by production of hydrogen peroxide (H$_2$O$_2$), and elevated activity of the pathogenesis-responsive and secondary metabolism genes (Schulz et al. 1999; Laukkanen et al. 2000, Koskimäki et al. 2009). As a countermeasure to these plant defenses, microbes rely on antioxidative products, including enzymes, catalases and superoxide dismutases (SOD), small proteins, thioredoxin and glutaredoxin, and small molecules, such as glutathione (GSH), to maintain cellular homeostasis under oxidative conditions (Cabiscol et al., 2010). Overall, innate plant immunity is regulated by a complex network of interconnected signaling-pathways, in which hormones play a central regulatory role (Pieterse et al. 2012). These interconnected pathways provide plants with a vast potential to efficiently regulate adaptive responses to diverse microbial communities that could include pathogenic, mutualistic and symbiotic species.

Fig. 1. Common traits for endophytic bacteria considered important for successful plant colonization and growth stimulation (modified from Hardoim et al., 2015).
1.6 Genomics of endophytic bacteria

Rapid advances in next-generation sequencing (NGS) have revolutionized genomic research with a continuous augmentation of genome sequence data (van Dijk et al., 2014). Comparative genomic analyses of whole-genome sequences have greatly facilitated research on the genetics of how organisms adapt to various environments. Comparison of whole-genome sequences between endophytic microbes and closely related taxa from other environments can reveal essential information for endophytic adaptation (Taghavi et al., 2009).

Among the vast number of sequenced bacterial genomes, there are several from endophytic species, including nitrogen-fixing diazotrophs Azoarcus sp. BH72 (Krause et al., 2006), Azospirillum sp. B510 (Kaneko et al., 2010), Herbaspirillum seropedicae SmR1 (Pedrosa et al., 2011), Klebsiella pneumoniae 342 (Fouts et al., 2008), Pseudomonas stutzeri A1501(Yan et al., 2008), phytohormone regulating or synthesizing species, such as Burkholderia phytofirmans PsJN (Weiharter et al., 2011) and Variovorax paradoxus S110 (Han et al., 2011), and endophytes associated with woody plant species, such as P. fluorescens PICF (Martinez-Garcia et al., 2015), Stenotrophomonas maltophilia R551-3, Enterobacter sp. 638 (Taghavi et al., 2010) and P. putida W619 (Taghavi et al., 2009). Comparative genomic analyses have defined core strategies, widespread among these species, associated with successful endophyte colonization, such as motility, chemotaxis, metabolic versatility, adhesion, ROS-detoxification, and plant-polymer degradation (Fig. 1). However, degree of colonization and niches occupied in individual host species often differ among endophytes. These differences make it difficult to determine a generalized set of traits required for adaptation to an endophytic existence.

1.7 Plant association and genomics of the genus Methylobacterium

The majority of methylotrophic bacteria belong to the genus Methylobacterium, gram-negative, obligate aerobes, often summarized as pink-pigmented facultative methylotrophs (PPFM) having a close association with plants (Lidstrom, 2006). These bacteria are highly resistant to various stressful environmental conditions, such as dehydration, freezing, ionizing radiation, UV light and elevated temperatures (Green, 1992). Methylobacterium spp. are ubiquitous in nature, found in soil, freshwater, as well as on surfaces of leaves of a wide variety of
plants (Corpe and Rheem, 1989; Gallego et al., 2005; Lidstrom and Chistoserdova, 2002). Some strains of *Methylobacterium* are endophytes and colonize intercellular spaces and vascular tissues of plants (Araujo et al., 2002; Idris et al., 2004; Prieto and Mercado-Blanco, 2008; Prieto et al., 2011). Pirttilä et al. (2000) previously identified a novel plant-endophyte association in the shoot tips of Scots pine (*Pinus sylvestris* L.). This association included bacteria of the genera *Methylobacterium* and *Pseudomonas*, and a yeast, *Rhodotorula minuta* (Pirttilä et al., 2003) inside meristematic tissues of Scots pine buds. *Methylobacterium extorquens* DSM13060 was found to be the most abundant endophytic species throughout the year. But, its particular abundance prior to bud elongation and differentiation (Pirttilä et al., 2005), suggested it may have a role in these processes. Unlike a number of epiphytic strains of *Methylobacterium* that produce plant-hormones (Ivanova et al., 2001; Koenig et al., 2002), *M. extorquens* DSM13060 does not produce any common phytohormones (Pirttilä et al., 2004). Additionally, unlike the legume nodule-inducing *M. nodulans* (Sy et al., 2001), *M. extorquens* DSM13060 does not fix N₂ (Pirttilä et al., 2000). However, this bacterium increases growth and viability of pine explants in vitro, indicating some unidentified factor was involved in the successful symbiosis of these two organisms (Pirttilä et al., 2004; Pohjanen et al., 2014).

There are currently 41 available genomic sequences of *Methylobacterium* bacteria, with several sequenced strains (total of 27) isolated from plant environments (rhizosphere, phyllosphere) or having a plant-association (Chistoserdova and Lidstrom, 2013). Many of the sequenced plant-associated *Methylobacterium* spp. are facultative plant mutualists, capable of surviving outside the plant environment, and have an average genome size of 6.5 Mb (4.9 - 8.9 Mb) (Chistoserdova and Lidstrom, 2013; Marx et al., 2012). A large genome size generally represents high phenotypic plasticity enabling bacteria to colonize a wide selection of unrelated plant hosts and habitats. Conversely, a reduction in genome-size often indicates high host-specificity (Mitter et al., 2013). Thus far, four of the sequenced *Methylobacterium* strains, *Methylobacterium* sp. L2-4 and GXF4, *M. mesophilicum* SR1.6/6, and *M. populi* BJ001, have been characterized as endophytes, originally isolated from woody plant species (Gan et al., 2012; Madhaiyan et al., 2014; Marinho Almeida et al., 2013).
1.8 Methanol assimilation and endogenous storage of carbon

Methylo- and methanotrophic bacteria are significant, on global level, due to their ability to fix the greenhouse gas, methane, in addition to many plant volatiles (Fall, 1996; Freyermuth et al., 1996; Kip et al., 2010). Methanol is one of the major organic volatiles formed in de-esterification of cell-wall pectin during active growth (Nemechek, 1995, Aalto et al., 2013). The interaction between plants and methylotrophs is mutualistic. Methanol and its subsequent oxidation products are toxic to plant cells. But, for methylotrophs, these compounds can serve as an effective source of energy. Thus, removal of cytotoxic methanol from plant tissues by these bacteria can directly provide growth benefits. In addition, many Methylobacterium species synthesize bioactive substances, phytohormones and vitamins, that stimulate plant growth and development (Ivanova et al., 2001; Koenig et al., 2002; Pirttilä et al., 2004). As the PPFM-host plant association promotes growth and simultaneous methanol production, the process is cyclically beneficial to both organisms.

The main trait enabling methylotrophic growth is methanol dehydrogenase (MDH), an enzyme responsible for oxidizing methanol to formaldehyde in the periplasmic space (Goodwin and Anthony, 1998). Formaldehyde is further oxidized to CO₂ for energy in the cytoplasm, or can be assimilated into cell biomass through the serine cycle (Chistoserdova et al., 2003). There are 15 genes involved in methanol oxidation in M. extorquens AM1 and PA1 (Chistoserdova et al., 2003; Nayak and Marx, 2014). The methanol-inducible promoter, upstream of the mxaF gene, is responsible for controlling transcription of the 14-gene cluster as a single operon (Zhang and Lidstrom, 2003). This operon includes genes encoding the large and small subunits (MxaFI) of MDH, and also genes encoding proteins involved in transport, assembly and electron transfer (Chistoserdova et al., 2003; Zhang and Lidstrom, 2003). Previous studies of methylotrophic bacteria revealed the mxaF promoter is highly active in epiphytic or nodule-bound lifestyles (Jourand et al., 2005; Sy et al., 2005). However, little is known regarding methanol fixation by endophytes in the plant interior.

Methylotrophic bacteria are capable of storing methanol-fixed carbon in high quantities as endogenous poly-3-hydroxybutyrate (PHB; 3-HB polymer) granules. The dry weight of a bacterial cell can consist of up to 90% PHB, depending on bacterial species. Since 1926, PHB has been commonly known as a carbon reserve of bacteria (Lemoigne, 1926), synthesized when nutrient status is low but a carbon source is available (Bourque et al., 1995; Khosravi-Darani et al., 2013).
Subsequently, bacteria can utilize the stored PHB as an energy source under conditions of starvation (James et al., 1999; Ratcliff et al., 2008). Generally, the ability to produce and store PHB is linked with improved survival under stress conditions, or in competitive environments (Aurass et al., 2009; Kadouri et al., 2003; Ratcliff et al., 2008; Tribelli et al., 2012; Zhao et al., 2007).

Because of its commercial importance in the manufacture of biodegradable plastics, biosynthesis of PHB has been thoroughly studied, especially involvement of phaC and phaZ genes (Jendrossek, 2009; Steinbüchel and Füchtenbusch, 1998). PHB biosynthesis is catalysed in a three-step reaction (Fig. 2a). The enzyme 3-ketothiolase (PhaA) initiates biosynthesis by coupling two acetyl-CoA molecules to form acetoacetyl-CoA, which is then converted to 3-hydroxybutyryl-CoA by acetoacetyl-CoA-reductase (PhaB). Finally, the poly-3-hydroxybutyrate synthase (PhaC) polymerizes the acyl moieties of 3-hydroxybutyryl-CoA to high-molecular weight PHB. PhaC is the key enzyme in biosynthesis of polyhydroxyalkanoates (PHAs), and in most α- and β-proteobacteria, this enzyme first forms short-chain-length (SCL) PHAs by utilizing units of three to five carbon atoms (C3–C5) as the substrate. PHB depolymerases (PhaZ) are responsible for degrading the endogenous polymer to the 3-hydroxybutyrate monomer (3-HB) and its oligomers (Fig. 2b) (Brandi et al., 1995; Doi et al., 1990). The copy number of genes involved in PHB accumulation and mobilization varies between bacterial species. But, in general, a high-copy number is associated with PHB production capacity. In the rhizosphere, a feasible capacity to store carbon is an important for survival of bacteria in contact with a plant host, providing a competitive advantage for bacterial colonization (Balsanelli et al., 2015).
Fig. 2. (a) A simplified model of pathways involved in bacterial PHB synthesis and degradation in connection to methylotrophy (Korotkova and Lidstrom, 2001; Orita et al., 2014). Arrows represent the main enzymatic steps of pathways contributing to PHB metabolism, and central intermediate compounds. Pathway enzymes corresponding to symbols: MDH, methanol dehydrogenase; PhaA, 3-ketothiolase; PhaB, NADPH-acetoacetyl-CoA reductase; PhaC, PHB synthase; PhaZ, PHB depolymerase. (b) Chemical structures of the poly-3-hydroxybutyrate and methyl-esters of the 3-hydroxybutyrate (ME-3HB) monomer, dimer, and trimer.
2 Aims of the study

The aim of the present study was to characterize the molecular basis governing the interaction between an endophyte, *Methylobacterium extorquens* DSM13060, and its host, Scots pine, based on the following: I) determine the colonization patterns of the endophyte; II) using genomics and bioinformatics, identify putative genes in the endophyte genome contributing to its successful establishment in the pine host; III) identify potential microbial factors benefitting growth and development of Scots pine.

The specific aims for the studies I-III were:

1. To detect the sites and mechanism involved in the *M. extorquens* DSM13060 host entry (I)
2. To identify potentially novel endophytic mechanisms for host colonization and apical meristematic localization (I)
3. To determine the temporal and spatial terms of the endophytic colonization of Scots pine seedlings in vitro (I).
4. To provide evidence on the requirements of endophytic life in forest trees by the means of comparative genomics (II)
5. To find essential genes for the plant-endophyte interaction in *M. extorquens* DSM13060 genome sequence (II)
6. To characterize bioactive compounds produced by *M. extorquens* DSM13060 and analyze their potential modes of action in the host-endophyte interaction (III)
3 Material and methods

3.1 Microbial strains and culture conditions (I, II, III)

The wild-type strain, *M. extorquens* DSM13060, was used in all studies. It was originally isolated from buds of mature Scots pine (*P. sylvestris* L.) trees growing in a natural stand in Oulu, Northern Finland (65°0’ N; 25°30’ E) (Pirttilä *et al.*, 2000). For isolation of genomic DNA and inoculant preparation, bacteria were routinely grown at 28°C in ammonium mineral salts (AMS) medium, supplemented with 125 mM methanol, or in M9 minimal malts medium, supplemented with 120 mM methanol and 18.5 mM sodium succinate (Sambrook and Russell, 2001). For production of bioactive compounds, culture conditions for *M. extorquens* DSM13060 were tested in various media, namely in DM1, LB, AMS, M9, supplemented with methanol and, or sodium succinate (see study III for detailed compositions). For some experiments, cultures were grown under conditions of carbon or nitrogen deficiency, in order to simulate starvation, or under oxidative stress, using H2O2 or Fenton’s reagents, added to the medium after 72 hours of culture. Growth conditions and media for culturing *Methyllobacterium* sp. IMBG290 and *Rhizobacterium leguminosarum* bv. *trifolii* are described in study III.

Derivate strains of *M. extorquens* DSM13060, tagged with fluorescent reporter constructs (Appendix 1), were used as an inoculant for colonization and gene expression studies performed with Scots pine. Bacterial cultures in late-logarithmic phase were diluted to 2.5 x 10^7 colony forming units (CFU/mL) in sterile water and transferred by pipette onto germinating pine seeds for inoculation. Yeast strains (*Saccharomyces cerevisiae*, strain BY4741; Appendix 2) were streaked from glycerol stocks onto YEPD agar plates and grown at 30°C. The Pre-cultures for yeast bioassays were initiated from plate cultures, by inoculation into liquid SG minimal medium at 30°C and cultured until reaching stationary phase. Yeast growth was monitored spectrophotometrically at 600 nm (OD600) and cell densities adjusted to 0.25 OD600 for each assay reaction.

3.2 Plant material and culture conditions (I, II, III)

Scots pine seed material used in the studies was collected from the Pudasjärvi region, Northern Finland (65°05’N – 65°28’N, 26°09’E – 27°43’E). For each
experiment, seeds were heat treated for 72 h at 55°C, incubated in sterile water at room temperature overnight, surface sterilized with 3% calcium hypochlorite for 15 min, carefully rinsed three times with sterile water and sown in moist vermiculite in sterile glass jars. Individual seedlings were germinated for 5 days in growth chambers under a photoperiod of 16/8 h (light/dark) and at 25±1°C, and inoculated with *M. extorquens* DSM13060 by pipette using a bacterial inoculum. Seedlings continued to grow in the vermiculite up to 5 months. Plant samples were collected twice a week for the first month and once a week for the second and third months (I, II, III). Then, sampling continued twice a month, until seedlings were 5 months old. For the colonization experiment (I), seedlings were transferred after the 5 month growth period to pre-fertilized nursery peat (Finnpeat, Kekkilä) in 10 liter sterile growth chambers, for the final sampling of 24 months.

### 3.3 Isolation of bacterial genomic DNA and total RNA (II, III)

Bacteria cells in late logarithmic phase were collected by centrifugation and ground in liquid nitrogen with a mortar and pestle. Genomic DNA was isolated, according to protocols outlined by the Joint Genome Institute (JGI) standard for bacterial DNA isolation, by using CTAB (Wilson, 2001) with minor modifications (II). The quantity and quality of DNA was assessed by electrophoresis in agarose gel against molecular weight standards to meet JGI specifications.

Total bacterial RNA was extracted according to Chomczynski and Sacchi (2006) with minor modifications (III). Each sample of bacterial culture was mixed with 0.1 volume of ice-cold stop solution (5% phenol in ethanol) and cells collected by centrifugation at 4°C. Cells were mixed with 10 volumes of (v/v) RNAlater reagent (Thermo Fisher Scientific) and lysed using modified TE-buffer (10 mM Tris-HCl, 10 mM EDTA, pH 7.5) with lysozyme (10 mg/µl) and incubation at 4°C for 30 minutes. The guanidinium thiocyanate–phenol–chloroform isolation protocol (Chomczynski and Sacchi, 2006) was utilized for extraction of total RNA in combination with DNA-free Kit (Thermo Fisher Scientific), according to the manufacturer’s instructions. RNA concentrations were determined by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Quality and integrity of total RNA was routinely assessed by agarose gel electrophoresis (Aranda *et al.*, 2012) or with RNA 6000 Nano LabChip on Agilent 2100 Bioanalyzer (Agilent Technologies)
3.4 Genome sequencing, assembly, annotation and analysis (II)

The genome draft of *M. extorquens* DSM13060 was completed at the U.S. Department of Energy, JGI-Oak Ridge National Laboratory (Oak Ridge, Tennessee, USA) using a combination of Illumina (Bennett, 2004) and 454 technologies (Margulies et al., 2005). All general features of library construction and sequencing performed at JGI can be found at https://img.jgi.doe.gov/ under IMG genome ID: 2507262015. The whole genome shotgun sequences have been deposited in GenBank, accession number: AGJK00000000. Details regarding genome assembly, gap bridging, error correction, annotation and analysis are described in more detail in study II.

3.5 Confocal Laser Scanning Microscopy (I, II, III)

Scots pine seeds were inoculated and seedlings were processed for confocal laser scanning microscopy. Detailed procedures are described in studies II and III.

3.6 Field Emission Scanning Electron Microscopy (FE-SEM) (I)

Pine seedlings were grown, prepared and inoculated with *M. extorquens* DSM13060 as described above. Samples were fixed under vacuum at 4°C in 4% paraformaldehyde and 0.1% glutaraldehyde, in 0.1 M sodium buffer pH 7.4 for 9 h, rinsed with PBS and dehydrated with a series of increasing alcohol concentrations (30–70%). Samples were exposed to critical-point drying with carbon dioxide in a BAL-TEC CPD 030 critical point dryer for scanning electron microscopic (SEM) studies, and sputter-coated with platinum, for imaging by field-emission scanning electron microscope (FE-SEM, Carl Zeiss), at an accelerating voltage of 5 kV.

3.7 Bacterial promoter-reporter constructs (I, II, III)

Bacterial strains and plasmids used in this study are described in Appendix 1. Promoter regions of putative ACC deaminase (*acdS*), cobalamin synthase (*cobS*), bacteriophytochrome (*bphP*), PHB synthase (*phaC*), PHB depolymerases (*phaZ1* and *phaZ2*) and methanol dehydrogenase (*mxaF*) genes were amplified with primers (I, Supplementary Table 1; II, Supplemental Table S2; III, Supplementary Table 2) containing sites for restriction enzymes (FastDigest; Thermo Fisher
Scientific). The mCherry gene was amplified from plasmid pMP7604 with primers including restriction sites to create compatible ends between promoter fragments and mCherry gene in pJET 1.2 plasmid (Thermo Fisher Scientific). The resulting insert was cloned into pME6031 to create plasmids pMExt054 (acdlS), pMExt870 (cobS), pMExt599 (bphP) and pMExt801 (phaC). For phaZ1, phaZ2 and mxaF reporter constructs, the promoter region and the mCherry gene were fused using overlap extension PCR. After digestion, the inserts were ligated into pME6031 to create plasmids pMExt730 (phaZ1), pMExt589 (phaZ2) and pMExt671 (mxaF). Validity of all promoter-reporter inserts was verified by sequencing before respective plasmids were electroporated into competent cells of M. extorquens str. 13061 (Pohjanen et al., 2014).

3.8 Identification of cell viability by AO-EB staining (I, II)

Roots and lower stems of M. extorquens-inoculated pine seedlings were sectioned as described in study II. After washing with PBS, tissues were treated with acridine orange–ethidium bromide (AO-EB) in PBS solution (25µg/mL each) for 15 min in the dark at room temperature (RT). Uninoculated pine root sections were used as controls, negative controls were treated with 20 mM H₂O₂ and positive controls were incubated in PBS for 2 hours prior to AO-EB staining (Byczkowska et al., 2013; Renvoize et al., 1998). Samples were then rinsed three times with PBS. Fixation, cryosectioning and imaging with CLSM were performed as described in study II, using appropriate excitation and emission wavelengths (488 nm/BP 505-530 nm for AO; 514 nm/BP 560-615 nm for EB; 488 nm/LP 650 nm for plant cell autofluorescence). Exposure and gain settings in the confocal laser scanning microscope were kept equivalent for all samples for consistent results.

3.9 Screening for cellulose hydrolysis and ACC utilization (II)

Ability of M. extorquens DSM13060 to hydrolyse cellulose was analyzed according to Kasana et al. (2008). Exponential phase M. extorquens DSM13060 cultures were transferred by pipette to the center of carboxymethyl cellulose (CMC, Sigma-Aldrich) agar plates and incubated at 28°C for 10 days. Appearance of a halo zone around the central colony was considered as positive indication of production of cellulose hydrolyzing enzymes. Activity of putative ACC deaminase was assayed according to Poonguzhali et al. (2006). Briefly, M.
M. extorquens DSM13060 was streaked onto AMS agar plates containing 1-aminocyclopropane-1-carboxylic acid (ACC, Sigma-Aldrich) as a replacement for NH₄Cl, as the nitrogen source. Plates were incubated at 28°C for 10 days and M. extorquens DSM13060 growth was compared to controls.

3.10 Bioactive fractions of M. extorquens DSM13060 and viability assays on pine shoot tips (III)

M. extorquens DSM13060 was cultured to stationary phase in DM1 medium, and bacterial cells were removed by centrifugation, as before (Pirttilä et al., 2004). The conditioned medium with bacterial products was freeze-dried (Heto PowerDry LL-1500, Thermo Fisher Scientific), suspended in sterile water, and fractionated (mono- to decamers of methyl-esterified 3-hydroxybutyrate) by flash chromatography (III, Supplementary fig. 2). Fractions were filter-sterilized through 0.2 µm filters (Schleicher & Schuell), respective filtrates added to 20 ml pre-cooled DM1 medium with 0.25% (w/v) Phytagel (Sigma-Aldrich) and 10µg/ml tetracycline (Sigma-Aldrich), and the mixtures poured into individual Petri plates. DM1 medium was used as the control. Buds of Scots pine were surface-sterilized as before (Pirttilä et al., 2004), peeled aseptically, placed onto the Petri plates containing the fractions and control (9-13 buds per plate) and grown in 16/8 h photoperiod at 25±1°C. Viability, assessed visually, was recorded after three weeks as the viability percentage (number of live buds / total number of buds × 100%). The screening experiment was repeated, except the fractions with ME-3HB dimers and trimers were collected and separated into individual fractions (III, Supplementary Fig. 2). The compounds present in the conditioned medium and in the bioactive fractions were analyzed by LC-MS and NMR, as described below (3.12 Identification of bioactive compounds).

3.11 Co-cultures of M. extorquens DSM13060 and pine callus (III)

Pine suspension cultures were initiated using 0.1 g of non-embryogenic callus tissue collected from surface-sterilized shoot tips, as described by Pirttilä et al. (2004). Co-cultures of pine and bacteria were produced using the following steps: (1) The suspension cultures were grown in D1 medium (Pirttilä et al., 2004) supplemented with 0.4 µM benzyl adenine and 9 µM 2,4-dichlorophenoxycetic acid by shaking at 120 rpm at 25±1°C in a 16/8 h photoperiod; (2) The pine cultures were inoculated with M. extorquens DSM13060 and the co-cultures were
grown for three days, after which cells were removed from the medium by centrifugation and sterile filtration; (3) The resulting cell-free, bacteria-primed suspension culture medium was inoculated for a second time with an *M. extorquens* DSM13060 late-log phase culture, as described above. These cultures were grown for seven days, sterile-filtered freeze-dried, and analyzed by LC-MS against the cell-free, bacteria-primed medium as the control.

3.12 Identification of bioactive compounds (III)

*Identification of methyl-esterified 3-hydroxybutyrate (ME-3HB) oligomers from culture media by mass spectrometry and NMR*

Mass spectrometric analysis was performed with an LCT time-of-flight mass spectrometer (TOF-MS, Micromass) equipped with an electrospray ionization source. Retention times and spectra were compared with those of the 3-HBA monomer (racemic DL-3-hydroxybutyric acid, Sigma-Aldrich). Quattro II triple quadrupole MS (Micromass) was used for MS/MS analysis. The NMR experiments were carried out at RT on a 500-MHz Bruker DRX spectrometer. Samples were prepared by dissolving the freeze-dried preparative HPLC fractions in CDC13.

*Analysis of ME-3HB oligomer biosynthesis by *M. extorquens* using 13C label*

*M. extorquens* DSM13060 was grown in DM1 medium supplemented with 1% (v/v) methanol 13C (Sigma-Aldrich). A culture with unlabelled methanol was grown as a control. Bacterial cells were harvested by centrifugation, and HPLC-(TOF)MS analysis was performed to compare chromatograms and spectra of the labelled and unlabelled media.

*Preparation of ME-3HB oligomers for antioxidant assays*

Methyl-esterified 3-HB di- and trimers were prepared, for use in antioxidant assays, from poly-(R)-3-hydroxybutyric acid (PHB, Sigma-Aldrich) by degradation. PHB was depolymerized according to Athlan *et al.* (1997) with minor modifications, fractionated, and purified by preparative HPLC. Oligomers
were identified by TOF-MS by direct injection, after each step, as described above. The ME-3HB di- and trimer were quantified by PULCON method (Wider and Dreier, 2006) on a 500 MHz Bruker DRX NMR spectrometer equipped with a 5 mm TXI-probe at 298 K. Samples and control of (-)-epicatechin (Sigma-Aldrich) were diluted in d4-MeOH for NMR analysis.

3.13 HOSC (HO• Scavenging Capacity) assay (II)

Assay reactions were prepared according to Moore et al. (2006) and analyzed with a Victor3 multilabel plate reader (Perkin-Elmer). All reagents were purchased from Sigma-Aldrich. Trolox standards, control solutions of L-glutathione, L-ascorbic acid, 3-HB monomer (3-HBA), and our synthesized ME-3HB oligomer samples were freshly prepared in 50% acetone for each assay. Each reaction was transferred by pipette, in triplicate, to 96-well plates. Fluorescence was measured at excitation of 485 nm and emission of 535 nm. Each well was measured for 0.1 s, and each plate was scanned once per minute for 3 h. Based on the area under the curve (AUC) of the Trolox concentrations and the net area under the fluorescein decay-curve the relative HOSC values were calculated by using the regression equation for the pure compounds (Moore et al., 2006; Ou et al., 2001). Values were expressed as Trolox equivalents (TE), where one micromole of TE corresponds to one micromole of sample.

Iron-catalyzed hydroxyl radical formation by Haber–Weiss driven Fenton reaction (Kehrer, 2000; Liochev, 1999):

\[ \text{Fe}^{3+} + \text{O}_2^- \rightarrow \text{Fe}^{2+} + \text{O}_2 \]  

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^- + \text{HO}• \text{ (Fenton reaction) } \]  

The net reaction:

\[ \text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{HO}^- + \text{HO}• \]

3.14 Hydroxyl-radical induced growth arrest bioassay for yeast (III)

Bioassays of yeast deletion-mutants were adjusted from original protocols (Kim et al., 2005) for yeast cells in phosphate-buffered SG medium to produce a sub-lethal level of constant flux of HO• radicals. Hydroxyl radicals were generated
under physiological pH (7.4) in a Fenton-like Fe$^{3+}$/H$_2$O$_2$ reaction. Hydroxyl radical stress was optimized to a concentration of 2.5 mM, and the reaction was supplemented with appropriate amounts of FeCl$_3$ (0.0646 mM). L-glutathione (reduced, Sigma-Aldrich) was used as the positive control. The 3-HB monomer (3-HBA) (Sigma-Aldrich) was tested in several concentrations for screening of yeast strains sensitive to HO·. The assay reactions were incubated at 30°C in an orbital shaker (195 rpm, 6 h). Five, 10-fold serial dilutions, resulting in six individual dilutions of 1×10$^6$ to 10 cells per sample, were prepared in sterile 96-well microtiter plates. These samples where then spotted, using a multi-channel pipette, onto SG agar plates, resulting in six individual, 4-µl cell-dilution spots per treatment. All treatments using the yeast cell dilution bioassays were performed in triplicate, and all bioassays were repeated three times, producing a total of nine observed bioassays per treatment. Cell growth was monitored at 30°C for 72 hours and Petri plates were scanned digitally after 48 and 72 hours.

3.15 Bacterial gene expression under hydroxyl-radical stress (III)

To generate HO· stress in cultures of *M. extorquens* DSM13060, a modified M9 medium (M9HO·) was used. The medium was supplemented with 250 mM methanol and 10 mM sodium succinate. Bacteria were cultured in M9 medium to mid-log phase. Test cultures, containing M9HO· medium, were inoculated with *M. extorquens* DSM13060 and grown by shaking at 160 rpm for 60 h at 28°C. Test cultures were then treated by adding the Fenton-like reagents (H$_2$O$_2$ and FeCl$_3$). Samples were taken before adding the Fenton-like reagents (0-hour), and subsequently after 30 min, 1 h, 3 h, 6 h, 12 h, 24 h and 72 h. Control cultures, grown without HO· stress, were sampled in parallel. Total RNA was isolated and reverse transcribed to cDNA using Superscript III First-Strand Synthesis System (Thermo Fisher Scientific) with genome-directed primers (III, Supplementary Table 3), instead of random hexamer primers. Genome-directed primers were designed specifically for the *M. extorquens* DSM13060 transcriptome according to Talaat *et al.* (2000). RT-qPCR primers (III, Supplementary Table 2) were designed for DNA recombinase A (*recA*), methanol dehydrogenase (*mxaF*), PHB synthase (*phaC*), and three intracellular and two putative extracellular PHB depolymerases (*phaZ1-5*) identified in the *M. extorquens* DSM13060 genomic sequence. Genes lacking expression were not monitored further. The RT-qPCR was performed by LightCycler 480 (Roche Diagnostics) and described in more detail in Study III. Relative expression levels were normalized to 16S rRNA and
*Ffh* (signal recognition particle) reference genes and results were calculated with LightCycler 480 software (release 1.5.0 SP3, Roche Diagnostics) using calibrator-normalized relative quantification with efficiency correction.

### 3.16 Detection of bacterial PHB under hydroxyl-radical stress (I, III)

HO· stress were mainly performed as described for RT-qPCR gene expression analysis. Bacteria were cultured in a total volume of 200 ml of M9HO· medium and 10 ml samples were taken prior to adding Fenton's reagents (0-hour), then, after 30 min, 1 h, 2 h, 3 h, and 6 h. Controls included cultures without Fenton’s reagents grown and sampled in parallel with treated cultures. Cells were centrifuged, mixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) and fixed at 4°C for 12 h. After washing with PBS, cells were stained according to Ostle and Holt (1982) using 1% (w/v) aqueous Nile blue A solution at 55°C for 10 min. Cells were de-stained with PBS and mounted on microscope slides and covered with a thin film of 2% (w/v) agar. Then, cells of *M. extorquens* DSM13060 were studied by CLSM. All images were acquired with identical exposure and gain settings in the CLSM for maintaining comparability between samples and analyzed by Zeiss ZEN lite 2012 software (Blue edition, Carl Zeiss). Intensity of PHB granule fluorescence was measured with ImageJ/Fiji image analysis software and the corrected total PHB fluorescence was calculated from each image as the integrated density − (area of selected PHB granule × mean fluorescence of background reading).

### 3.17 Detection of bacterial PHB in pine roots during infection (I, III)

Sixty days after inoculation, roots of pine seedlings inoculated with *M. extorquens* DSM13060, as well as control seedlings, were cut into 3-mm segments and fixed with 4% paraformaldehyde (w/v), 0.1% glutaraldehyde (v/v), 20% glycerol (v/v) and 0.1 M Na-phosphate buffer (pH 7.4) for 4 hours under vacuum at 4°C. Root tissues were stained with Nile blue A, as described above. After washing with PBS, samples were cut into 25-35 µm sections with a cryomicrotome and mounted on microscope slides with 0.1 M Na-phosphate buffer (pH 7.4), 10% glycerol (v/v). Tissues were studied using CLSM using Plan-Neofluor 40x/1.3 oil objective. Nile blue A-stained PHB granules were excited at 514 nm with an argon ion laser and emission was detected through a 560-to-615-nm band-pass (BP) filter. Endogenous autofluorescence resulting from plant cell walls after Nile
blue A staining was exited at 633 nm by HeNe laser and detected with a 650-nm long-pass (LP) filter, to illustrate the outline of root morphology. For multichannel images of Nile blue A and root autofluorescence, an HFT (HauptFarbTeiler) 488/543/633-nm beam splitter was used with a secondary NFT (Neben-FarbTeiler) 635 dichromic mirror to discriminate between emissions. For consistent results, laser power and channel settings were kept equivalent throughout the experiment. Projections of both channels were processed and analyzed with ZEN lite software (Carl Zeiss).

3.18 Detection of iron and H$_2$O$_2$ during infection of host plant (III)

Seedlings of Scots pine were inoculated with *M. extorquens* DSM13060 as described in study III. Sixty days after inoculation, roots of controls and *M. extorquens*-inoculated seedlings were cut into 3-mm segments and fixed with Metacarn for 15 h at 4°C. Pine roots were double stained for H$_2$O$_2$ and iron species (Fe$^{2+}$ or Fe$^{3+}$) as described by Smith *et al.* (1997) and Thordal-Christensen *et al.* (1997). Samples were incubated in 7% potassium ferrocyanide for 24 h for detection of Fe$^{3+}$, and in 7% potassium ferricyanide in an aqueous 3% hydrochloric acid for 48 h at RT for detection of Fe$^{2+}$. Root samples were then incubated in 1 mg/mL 3,3'-diaminobenzidine (DAB, pH 3.8) for 75 min to detect H$_2$O$_2$. After staining, root tissues were cut into 20-25 µm sections using a cryomicrotome and mounted on microscope slides, as described above. Samples were studied by light microscopy (LSM 5 Pascal) using Plan-Neofluor 40x/1.3 and Plan-Apochromat 63x/1.4 oil objectives according to Liu *et al.* (2007) and Mucha *et al.* (2012).

3.19 Phylogenetic analysis (III)

Homologues of *M. extorquens* DSM13060 genes phaC, phaZ, and mxaF were studied in genomes available through the JGI database (Markowitz *et al.*, 2014) using programs Find Gene, IMG Genome Blast (E-value<1e-5, identity>30%), and BLAST, available at IMG/ER (https://img.jgi.doe.gov/cgi-bin/er). A phylogenetic analysis of representative bacteria carrying these genes was performed using the Distance Tree program of IMG/ER. Trees were generated by IMG/ER based on alignment of 16S rRNA genes available from the SILVA database and using dnadist and neighbor tools of the PHYLIP package (Felsenstein, 1989).
3.20 Statistical analyses (III)

Data were analyzed using SPSS v.16.0 (SPSS Inc.). Equality of means was tested with Welch’s ANOVA, and pairwise comparisons were analyzed using the Mann-Whitney U-test. To correct false discovery rates, the Benjamini & Hochberg procedure (FDR correction) was used with a cut-off value of 0.05 (type I error) when multiple comparisons were made (Benjamini and Hochberg, 1995; Verhoeven et al., 2005).
4 Results

4.1 Endophytic colonization of Scots pine by *M. extorquens* DSM13060 (I, II)

A fluorescent GFP-reporter strain, *M. extorquens* 13061, was used for studying spatio-temporal dynamics of colonization of the host plant by *M. extorquens* (Study I). In nature, bacteria of *M. extorquens* colonizing Scots pine most likely come from the rhizosphere. Using the GFP-reporter strain, it was observed that during the first week after inoculation, bacterial cells and small colonies were detected on root surfaces. After colonization progressed further, bacteria formed infection pockets, 7-14 days post-inoculation (dpi), within cylindrical sheaths, covering the primary root (Fig. 3; I, Fig. 1a). Similarly, infection pockets were observed in the root cap 40-60 dpi (I, Fig. 1d-f). Bacteria colonization progressed further on root surfaces, in the cells of cylindrical sheaths, and root epiderm 50-80 dpi, by penetrating directly through plant cell walls and then invading adjacent cells of the same tissue vertically (Fig. 3; I, Fig. 1h-j). At this point in time, individual bacterial cells were regularly detected inside xylem vessels (I, Supplementary Video S4). At ≥80 dpi, *M. extorquens* formed a thick structure, resembling a biofilm, between cylindrical sheaths and epiderm in primary roots, (Fig. 3; I, Fig. 1l). Internal plant invasion continued by radiating infection of adjacent cells in the cortex until the bacteria started horizontal spread, past endoderm towards the xylem, during the first weeks of colonization (Fig. 3). In addition to gradual invasion of cortical tissues, *M. extorquens* DSM13060 formed thread-like infection structures, arising from infection pockets, intersecting within the cortex and endoderm (Fig. 3; I, Fig. 4a, b) and reaching into vascular tissues. Intracellular bacteria were often observed inside parenchymal cells (I, Fig. 4c), and from 90 dpi onwards, dividing bacteria were observed in parenchymal cells of the xylem (I, Fig. 4d, e).

The cylindrical sheath played a central role in vertical colonization (I, Supplementary Fig. S2). The sheath extends from the root to the transition zone of the lower part of the stem, and presents a morphology of elongated, randomized cells in diverse orientations. Such a tissue structure provided numerous gaps and encasements for bacterial colonization. Formation of infection pockets was especially abundant in belowground parts of the cylindrical sheath, visible in cross-sections of the transition zone (I, Supplementary Fig. S2e).
Colonization of the transition zone initially progressed vertically, but when the number of infection pockets significantly increased in the cylindrical sheath, *M. extorquens* DSM13060 directed its growth deeper into host tissues (I, Fig. 1j; Supplementary Fig. S2f). Compared to other plant parts (root, stem, and needles), endophytic bacteria were most abundant in the transition zone at 60 dpi and throughout the study (I, Supplementary Table S3).

Similar to roots, bacteria actively penetrated the epiderm of the stem and formed infection pockets in the outermost tissues. Endophytic colonization of the upper stem was clearly slower than in the lower stem, or roots, occurring after 80 dpi. *M. extorquens* DSM13060 formed round-shaped microcolonies on the smooth stem surface ≥ 40 dpi (I, Fig. 2g, h; I, Fig. 3a). Eventually, colonies grew into thick microcolonies, forming a dense, raft-like bacterial mat at sites of penetration (I, Fig. 3b). Morphology of colonies on the upper stem surfaces differed clearly from previously observed bacterial colonies in the roots. Based on GFP fluorescence, these colonies contained a large portion of dead or metabolically inactive cells. After penetration of the epidermal layer (I, Fig. 3c), bacteria formed infection pockets in the invaded cells (I, Fig. 3d), and continued vertically to colonize neighboring cells. Similar to roots, formation of infection pockets and infection thread-like structures were regularly detected during the invasion process of the stem, and colonization progressed first mainly in a vertical direction (I, Fig. 3g) and then horizontally towards vascular tissues (I, Fig. 3h, i). Multicellular bacterial aggregates were observed in stomatal apertures of the stem (I, Fig. 3e), and later, larger colonies were observed in stomatal airspaces (I, Fig. 3f) and in the underlying cortex (I, Fig. 3g, h), indicating stomatal openings were another route for colonization. High autofluorescence complicated detection of bacteria in the apical tissues. However, individual bacterial cells were detected in buds ≥ 90 dpi, residing near xylem vessels (I, Fig. 4g), or colonizing the cytoplasm of meristematic cells (I, Fig. 4h, i). In the final sampling at 24 months post-inoculation (mpi) (I, Supplementary Fig. S1), the majority of bacteria persistently colonized non-vascular parenchymal cells, with a similar pattern of colonization as observed in younger seedlings.

Assessment of viability of colonized pine tissues was performed using acridine orange-ethidium bromide (AO-EB) staining accompanied by examination of cell morphology. Use of double-staining of tissues, with acridine orange (AO) and ethidium bromide (EB), provides the ability to distinguish between living and dead cells, and enables the differentiation among stages in the progress of programmed cell-death (PCD) (Byczkowska et al., 2013; Renvoize et
AO emits green fluorescence when binding to double-stranded nuclear chromatin. AO stains both living and dead cells, whereas EB can only penetrate dead cells that have lost cytoplasmic membrane integrity. In these cells, incorporation of EB can be detected as red fluorescence. Cells in early-PCD present irregular nuclei having a green-yellow stain, where slightly condensed or fragmented chromatin is visible in the form of bright green patches. Cells having disrupted cytoplasmic membranes incorporate EB into the chromatin during late-PCD, and the EB stain dominates the AO stain, rendering cells an orange-red color. Similar to healthy viable cells, necrotic cells have structurally normal morphology, but their nuclei are stained a bright orange. Overall, the predominant lifestyle of *M. extorquens* DSM13060 was intracellular. During the study, symptoms of necrosis or late-stage PCD were rarely detected, demonstrating *M. extorquens* DSM13060 colonizes intact, living pine cells (I, Supplementary Fig. S5; II, Fig. 3). Intracellular colonies were most abundant in parenchymal cells of the transition zone, where bacteria were most frequently observed to aggregate near the nucleus healthy cells (II, Fig. 2d-g, Fig. 3d, e, g; Supplementary Movie S2, S3).
Fig. 3. A model of Scots pine showing stepwise colonization by *M. extorquens* 13060 from the rhizosphere, with a representation of seedling anatomy and morphologies of the tissues. (1) Bacteria initiated the colonization from the root surface by forming microcolonies on the cylindrical sheath or the epiderm at ≥ 7 dpi. (2) Penetration through epidermal cell walls was established via formation of infection pockets and biofilm-like structures between the cylindrical sheath and root epiderm (≥ 30 dpi) (dashed arrows) (≥ 50 dpi). Once endophytic, bacteria formed multicellular aggregates to colonize adjacent cells in the epiderm and outer cortex (≥ 50 dpi). Eventually this led to invasion of surrounding tissues (dashed arrows). (3) To access the vascular tissues, bacteria formed infection thread-like structures through the pine cortex and past the endoderm (arrow) (≥ 90 dpi). (4) The endophytic lifestyle was frequently characterized by formation of intracellular aggregates. Bacteria proliferated in parenchymal cells around (dashed circle) xylem vessels (≥ 90 dpi-24 mpi). (5) Systemic colonization of pine was potentially enabled through the transpiration stream. After accessing xylem vessels of the roots (≥ 50 dpi), the first bacterial cells accessed apical tissues by 90 dpi. Cs, cylindrical sheath; Co, cortex; E, epiderm; En, endoderm; NVp, non-vascular parenchyma; Tz, transition zone; Xy, xylem vessels.
4.2 Genome sequence and key characteristics (II)

The final, assembled *M. extorquens* DSM13060 genome was approximately 6.7 Mb consisting of 12 scaffolds (240 contigs). The genome was closely related to that of *M. extorquens* AM1 (II, Supplemental Fig. S1). The draft chromosome backbone of *M. extorquens* DSM13060 (approximately 5.44 Mb) was almost entirely composed of gene orthologs found in other *Methylobacterium* genomes (II, Fig. 4), whereas 37% of the gene content of the megaplasmid was unique to DSM13060 and AM1 (II, Fig. 4; Supplemental Table S1). Approximately 75% of megaplasmid genes were syntenic with the AM1 megaplasmid and likely a result of horizontal gene transfer (HGT). The remaining 25% of the DSM13060 megaplasmid had additional genes homologous with other *M. extorquens* strains.

Genome analysis verified DSM13060 lacks key genes for synthesis of common plant hormones and growth-promoting volatiles. The reporter construct analysis with fluorescent strains of *M. extorquens* DSM13060 was used to evaluate significance of known growth promotion genes in the current host-endophyte interaction. The analysis showed the genome contained a homolog of the aminocyclopropane-1-carboxylate (ACC) deaminase (AcdS), which typically promotes plant growth by lowering ethylene levels in plant-associated bacteria. However, the bacterium was unable to utilize ACC as the sole nitrogen source in *in vitro* plate assays, and the reporter construct controlled by the promoter of this gene exhibited no activity (II, Supplemental Fig. S3). Bioinformatic analysis revealed the encoded protein of the AcdS homologue lacks key amino acid residues in the active center and, instead, potentially fulfills the function of the highly similar D-cysteine desulphhydrase enzyme (Appendix 3). Similarly, production of vitamin B₁₂ is considered to promote plant growth by epiphytes, and genome analysis revealed *M. extorquens* DSM13060 harbors the complete pathway for its synthesis. Bacterial B₁₂ synthesis was studied during pine colonization with a reporter gene construct for cobalamin synthase (*cobS*). This reporter gene did not demonstrate any activity inside the plant during the course of the study (II, Supplemental Fig. S3), indicating vitamin B₁₂ synthesis is not a significant mechanism promoting host growth by *M. extorquens* DSM13060. Another reporter strain controlled by bacteriophytochrome (BphP) was constructed to study bacterial light sensing during various stages of endophytic colonization. In stem-nodulating *Bradyrhizobium* strains, higher infection rates are linked with far-red light activation of photosynthesis machinery through the photosensory receptor, bacteriophytochrome (BphP). The *

bphP* gene promoter
was activated in *M. extorquens* DSM13060 during infection of roots (II, Supplemental Fig. S3A), but not shoots (II, Supplemental Fig. S3B). No activity by this promoter was detected when the bacteria colonized the inner tissues of Scots pine seedlings.

The finding of *M. extorquens* DSM13060 colonizing the vicinity of nuclei in host cells indicated the symbiont possibly modified host nuclear processes. This finding prompted the analysis of the genome for potential bacterial candidate genes encoding eukaryotic effector-like proteins. Such effectors could target host nuclear functions with novel molecular mechanisms, potentially involved in intracellular colonization and plant growth promotion. A simple chi-square test was used to identify 233 eukaryote-like Pfam domains (Bateman *et al.*, 2004) from the *M. extorquens* DSM13060 genome. As a search criterion, the domains had to be present more often than probable in eukaryotes ($P < 0.05$) and less often than expected in bacteria ($P < 0.05$), hence eukaryote-like. Genome analysis identified a set of genes having predicted eukaryote-like functions (Table 1), common as effectors in intracellular bacterial pathogens, supporting intracellular function in *M. extorquens*. These genes encode proteins with ankyrin repeats (Ank) (Table 1, protein 16), putative transcription factors, and putative host-defense silencing functions. Moreover, these proteins are potentially secreted by a Type-IV secretion system (T4SS) identified in the *M. extorquens* DSM13060 megaplasmid. Other putative factors involved in enhancement of host growth include three copies of Phospholipase A2 (PLA$_2$) (Table 1, protein 2-4), an enzyme rare in bacteria but implicated in a range of plant cellular processes (II, Fig. 5), and proteins putatively involved in gibberellin biosynthesis (Table 1, proteins 21, 22, 29-34).
Table 1. Proteins of *M. extorquens* DSM13060 with eukaryote-like domains.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Pfam name</th>
<th>Bacterial sequences in Pfam (%)</th>
<th>Proposed function in the host cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>pfam00777</td>
<td>Glyco_transf_29</td>
<td>1.6</td>
<td>Sialytransferase; host immune evasion</td>
</tr>
<tr>
<td>pfam00068</td>
<td>Phospholip_A2_1</td>
<td>2.1</td>
<td>Host growth promotion</td>
</tr>
<tr>
<td>pfam06839</td>
<td>zf-GRF</td>
<td>2.8</td>
<td>DNA binding; host transcription</td>
</tr>
<tr>
<td>pfam05686</td>
<td>Glyco_transf_90</td>
<td>4.4</td>
<td>None/Various</td>
</tr>
<tr>
<td>pfam02229</td>
<td>PC4</td>
<td>4.6</td>
<td>DNA binding; host transcription</td>
</tr>
<tr>
<td>pfam00400</td>
<td>WD40</td>
<td>4.8</td>
<td>Various (possibly host growth and development)</td>
</tr>
<tr>
<td>pfam13964</td>
<td>Kelch_6</td>
<td>4.8</td>
<td>None/Various</td>
</tr>
<tr>
<td>pfam13202</td>
<td>EF_hand_3</td>
<td>5.4</td>
<td>Signaling; cell division, cell elongation, cell differentiation, and plant defense and stress responses</td>
</tr>
<tr>
<td>pfam03098</td>
<td>An_peroxidase</td>
<td>5.8</td>
<td>Systemic resistance</td>
</tr>
<tr>
<td>pfam00069</td>
<td>Pkinase</td>
<td>6.5</td>
<td>Protein kinase; host signal transduction</td>
</tr>
<tr>
<td>pfam12796</td>
<td>Ank_2</td>
<td>7.4</td>
<td>Protein or DNA binding, possibly host transcription</td>
</tr>
<tr>
<td>pfam02201</td>
<td>SWIB</td>
<td>8.8</td>
<td>Host chromatin remodeling</td>
</tr>
<tr>
<td>pfam00782</td>
<td>DSPc</td>
<td>9.3</td>
<td>Tyrosine phosphatase; host signal transduction</td>
</tr>
<tr>
<td>pfam00450</td>
<td>Peptidase_S10</td>
<td>9.5</td>
<td>Defense</td>
</tr>
<tr>
<td>pfam14226</td>
<td>DIOX_N</td>
<td>10.4</td>
<td>Gibberellin biosynthesis</td>
</tr>
<tr>
<td>pfam11721</td>
<td>Malectin</td>
<td>10.6</td>
<td>None/Various</td>
</tr>
<tr>
<td>pfam11523</td>
<td>DUF3223</td>
<td>10.9</td>
<td>None/Various</td>
</tr>
<tr>
<td>pfam07250</td>
<td>Glyoxal_oxid_N</td>
<td>12.3</td>
<td>Lignin degradation</td>
</tr>
<tr>
<td>pfam04577</td>
<td>DUF563</td>
<td>13.1</td>
<td>None/Various</td>
</tr>
<tr>
<td>pfam00067</td>
<td>p450</td>
<td>13.7</td>
<td>Gibberellin biosynthesis</td>
</tr>
<tr>
<td>pfam12799</td>
<td>LRR_4</td>
<td>17.3</td>
<td>Host defense suppression</td>
</tr>
</tbody>
</table>

4.3 Bioactive compounds of *M. extorquens* DSM13060 (III)

When products of *M. extorquens* DSM13060 were separated into ten fractions and tested for activity on pine tissue *in vitro*, only two of the fractions showed...
activity (III, Supplementary Fig. 1). In these fractions 70-78% of bud tissues were alive compared to 30-54% of control bud samples after three weeks. Bioactive compounds in the fractions were identified as methyl-esterified di- and trimers of 3-hydroxybutyrate by LC-MS and NMR analysis (III, Fig. 1a-c). ME-3HB di- and trimers were then individually tested for their capacity to increase viability of pine buds in vitro. As a result, ME-3HB di- and trimer-treated bud explants showed 75% and 80% survival, respectively, in contrast to 34% of control. Besides M. extorquens DSM13060, di- and trimers of ME-3HB were detected in liquid cultures of Methylobacterium sp. IMBG290, an endophyte of potato, and Rhizobium leguminosarum bv. trifolii str. 8-9, a strain nodulating trifolium, and in the co-cultures of M. extorquens DSM13060 and pine cells. Methanol has previously been identified as the main carbon source for methylotrophic bacteria, such as M. extorquens, living on plant surfaces (Sy et al., 2005). Bacteria were cultured in the presence of 13C-labeled methanol to determine if methanol served as a source compound for biosynthesis of the methyl-esterified 3-HB oligomers. ME-3HB oligomers with single to all carbon atoms labeled with 13C were identified from the bacterial cultures, showing that methanol serves as a starting material in biosynthesis of the compounds (III, Fig. 1d).

Fluorometric hydroxyl-radical (HO•-) scavenging capacity (HOSC) assay was utilized to study antioxidant activities of ME-3HB oligomers (Moore et al., 2006). In the HOSC assay, scavenging capacities of ME-3HB di- and trimers, 3-hydroxybutyric acid (3-HBA), and known antioxidants, glutathione (GSH) and ascorbic acid (AA), were compared under hydroxyl-radical stress/Fenton-like reaction, against Trolox, an analog of vitamin E (III, Fig. 2b-c). Both ME-3HB di- and trimers showed 3- and 2.8-times higher HO•- scavenging activity than GSH, respectively, and > 10.9-times higher than AA. The ME-3HB oligomers had 11.3-times higher activity than 3-HBA. Integration analysis (Area Under the Curve; AUC) of HOSC assay confirmed scavenging activity of ME-3HB oligomers increased linearly according to time and concentration (III, Supplementary Fig. 2).

In the developed hydroxyl-radical growth-arrest bioassay for yeast, sensitivities of various Saccharomyces cerevisiae strains, having singular gene deletions related to yeast oxidative stress response, were evaluated under HO•-stress (III, Fig. 2d; Supplementary Fig. 3). Strains with gene deletions for glutathione biosynthesis (gsh1A and gsh2A) were hypersensitive to HO•-, which was reversed by treatment with ME-3HB di- and trimers at concentrations of 50-200 µM.
4.4 Expression of genes involved with PHB processing under hydroxyl-radical stress and host infection (III)

The genome sequence of *M. extorquens* DSM13060 was used to design primers for three putative intracellular depolymerases (i-nPHA<sub>SCL</sub>; phaZ1-3), two putative extracellular depolymerase family esterases (e-dPHAS<sub>SCL</sub>; phaZ4-5), and PHB synthase (phaC) for the real-time quantitative PCR analysis (RT-qPCR). In addition, genes responsible for methanol utilization (methanol dehydrogenase α subunit; mxaF) and DNA repair (DNA recombinase A; recA), were included in the RT-qPCR study (III, Fig. 3a; Supplementary Table 4). Expression levels of these genes were compared to that of constitutive expression of 16S rRNA and ffh signal recognition particle genes, used as reference. When stressed by HO<sup>·</sup>, there was rapid induction of the phaZ genes responsible for PHB polymer degradation. Activities of depolymerase genes phaZ1 and phaZ2 were also significantly increased at 3.0- to 1.9-fold higher, respectively, than reference genes, after an hour. Similar to the depolymerases, expression of PHB synthase phaC increased by 1.3-times, and expression of the recA gene, responsible for DNA repair, was rapidly induced by oxidative stress. Carbon was not a limiting factor for the bacteria, because oxidative stress had no effect on expression of mxaF, required for methanol fixation, and methanol was present in the culture medium.

Expression of genes involved with PHB synthesis and degradation, namely synthesis of ME-3HB oligomers, was examined in *M. extorquens* DSM13060 in vivo during infection of host pine tissues. Promoter-reporter strains of *M. extorquens* DSM13060 were constructed (III, Fig. 4a) for genes phaC, phaZ1, phaZ2 and mxaF. The promoters of phaC, phaZ1 and mxaF were clearly active during initial stages of host colonization (III, Fig. 4b-c). Promoters of phaZ1, phaC and mxaF were activated primarily during infection of outermost tissues of pine seedlings, cylindrical sheath and epiderm and outer cortex. Activity of the phaZ2 promoter was not detected during colonization experiment (data not shown). Bacteria colonizing deeper tissues, inner cortex, non-vascular parenchyma and xylem, had low, or undetectable activity of phaC, phaZ1 and mxaF promoters, (III, Supplementary Fig. 6).
4.5 Mobilization of bacterial PHB granules under hydroxyl-radical stress (III)

To confirm accumulation and mobilization of PHB, *M. extorquens* DSM13060 was subjected to HO\cdot stress under the same conditions as previously applied in the gene expression RT-qPCR study. PHB granules in bacterial cells were stained with Nile blue A and analyzed by CLSM at different time points (III, Fig. 3b). Within the 6-h exposure to HO\cdot stress, fluorescence intensity of Nile Blue A-stained PHB granules was significantly reduced in bacterial cells, especially after the 2-h time point, in relation to untreated controls. Control cultures, treated with either H\textsubscript{2}O\textsubscript{2} or iron, separately, showed no evidence of degradation of PHB (III, Supplementary Fig. 4). Intensity of fluorescent signals of PHB granules was further analyzed using ImageJ/Fiji imaging software. In the image analysis, background fluorescence, total area of the cell PHB granules (\text{\mu m\textsuperscript{2}}), and fluorescence intensity were considered when quantifying the relative PHB amounts in treated and control *M. extorquens* DSM13060 cells. The corrected total PHB fluorescence calculated from the images at each time point confirmed that PHB is significantly degraded upon hydroxyl radical stress relative to controls (III, Fig. 3b).

4.6 Detection of bacterial PHB and local accumulation of Fenton’s reagents in pine tissues during endophytic colonization (I, III)

Pine roots were stained with Nile blue A to determine the location and aggregation of bacterial PHB granules during host colonization (I, Fig. 7; III, Fig. 5). Based on the colonization pattern of GFP-tagged *M. extorquens* DSM13060 cells, at 60 dpi, there were numerous bacteria present in the tissues between cylindrical sheath and xylem of roots (III, Fig. 5a). Compared to the uninoculated control (I, Fig. 7d, e; III, Fig. 5b), fluorescent PHB granules were observed mainly in bacterial cells penetrating the cylindrical sheath and epiderm (I, Fig. 7f-i; III, Fig. 5c-f). In bacteria colonizing deeper pine tissues including, inner cortex, endoderm, and non-vascular parenchyma (III, Fig. 5a), PHB granules were rarely detected. This rarity coincides with the low or undetectable expression of *phaC* and *phaZ* genes in these deeper tissues (III, Supplementary Fig. 6).

To analyze formation of hydroxyl radicals in response to infection by *M. extorquens* DSM13060 in pine seedling tissues, the presence and distribution of Fenton reagents, H\textsubscript{2}O\textsubscript{2} and Fe\textsuperscript{2+}, along with Fe\textsuperscript{3+}, were analyzed (III, Fig. 6).
uninoculated control seedlings, Fenton reagents were weakly present and evenly dispersed within the outermost tissues (III, Fig. 6c, d), but highly concentrated and localized in the cells of the cylindrical sheath and epiderm, and in few cases in cortex, in the seedlings infected by *M. extorquens* DSM13060 (III, Fig. 6e-h). Similarly, clusters of Fe$^{3+}$ were primarily localized in epidermal or outer cortex cells of infected seedlings (III, Fig. 6i-l). The Fenton reagents and Fe$^{3+}$ were not observed in the deeper tissues, xylem and non-vascular parenchyma, of infected seedlings.

### 4.7 Analysis for presence of genes involved in PHB biosynthesis among bacterial taxa (III)

To study prevalence for a potential to produce ME-3HB oligomers, occurrence of homologous key genes involved in PHB processing and methanol utilization, namely *phaC*, *phaZ* and *mxaF*, were screened among bacterial genomes deposited in the Joint Genome Institute databases (III, Supplementary Fig. 7). Based on this analysis, the majority of bacterial species carrying these genes belong to plant-associated families, such as *Methylobacteriaceae*, *Rhizobiaceae*, *Bradyrhizobiaceae*, *Aurantimonadaceae*, *Phyllobacteriaceae*, *Xanthobacteraceae*, *Hyphomicrobiaceae*, *Rhodospirillaceae*, *Burkholderiaceae* and *Pseudomonadaceae*. Results indicated that evidence for methanol utilization was especially high in plant-associated bacteria, both beneficial and pathogenic species. Alternatively, evidence for PHB synthesis and degradation was mainly present in several families adapted to extreme environments, such as drought, high salinity, high metal concentrations, low pH, and high or low temperatures. These extremophiles consisted of genera *Acidiphilium*, *Cupriavidus*, *Glaciecola*, *Halomonas*, *Janthinobacterium*, *Magnetospirillum*, *Marinobacter*, *Oceanicola*, *Polaromonas*, *Sulfitobacter* and *Thiothrix*. In addition, PHB biosynthesis genes were present in human pathogens, interestingly in highly specialized intracellular pathogens of genera *Bordetella*, *Burkholderia*, *Legionella*, *Mycobacterium*, *Rickettsia* and *Vibrio.*
5 Discussion

5.1 Colonization of Scots pine by *M. extorquens* DSM13060

In general, our detailed knowledge of the biological interaction of bacterial endophytes and forest trees is limited (Izumi, 2011). A majority of endophyte studies have focused on the diversity of endophytic species in a specific plant host. However, few studies have even attempted detailed microscopic analysis of characteristics of endophytic colonization in a woody plant species (Germaine *et al.*, 2004; Prieto *et al.*, 2011; Rincón *et al.*, 2005; Weyens *et al.*, 2012). This shortage is partly a result of technical challenges related to detection of bacteria in plant tissue. In earlier colonization studies performed on a coniferous species, high levels of autofluorescence of plant tissues complicated detection of endophytic bacteria (Anand and Chanway, 2013; Timonen, 1995). In the current study, this problem was overcome by using a reproducible *in vitro* inoculation system in combination with a mild fixation process and cryosectioning. This combination of microtechniques produced optimal conditions for detection of bacteria carrying fluorescent reporters in Scots pine. In addition, a combination of fluorescent protein variants, with high intensity and minimal overlap of excitation and emission spectra, enabled effective fluorophore detection apart from host background autofluorescence. Combining and optimizing these methods, provided an ability to make a comprehensive analysis of colonization patterns of *M. extorquens* DSM13060 in pine tissue in the present studies (I, II, III). Thus, the series of methods outlined, here, are a practical choice for a wide-range of endophyte colonization studies of woody plants, or other species, where autofluorescent metabolites present a challenge to imaging.

Bacterial colonization initiated in the cylindrical sheath and epiderm of the host plant through formation of infection pockets. Bacteria eventually gained access to the pine interior by secretion of cell-wall hydrolyzing enzymes. The cylindrical sheath (Tillman-Sutela *et al.*, 2008), covering the epiderm of the primary root and partially the lower stem, was a central structure for colonization. The importance and involvement of the cylindrical sheath in plant-endophyte interactions appears novel and is presumably utilized by other rhizospheric microbes associated with coniferous trees. Formation of infection pockets, itself, shared characteristics with colonization of legumes by *Rhizobium* species, prior to nodule initiation (D'Haeze *et al.*, 2003; Gage, 2002). Moreover, similar to another
The colonization mechanism common to rhizobium-legume symbiosis (D’Haeze et al., 2003; Gage, 2002), *M. extorquens* DSM13060 formed infection thread-like structures during the course of endophyte establishment in the host plant. The bacteria used these structures to further infect its host, passing through the pine cortex and endoderm, and eventually accessing vascular tissues. Both of these types of rhizobia-like colonization mechanisms were detectable by *in situ* hybridization in meristematic tissues of Scots pine buds, advancing from scale primordia to further meristematic tissues (I, Supplementary Fig. S3). However, CLSM analysis with fluorescent reporter strains conclusively confirmed the capacity of *M. extorquens* DSM13060 to actively utilize these infection structures during the course of host tissue colonization.

Endophytic *Azoarcus*, *Azorhizobium*, *Acetobacter*, *Burkholderia* and *Herbaspirillum* species have been reported to primarily colonize intercellular spaces of roots (Compant et al., 2005; Hurek et al., 1994; James et al., 1994; Webster et al., 1998). Alternatively, in aerial parts of plants, these bacteria are mostly observed inside xylem vessels (James et al., 1997; Reinhold-Hurek and Hurek, 1998b). The transpiration stream of xylem vessels has previously been proposed as the main route for competent endophytes to gain access to aerial tissues (Compant et al., 2005; Hurek et al., 1994; James et al., 2002). *M. extorquens* DSM13060 was observed in xylem vessels of roots after 50 dpi, and at 90 dpi bacteria were detected in the apical meristems, likely transported by the transpiration stream. The endophytic colonization by *M. extorquens* DSM13060 is clearly different from that of other endophytes. This difference is represented by *M. extorquens* DSM13060 colonies being most abundant intracellularly in parenchymal cells of roots and stem following initial stages of colonizing plant surfaces. Colonization of pine seedlings by *M. extorquens* DSM13060 was systemic and, interestingly, bacteria were frequently detected in the host-cell cytoplasm, forming aggregates around nuclei. Based on the fairly limited scientific literature regarding plant colonization, intracellular colonization is either uncommon or understudied in bacterial endophytes (Chaintreuil et al., 2000; Cocking et al., 2006). Furthermore, aggregation of bacteria near the host nucleus has not previously been reported for beneficial plant-associated bacteria, despite the nucleus is known as a common effector target for pathogenic plant bacteria (Canonne and Rivas, 2012).
5.2 Characteristics of the *M. extorquens* DSM13060 genome

The draft genome of *M. extorquens* DSM13060 and its general features were compared with 11 previously sequenced *Methyllobacterium* genomes (Vuilleumier *et al.*, 2009; Marx *et al.*, 2012). The genome sequence of the epiphyte *M. extorquens* AM1, a model organism of methanol assimilation and carbon metabolism in methylotrophic bacteria, showed especially high homology with *M. extorquens* DSM13060 (e.g., the 16S rRNA gene sequences are 100% identical). *M. extorquens* DSM13060 carries a megaplasmid approximately the same size as found in *M. extorquens* AM1 (Vuilleumier *et al.*, 2009). Genomes of *M. extorquens* strains, described to date, have highly syntenic chromosomes and strain-specific plasmids (Vuilleumier *et al.*, 2009; Marx *et al.*, 2012). Indeed, as the strain AM1 carries three smaller plasmids (Vuilleumier *et al.*, 2009), and despite high similarities between the strains, no additional plasmids were identified in *M. extorquens* DSM13060. Mobile elements, such as plasmids and specific genomic islands, can transfer essential gene content from other bacterial species and accelerate bacterial adaptation to its environment (Ochman *et al.*, 2000; Van Elsas *et al.*, 2003). This is commonly referred to as horizontal gene transfer (HGT), which functions as an important mechanism for endophytic bacteria for acquiring essential traits for colonization of plants (Hardoim *et al.*, 2008; Ryan *et al.*, 2008). Many of the putative HGTs of *M. extorquens* DSM13060 genome are similar to proteins from plant- and soil-associated Gamma- and Betaproteobacteria. The imported functions of these HGTs can potentially contribute to endophytic success, including secretion systems, exopolysaccharide biosynthesis, catalases, polyketide synthases and glycosyltransferases (Frank, 2011; Mitter *et al.*, 2013; Monteiro *et al.*, 2012).

Large-scale genome reduction is commonly observed in endosymbiotic bacteria (Shigenobu *et al.*, 2000) and intracellular pathogens (Andersson *et al.*, 1998; Chien *et al.*, 2004) of eukaryotes. However, as an exception, there are few examples of genome reduction in plant symbionts and pathogens (Carlier and Eberl, 2012; Hartung *et al.*, 2011; Ran *et al.*, 2010). Intracellularity, together with obligate host interaction, is often associated with a small genome size due to a massive gene loss from purifying selection (Merhej *et al.*, 2009; Sakharkar *et al.*, 2004). Restriction to an intracellular environment limits the extreme environmental fluctuations commonly encountered by free-living bacteria. For bacteria, life inside a eukaryotic cell usually represents a high level of specialization in that the intracellular microbe needs only to retain the most
essential functions, which could also be complemented by host-encoded factors (McCutcheon and Moran, 2012). Despite the ability of *M. extorquens* DSM13060 to colonize the cell interior of the host, its genome is not reduced relative to other *Methylobacterium* species. On the contrary, together with the shared megaplasmids, the DSM13060 and AM1 genomes are among the largest of the *M. extorquens* strains (II, Supplemental material, Fig. S1). The large genome size, substantial amount of horizontally transferred genes, strong purifying selection, and wide distribution of functional COG categories on chromosome genes in *M. extorquens* DSM13060 indicates that it only spends part of its life cycle inside host cells. The fact *M. extorquens* DSM13060 can be cultured on artificial media and can actively colonizes its host systematically from the plant surface, shows it is not an obligate intracellular organism. The characteristics of its genome are common to a free-living bacterium with a versatile lifestyle and large population size, and further suggest that between hosts, *M. extorquens* DSM13060 spends time in the environment e.g., the soil, water, phyllosphere, and endosphere of other plant species.

### 5.3 Intracellular host association and eukaryotic-like effectors

Bacterial endophytes have been traditionally shown to enhance plant growth by increasing stress tolerance and growth via production of phytohormones. However the *M. extorquens* DSM13060 genome sequence did not contain any known genes involved in these processes. Therefore we hypothesized the genome encoded genes rarely documented or previously unknown to be involved in host growth promotion. Its unique lifestyle within host cells and its close association with the nucleus may provide an advantage for *M. extorquens* DSM13060 to directly affect host processes in the cytoplasm or nucleus. Moreover, its location in the shoot meristems, considered one of the most important tissues of the plant, responsible for growth and development of new leaves and stems (Pirttilä *et al.*, 2000), provides an opportunity for the bacterium to manipulate these host processes. The nucleus is a well-known target for animal (Agbor and McCormick, 2011; Christie and Vogel, 2000; Rennoll-Bankert *et al.*, 2015) and plant pathogens (Canonne and Rivas, 2012; Deakin and Broughton, 2009; Jiang *et al.*, 2013) as a means to manipulate host transcription through production of effectors. Type-IV secretion systems are especially common among pathogens, where they are important for enabling delivery of effector molecules and exchange genetic material to eukaryotic target cells (Christie and Vogel, 2000; Rennoll-Bankert *et al.*, 2015).
The unusual lifestyle of \textit{M. extorquens} DSM13060 triggered the search for genes encoding potential bacterial effector proteins and ‘nucleomodulins’ (Bierne and Cossart, 2012; Bierne, 2013; Nougayrède \textit{et al.}, 2005) that could target nuclear processes of the pine host. Interestingly, a T4SS was identified on the \textit{M. extorquens} DSM1360 megaplasmid scattered in four locations (II, Fig. 5; TFSS1-4). The T4SS was closely related to the \textit{Legionella dot/icm} system, and appears to have been imported together along with a set of genes associated with their replication- and recombination. Aside from \textit{M. extorquens} AM1, the other \textit{Methylobacterium} genomes, to which DSM13060 was compared, do not share the dot/icm-type T4SS.

Several of the identified proteins with eukaryote-like Pfam domains shared homology with eukaryotic transcription factors (TFs) (II, Table 1.). Two of the putative TFs were located in the \textit{M. extorquens} DSM1360 megaplasmid region adjacent to genes encoding the T4SS, suggesting the secretion system is used for the translocation of the TFs to the plant host (II, Fig. 4). The first effector consisted entirely of a SWIB domain (II, Table 1, protein #17), usually involved in chromatin remodeling in eukaryotes. SWIB domain proteins are widespread among \textit{Chlamydia} spp., where it is a separate protein or fused to a chlamydial DNA topoisomerase (Stephens \textit{et al.}, 1998). SWIB domain effectors function in remodeling chromatin condensation-decondensation during the chlamydial life cycle (Barry \textit{et al.}, 1992; Hackstadt \textit{et al.}, 1991). The second putative TF effector, located in the vicinity T4SS, had two GRF zinc finger (zf-GRF) domains (II, Table 1, protein #5). This domain is very unusual in bacteria (pfam database: 854 eukaryotic sequences and 23 bacterial sequences possess this domain). Interestingly, in \textit{M. extorquens} DSM13060 proteins and \textit{M. extorquens} AM1 orthologs, the two zf-GRF domains appear to be fused to a C-terminus of bacterial topoisomerase, thus forming an unusual domain architecture. Given the role of topoisomerases in the transcriptional activation of genes (Pedersen \textit{et al.}, 2012) and the likely association of zf-GRF in DNA binding, the putative protein may be an effector secreted by the adjacent secretion system, having a role in host transcriptional activation.

Ankyrin repeats (Ank), containing eukaryotic motifs, are common effector proteins found in many human intracellular bacterial pathogens, such as \textit{Rickettsia}, \textit{Coxiella} and \textit{Legionella} species (Al-Khodor \textit{et al.}, 2010). \textit{M. extorquens} DSM13060 encodes a putative Ank (II, Table 1, protein #16) widespread in the genus, but has no homologs in other \textit{Rhizobiales} species. Homologs of this protein are most common in bacteria that colonize eukaryotic
cells (Mosavi et al., 2004). During host infection by the *Legionella pneumophila*, ankyrin effectors are translocated by the Dot/icm TFSS and required for intracellular proliferation (Al-Khodor et al., 2010; Habyarimana et al., 2010). The Ank proteins are known to mediate protein–protein interactions in several host processes, including cell-cycle progression, cytoskeletal organization, and transcriptional regulation (Jernigan and Bordenstein, 2014; Mosavi et al., 2004). The motif containing proteins can bind directly to host chromatin in the nucleus of infected cells (Park et al., 2004a; Rennoll-Bankert et al., 2015; Zhu et al., 2009).

Among the identified putative effectors was phospholipase A2 (PLA2) (II, Table 1, proteins #2-4), an extremely rare enzyme in bacteria but implicated in a range of plant cellular processes, including growth. The PLA2 domain is abundant in the genus *Methylobacterium*, where most genomes have 2-3 copies. All three PLA2 proteins found in *M. extorquens* DSM1360 were predicted to possess a signal peptide, and therefore are likely secreted out of the cell. PLA2 homologs are enzymes that catalyze hydrolysis of membrane phospholipids, producing free fatty acids and lysophospholipids (Chen et al., 2011). These enzymes and their catalyzed products are involved in a range of cellular processes in plants, such as growth, development, stress response and defense signaling (Chapman, 1998; Chen et al., 2011; Lee et al., 2005; Polkowska-Kowalczyk et al., 2011). PLA2 enzymes are thus potential candidates for contributing to a growth-promoting effect of *M. extorquens* DSM13060 on *P. sylvestris* seedlings in situ. Given the widespread association between *Methylobacterium* spp. and plants, PLA2 domain homologs are interesting candidates for involvement in a range of interactions between *Methylobacterium* species and plants.

### 5.4 Bacterial methanol utilization and PHB synthesis during pine colonization

The association of methylotrophic bacteria (Corpe and Basile, 1982; Corpe and Rheem, 1989) with plants was recognized prior to discovery of methanol emissions from plants (MacDonald and Fall, 1993). The methanol emission discovery showed epiphytic methylotrophs could serve as a methanol sink, benefiting the host plant (Holland and Polacco, 1994). Capacity for methanol assimilation provides a competitive advantage to facultative methylotrophs during epiphytic colonization of *Medicago truncatula* despite availability of other plant-derived carbon sources (Sy et al., 2005). Experimental evidence suggests
methanol is emitted primarily through stomata in leaves (MacDonald and Fall, 1993; Nemecek-Marshall et al., 1995). This finding suggests methanol concentration should higher in the plant interior where a majority of pectin demethylation occurs. This hypothesis was later supported by detection of aggregates of methylotrophic bacteria in plant leaves to be localized in stomatal openings and, intercellularly, in sub-stomatal cavities (Sy et al., 2005). During endophytic colonization of Scots pine the mxaF promoter-reporter strain of \textit{M. extorquens} DSM13060 displayed high levels of activity on outer root tissues, an indicator of ongoing methanol assimilation. These results demonstrated methanol was available to bacteria in the cylindrical sheath, epiderm and outer cortex of pine seedlings. As a departure from that hypothesized by Sy et al. (2005), the current study suggests availability of methanol clearly declined, or was non-existent, when endophytic colonization penetrated into deeper pine tissues. Therefore, \textit{M. extorquens} DSM13060 can likely utilize alternative carbon sources, apart from methanol, for energy when colonizing deeper tissues, as hypothesized for endophytes (Podolich et al., 2015). In summary, the current results suggest, prior to shifting to endophytic lifestyle, methanol fuels the initial steps of plant invasion by bacteria. On the other hand, availability of methanol in outer tissues can favor polyhydroxybutyrate (PHB) accumulation, which could be used as an alternate energy source when other carbon sources are limited or unavailable.

Many aerobic bacteria are able to synthesize PHB as an intracellular carbon reserve (Bourque et al., 1995; Jendrossek, 2009; Ochsner et al., 2015). \textit{Methylobacterium} spp. naturally synthesize PHB from methanol. In optimized nitrogen-limited fed-batch cultures PHB production by \textit{Methylobacterium} can reach up to 149 grams per liter (64% of dry cell weight) (Bourque et al., 1995; Ochsner et al., 2015; Suzuki et al., 1986). Early in the present study, CLSM analysis in GFP-tagged \textit{M. extorquens} DSM13060 growing on plant surface revealed dark spherical inclusion bodies (no GFP fluorescence) resembling PHB granules (I, Fig. 3a). These structures were later confirmed by Nile blue A staining as bacterial PHB granules. The fluorescent PHB granules were detected mainly in outer tissues, cylindrical sheath and epiderm, during bacterial invasion of pine seedlings. In accordance with these observations, activity of \textit{mxaF} and \textit{phaC} promoter-reporter strains was localized in the same tissues. Overall, results from fluorescent-labeling analyses indicate \textit{M. extorquens} DSM13060 directs carbon flow from methanol to production of endogenous PHB granules. In rhizobia, presence and subsequent depletion of PHB in bacterial cells during plant invasion fostered the hypothesis bacteria accumulate PHB before initiating plant
invasion (Charles et al., 1997; Hirsch et al., 1983). It has been suggested rhizobia utilize PHB to support cell proliferation within the infection thread prior to establishment of nodule symbiosis (Charles et al., 1997; Trainer and Charles, 2006; Wang et al., 2007).

5.5 Induction of host defense-response during endophytic colonization

Endophytic colonization by *M. extorquens* DSM13060 in Scots pine was significantly slower in comparison to previous reports of symbionts in other plant species (Compant et al., 2005; D'Haeze et al., 2003; James et al., 2002; Sathyapriya et al., 2012). The moderate capacity for cell-wall degradation, and overall, slow progression of colonization by *M. extorquens* DMS13060 explains why invasion of pine tissues by this endophyte appears to be symptomless. It has been proposed that low levels of cell-wall degrading enzymes produced by root-colonizing bacteria differentiate endophytes from phytopathogens, which produce these enzymes often at deleteriously high levels (Elbeltagy et al., 2000; James et al., 2002). In several other reports of plant-endophyte interactions, host entry is achieved through fissures in lateral roots (James et al., 2002; Reinhold-Hurek and Hurek, 1998b). However, the current study showed colonization of pine by *M. extorquens* DSM13060 was initiated by formation of tightly packed multicellular aggregates, resembling previously described structures of rhizobial infection pockets (D'Haeze et al., 1998; Tsien et al., 1983) or symplasmata in *Pantoea* spp. (Achouak et al., 2004; Zhang et al., 2010).

As an early defense event, plants respond to invading microbes, whether beneficial or pathogenic, by accumulating various antimicrobial compounds and reactive oxygen species at the penetration site (Lamb and Dixon, 1997; Santos et al., 2001). Co-localization of ROS at high concentrations has widely been described in association with host entry and infection pocket initiation during initial stages of rhizobial-legume symbiosis (D'Haeze et al., 2003). In addition, several reports describe release of redox-active iron, from cell-wall appositions, simultaneous to systemic distribution of hydrogen peroxide, during oxidative burst, in plant cells at sites of pathogen penetration (Aznar et al., 2015; Hückelhoven and Kogel, 2003; Liu et al., 2007; Mucha et al., 2012). To assess host defense responses, pine tissues were stained to detect Fenton’s reagents (Fe$^{2+}$ and H$_2$O$_2$) along with Fe$^{3+}$, which could initiate localized accumulation of hydroxyl radicals. In the early stages of pine colonization by *M. extorquens*
DSM13060, co-localization of $\text{H}_2\text{O}_2$ with deposits of $\text{Fe}^{2+}$ and $\text{Fe}^{3+}$ were observed in root tissues invaded by the endophytic bacteria, i.e. in the cylindrical sheath, epiderm, and outer cortex. Consistent with the present study, similar responses have previously been reported in Scots pine infected by pathogenic fungi (Mucha et al., 2015; Mucha et al., 2012).

Despite iron is an essential component for all living cells, the majority of cellular iron is in the non-bioavailable ferric form ($\text{Fe}^{3+}$), bound to proteins to avoid its toxicity (Briat et al., 1995; Miethke and Marahiel, 2007; Valko et al., 2007). The redistribution of redox-active iron to the invasion sites has been shown to modulate host cellular iron homeostasis, leading to activation of defense genes and promotion of further iron efflux with hydrogen peroxide production (Liu et al., 2007; Mucha et al., 2015). From the bacterial perspective, iron is needed for growth, pathogenicity and biofilm formation. Some host strategies to limit iron availability have been well documented in clinical studies in mammalian systems (Nairz et al., 2010; Ward and Conneely, 2004). High iron concentrations have been shown to stimulate aggregation and biofilm formation, while low iron concentration induces free-living forms and motility in opportunistic human pathogens (Berlutti et al., 2005). Metals are also essential for cellular plant and animal defenses in generation of reactive oxygen species (ROS), which are directly used for limiting microbial invasion (De Gara et al., 2003; Fones and Preston, 2013; Lamb and Dixon, 1997; Lambeth, 2004). Interestingly, during the host interaction with $M. \text{extorquens}$ DSM13060, Fenton reagents and $\text{Fe}^{3+}$ were not observed in deeper pine tissues, xylem, and non-vascular parenchyma of infected seedlings. This observation potentially indicates attenuation of host defenses and reprogramming of the interaction towards symbiosis in these host tissues, typical for beneficial microbes (Shaw and Long, 2003).

5.6 Identification of bacterial bioactive compounds

LC-MS and NMR analyses showed the bioactive compounds of $M. \text{extorquens}$ DSM13060 to be methyl-esterified dimers and trimers of 3-hydroxybutyrate (ME-3HB). These compounds demonstrated a capacity to increase viability of pine buds in vitro. Moreover, the ME-3HB di- and trimers were present in the bioactive fractions of $M. \text{extorquens}$ DSM13060 liquid cultures exposed to stress, when co-cultured with plant cells, and in cultures of $\text{Methylobacterium}$ sp. IMBG290, an endophyte of potato, and $\text{Rhizobium}$ leguminosarum bv. trifolii str.
8-9, a strain nodulating trifolium. In mammalian cells, 3-HB is regarded as a common ‘ketone body’, produced in the liver as an energy source during starvation, or in human metabolic diseases, such as ketosis in diabetic patients (Klocker et al., 2013). Besides direct involvement in protection from oxidative stress (Haces et al., 2008), monomeric 3-hydroxybutyrate has recently been linked in mammalian cells with epigenetic regulation (Shimazu et al., 2013), anti-inflammatory signaling (Youm et al., 2015), mitochondrial protection (Maalouf et al., 2007) and prevention of apoptosis (Cheng et al., 2013). In addition to the diverse cytoprotective capacities observed for 3-HB, the methylated form (ME-3HB; HBME) of the monomeric compound shares similar capacities (Zhang et al., 2013). ME-3HB oligomers were produced by *M. extorquens* DSM13060 at high concentrations (200-500 µM) even in suboptimal conditions. Earlier papers reported depolymerized PHB content of some bacterial strains can reach 146 mM in optimized culture conditions (Kawata et al., 2012; Lee et al., 1999). Recently, Obruca et al., (2015) estimated that a functional PHB cycle could maintain an endogenous 3-HB (and oligomer) pool higher than 100 mM in PHB-producing bacteria, providing a protective buffer against stress.

### 5.7 Hydroxyl-radical scavenging capacity of ME-3HB oligomers

Simultaneous accumulation of ROS (namely H$_2$O$_2$ and O$_2^-$) and transition metals, such as iron, copper or zinc, potentially results in a localized cycle of production of hydroxyl radicals (HO·), through Fenton/Haber-Weiss reactions, at host infection sites (Halliwell and Gutteridge, 1999; Liochev, 1999; Smith et al., 1997; Valko et al., 2007). The hydroxyl radical is the most cytotoxic of ROS species, with no known enzymatic system for its detoxification (Halliwell and Gutteridge, 1999; Liochev, 1999; Valko et al., 2007). Instead, bacterial defenses are mainly founded on preventing formation of this radical by restricting accumulation of the Fenton/Haber-Weiss reaction constituents (Imlay, 2008; Lushchak, 2011; Valko et al., 2007). In stressful conditions, these cellular ROS defenses can be overwhelmed, resulting in oxidative damage (Cabiscol et al., 2010; Kehrer, 2000; Lushchak, 2011).

The HOSC assay (Moore et al., 2006) was utilized to assess potential hydroxyl radical scavenging activity of ME-3HB oligomers and for comparison of known antioxidants, relevant in bacterial ROS defense (Cabiscol et al., 2010; Lushchak, 2011). In the HOSC assay, ME-3HB di- and trimers showed 3- and 2.8-times higher HO· scavenging activity than glutathione (GSH), respectively.
The antioxidative activity of ascorbic acid (AA) and 3-hydroxybutyric acid (3-HBA) was found to be more than 10-times weaker than ME-3HB oligomers. The surprisingly low antioxidant activity of AA is likely explained by a pro-oxidant effect, previously observed when AA was applied in low ratio (≤ 1 mM) to metal ions (Buettner and Jurkiewicz, 1996; Yin et al., 2012). The hydroxyl group present in the 3-HB has been suggested to be the main contributor to the antioxidant capacity of the molecule (Haces et al., 2008). However, ME-3HB dimers and trimers have only one hydroxyl group, like 3-HB, yet have a significantly higher hydroxyl-radical scavenging capacity than 3-HB. Hence, there must be some other explanation for the higher activity the oligomeric forms. Thus, the cytoprotective potential of ME-3HB oligomers was tested using hydroxyl radical growth-arrest bioassays on yeast cells. This bioassay provided direct evidence the ME-3HB oligomers have a significant physiological role in protecting yeast cells from HO· stress when present at 50-200 µM. Yeast mutants deficient in GSH synthesis (gsh1Δ and gsh2Δ), displayed approximately a ten-fold higher hypersensitivity to hydroxyl radical stress when cultured in a medium not supplemented with antioxidants.

5.8 Intracellular PHB depolymerization in *M. extorquens* DSM13060

According to literature, bacterial PHB depolymerases are responsible for production of 3-HB oligomers (Kasuya et al., 1995; Wang et al., 2002). Unlike well-characterized PHB-producing strains, *Ralstonia eutropha* and *Azohydromonas lata*, PHB depolymerases (PhaZ) of plant-associated bacteria, such as *Methylobacterium* spp., have not been characterized in detail. Based on the amino acid and nucleotide sequence BLAST analysis, depolymerases phaZ1 and phaZ2 of *M. extorquens* DSM13060 showed greatest homology to other intracellular PHB depolymerases. Furthermore, sequence alignment with the intracellular PHB depolymerases of *R. eutropha* str. H16 enabled identification of putative active sites of the PhaZ1 and PhaZ2 depolymerases of *M. extorquens* sp. (Appendix 4), consistent with previously published data (York et al., 2003).

Depolymerases hydrolyze ester bonds of PHB polymers producing water-soluble free 3-hydroxybutyric acid and shorter oligomers of PHB (Jendrossek and Handrick, 2002). Structural characteristics of PHB depolymerases in different bacterial strains determine their specific enzymatic activity and ratio of different oligomeric lengths of the end products. Depending on bacterial species, enzymatic hydrolysis products are usually only monomers, monomers and
dimers, or a mixture of oligomers (Jendrossek and Handrick, 2002; Lee et al., 1999). In addition, some strains of bacteria have specific hydrolases that cleave the oligomers and dimers to monomeric end-products (Sugiyama et al., 2004). Homologs of these hydrolases were not found in the M. extorquens DSM13060 genome, which could explain the higher levels of ME-3HB di- and trimers detected in M. extorquens DSM13060. Methyl-esterification can prevent re-polymerization of 3-HB oligomers and, thus, increase the stability of these esters (Park et al., 2004b).

5.9 Bacterial PHB degradation in response to hydroxyl-radical stress

Importance of PHB accumulation to bacterial survival in carbon-limited environments is well documented (Handrick et al., 2000; James et al., 1999; Jendrossek and Handrick, 2002; López et al., 1995; Matin et al., 1979). Also the association of this polymer with bacterial stress-resistance in unfavorable ecosystems, and during plant colonization, has been reported in several studies (Aneja et al., 2005; Ayub et al., 2009; Kadouri et al., 2003; Ratcliff et al., 2008; Tal and Okon, 1985). The capacity for PHB biosynthesis is a notably typical trait for bacteria adapted to abiotic (Kadouri et al., 2003; Tribelli et al., 2012; Zhao et al., 2007) and biotic stresses (Aneja et al., 2005; Aurass et al., 2009; Kim et al., 2013).

To further confirm the link between PHB biosynthesis and depolymerization in response to host-derived stress, gene expression of M. extorquens DSM13060 PHB synthase (phaC) and depolymerases (phaZ1-phaZ5) was analyzed under hydroxyl-radical stress. The results demonstrated that under HO· stress M. extorquens DSM13060 depolymerizes endogenous PHB reserves to produce ME-3HB oligomers as a mechanism of protection against oxidative agents. With a carbon source available, the bacterium is capable of concurrent polymerization and depolymerization of PHB polymers under stress. Similar to these current findings, parallel degradation and biosynthesis of PHB has previously been reported in Pseudomonas putida and Ralstonia eutropha (Doi et al., 1990; Ren et al., 2009). However, in M. extorquens DSM13060 carbon fixation was not enhanced when oxidative stress was introduced, indicating stress tolerance resulted from PHB mobilization and antioxidant activity of ME-3HB oligomers. Under the same conditions, degradation of intracellular PHB granules in M. extorquens DSM13060 cells was detected with Nile blue A staining and confocal
microscopy. In pine tissues, high activity of \textit{phaC} and \textit{phaZ1} in corresponding promoter-reporter strains in outermost tissues of pine seedlings suggest \textit{M. extorquens} DSM13060 concurrently accumulates and depolymerizes intracellular PHB reserves. During in early stages of colonization, this concurrent activity is a putative response against the accumulation of hydroxyl radicals in host tissue (Fig. 4).

\textbf{Fig. 4.} A model of early stages of Scots pine infection by \textit{M. extorquens} DSM13060. (a) Bacteria utilize plant-produced methanol as a carbon source to accumulate endogenous polyhydroxybutyrate (PHB) granules for carbon storage (Fall, 1996; Khosravi-Darani \textit{et al.}, 2013). (b) As an initial host defense response, pine cells concentrate ferrous (Fe$^{2+}$) and ferric (Fe$^{3+}$) iron, together with systemically accumulated ROS (H$_2$O$_2$, O$_2^\cdot$), at the infection site (dashed line)(Liu \textit{et al.}, 2007; Mucha \textit{et al.}, 2012). Co-localization of redox-active iron with oxidative H$_2$O$_2$ potentially catalyzes local generation of toxic hydroxyl radicals (HO·) through the Fenton/Haber–Weiss reaction during initial stages of infection (Liochev, 1999; Pierre and Fontecave, 1999). (c) As a protective response, bacteria rapidly depolymerize PHB and produce antioxidant methyl-esterified 3-hydroxybutyrate oligomers (ME-3HB) to overcome oxidative stress associated with production of HO· at the infection site.

In accordance with these results, a transcriptome sequencing study of a grass endophyte \textit{Herbaspirillum seropedicae} sp., showed 12-16 fold induction of \textit{phaZ} gene expression at early stages of host infection (Balsanelli \textit{et al.}, 2015). In the same study, mutants incapable of PHB synthesis demonstrated 32-fold and 18-fold lower capacity for epiphytic and endophytic colonization, respectively, highlighting the importance of PHB mobilization during this particular stage. Induction of \textit{phaC} and \textit{phaZ}, has also been found under stress in \textit{Aromatoleum aromaticum}, \textit{Dinoroseobacter shibae}, \textit{Ensifer meliloti} and \textit{Pseudomonas oleovorans} (Krol and Becker, 2004; Ruiz \textit{et al.}, 2001; Trautwein \textit{et al.}, 2008; Wang \textit{et al.}, 2014).
In cold environments, increased solubility of oxygen and stability of its radicals force bacteria to adapt and cope with high levels of oxidative stress (Ayub et al., 2009; D'Amico et al., 2006; Medigue et al., 2005). Ayub et al. (2009) demonstrated phaCA deletion mutants of cold-resistant Pseudomonas extremaustralis sp. were unable to grow in conditions below 10°C. When compared to wild-type bacteria, cold-shock induced oxidative stress increased lipid peroxidation by 25-fold in the mutants. Cold-sensitivity of the mutants was reversible by addition of antioxidants, such as glutathione, to the growth media. Moreover, a cold-shock induced, rapid mobilization of intracellular PHB reserves in the wild-type, was used to overcome the oxidative stress. Similarly, Escherichia coli, normally incapable of PHB synthesis, showed an improved tolerance against various stresses when bioengineered for PHB biosynthesis and degradation (Wang et al., 2009). It has been suggested PHB functions as a sink for reducing equivalents in maintaining redox homeostasis by control of cellular NADH/NAD⁺ ratios and NADPH content (De Eugenio et al., 2010; López et al., 2015; Ren et al., 2009; Schubert et al., 1988), which does not explain the drastic antioxidant effects observed. Generally, conditions for accumulation of bacterial PHB reserves are associated with moderate stress, which can potentially act as an initial cue for arming the cell for upcoming adverse conditions. Previously, moderately elevated levels of oxidative stress (Obruca et al., 2010a; Obruca et al., 2010b) and heavy-metal stress (Kamnev et al., 2012) have been reported to increase PHB accumulation in bacterial cultures. However, after a certain threshold, decrease of PHB content could be observed in response to greater severity of the stress (Obruca et al., 2010b).
6 Conclusions and future prospects

The current study characterizes the interaction of an intracellular endophyte, *M. extorquens* DSM13060, and Scots pine. The study combines a number of diverse scientific methods used in the fields of chemistry, molecular biology, microscopy and genomics. The study shows *M. extorquens* DSM13060 is able to colonize Scots pine seedlings *in vitro*, systemically, via active mechanisms, penetrating through epidermis of roots and shoots. The cylindrical sheath, covering the epiderm of the primary pine root and partially lower stem, was discovered to be an important tissue for microbial colonization. This endophytic bacterium migrated, using rhizobia-like colonization mechanisms, efficiently to shoot meristems, thus explaining how original findings showed the presence of bacteria in shoot tips of adult trees. Despite the fact this bacterium was first observed to be mainly in the rhizosphere, 90 days after inoculation it was detectable in apical meristematic tissue, most likely transported there by the transpiration stream.

Mechanisms used by *M. extorquens* DSM13060 for colonization are novel among previously described plant-endophyte interactions. One of this study's most interesting findings with its intracellular infection of pine cells was the unusual aggregation of bacteria near the host nucleus. The intracellular lifestyle of this bacterium provides several advantages to its success and a capability to manipulate host biological processes. The finding of putative nucleomodulins and a Type-IV secretion system in its genome attests to its intracellular lifestyle. Because the genome lacks currently known key genes for plant growth promotion, the growth enhancement of the host by *M. extorquens* could result from intracellular modulation of host metabolism through production of effectors, like PLA2. Future research could focus on aspects of the intracellular lifestyle of *M. extorquens* DSM13060, and on determining if these bacteria secrete eukaryote-like effectors to stimulate host growth and development. Intracellular endophytes may provide more persistent advantages to host plants than the common apoplastic endophytes. Furthermore, a better understanding of this endosymbiosis can have broad implications for plant biotechnology, forestry, and agriculture. The recent availability of the genome sequence of loblolly pine (Neale *et al.*, 2014), together with advances in NGS technologies, should facilitate utilization of dual transcriptome sequencing in order to reveal essential gene expression in both organisms of this endosymbiotic interaction.

The current study focused on using experiments to reveal bioactive compounds and target genes responsible in managing the Scots pine-
Methylobacterium interaction. The identified bioactive compounds, methylated 3-hydroxybutyrate (ME-3HB) di- and trimers, are also produced by other Methylobacterium and Rhizobium species. Collectively, the results demonstrated ME-3HB di- and trimers prolong pine-explant viability and protect eukaryotic yeast cells from oxidative stress. Moreover, transcription of genes responsible for the generation of these compounds is induced by hydroxyl-radical stress, accompanied by PHB degradation in vitro and also in vivo, bacteria penetrate the pine host. ME-3HB oligomers are structurally similar to 3-hydroxybutyric acid (3-HB), a common ketone body in mammalian systems, also possessing antioxidant activity against hydroxyl radicals (Haces et al., 2008) and cytoprotective effects through epigenetic modifications, prevention of oxidative damage and inhibition of apoptosis via mitochondrial protection (Cheng et al., 2013; Maalouf et al., 2007; Shimazu et al., 2013; Zhang et al., 2013). Besides its importance in mammalian physiology, polymerized 3-HB, poly-3-hydroxybutyrate (PHB), is a widespread and well-known endogenous carbon-storage compound in aerobic bacteria. Based on the current study, endogenous PHB represents a huge antioxidant reservoir that can be rapidly released upon hydroxyl-radical stress. This novel finding drastically changes our understanding of bacterial physiology regarding this polymer and potentially explains how PHB-producing bacteria can retain their cellular homeostasis in extreme conditions. The study combines novel experimental data, supported by a comprehensive body of evidence from animal and bacteriological research, providing new insights on bacterial PHB metabolism and its widespread use as an agent against oxidative stress. Similarities between plant and animal early defense responses to microbial infection potentially permit extending results of the current study to the PHB-producing bacteria involved in human diseases. Many such intracellular pathogens belonging to genera Bordetella, Burkholderia, Legionella, Mycobacterium, Rickettsia and Vibrio are capable of causing persistent intracellular infections in humans despite the existence of an innate immune system (Grant et al., 2012; James et al., 1999; Sikora et al., 2009). This study revealed a new mechanism behind bacterial adaptation and persistent survival not only in plant tissue, but also in the human body, and therefore can provide new targets for antibacterial compounds.
References


Appendices

Appendix 1. Bacterial strains used in the studies
Appendix 2. Yeast strains used in the study III
Appendix 3. Alignment of putative amino acid sequences of Methylobacte-
rium sp. ACC deaminase
Appendix 4. Alignment of amino acid sequences of M. extorquens DSM13060
and R. eutropha str. H16 intracellular PHB depolymerases
Appendix 1. Bacterial strains used in the studies

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference and/or Study</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td><em>endA1 gyrSA96 hrDR17 (rK-mK-)</em> supE44 recA1; general host strain used for transformation and propagation of plasmids</td>
<td>Boyer &amp; Roulland-Dussoix (1969); I, II, III</td>
</tr>
<tr>
<td><em>Methylobacterium extorquens</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSM13060</td>
<td>Wild type; a plant growth-promoting conifer endophyte</td>
<td>Pirttilä <em>et al.</em> 2000; I, II, III</td>
</tr>
<tr>
<td>13061</td>
<td>DSM13060 containing mTn5gusA-pgf21-Km cassette (pFAJ1820)</td>
<td>Pohjanen <em>et al.</em> 2014; I, II, III</td>
</tr>
<tr>
<td>13062</td>
<td>13061 containing mTn5gusA-pgf21-Km cassette (pFAJ1820) and pMP7604 containing mCherry gene under the control of the tac promoter, Tc'</td>
<td>II</td>
</tr>
<tr>
<td>13061-acdS</td>
<td>Strain 13061 containing pMExt054 plasmid, Tc'</td>
<td>II</td>
</tr>
<tr>
<td>13061-bphB</td>
<td>Strain 13061 containing pMExt599 plasmid, Tc'</td>
<td>II</td>
</tr>
<tr>
<td>13061-cobS</td>
<td>Strain 13061 containing pMExt870 plasmid, Tc'</td>
<td>II</td>
</tr>
<tr>
<td>13061-phaC</td>
<td>Strain 13061 containing pMExt801 plasmid, Tc'</td>
<td>III</td>
</tr>
<tr>
<td>13061-phaZ1</td>
<td>Strain 13061 containing pMExt730 plasmid, Tc'</td>
<td>III</td>
</tr>
<tr>
<td>13061-mxaF</td>
<td>Strain 13061 containing pMExt801 plasmid, Tc'</td>
<td>I</td>
</tr>
<tr>
<td><em>Methylobacterium sp.</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMBG290</td>
<td>Wild type; a plant growth-promoting potato endophyte</td>
<td>(Pirttilä <em>et al</em>., 2000); III</td>
</tr>
<tr>
<td><em>Rhizobium leguminosarum bv. trifolii</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>str. B-9</td>
<td>Wild type; a plant growth-promoting white clover nodule bacterium</td>
<td>(Svenning <em>et al</em>., 2001); III</td>
</tr>
</tbody>
</table>
# Appendix 2. Yeast strains used in the study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference; Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
<td>Wild type; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, YIR038c::KanMX4</td>
<td>EUROSCARF; III</td>
</tr>
<tr>
<td>ccplΔ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, YKR066c::KanMX4</td>
<td>EUROSCARF; III</td>
</tr>
<tr>
<td>YIR038c::KanMX4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cta1Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, YDR256c::KanMX4</td>
<td>EUROSCARF; III</td>
</tr>
<tr>
<td>YDR256c::KanMX4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ctf1Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, YGR088w::KanMX4</td>
<td>EUROSCARF; III</td>
</tr>
<tr>
<td>YGR088w::KanMX4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gfr1Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, YPL091w::KanMX4</td>
<td>EUROSCARF; III</td>
</tr>
<tr>
<td>YPL091w::KanMX4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gpx1Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, YKL026c::KanMX4</td>
<td>EUROSCARF; III</td>
</tr>
<tr>
<td>YKL026c::KanMX4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>grx2Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, YDR513w::KanMX4</td>
<td>EUROSCARF; III</td>
</tr>
<tr>
<td>YDR513w::KanMX4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>grx5Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, YDL010w::KanMX4</td>
<td>EUROSCARF; III</td>
</tr>
<tr>
<td>YDL010w::KanMX4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gsh1Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, YJL101c::KanMX4</td>
<td>EUROSCARF; III</td>
</tr>
<tr>
<td>YJL101c::KanMX4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gsh2Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, YOL049w::KanMX4</td>
<td>EUROSCARF; III</td>
</tr>
<tr>
<td>YOL049w::KanMX4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nde1Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, YMR145c::KanMX4</td>
<td>EUROSCARF; III</td>
</tr>
<tr>
<td>YMR145c::KanMX4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ndf1Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, YML120c::KanMX4</td>
<td>EUROSCARF; III</td>
</tr>
<tr>
<td>YML120c::KanMX4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fn2Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, YGR209c::KanMX4</td>
<td>EUROSCARF; III</td>
</tr>
<tr>
<td>YGR209c::KanMX4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix 3. Alignment of putative amino acid sequences of *Methylobacterium* sp. ACC deaminase.

Alignment of amino acid sequence of *M. extorquens* DSM13060 (WP_003602931.1) putative D-cysteine desulphydrase (DcyD) with *M. extorquens* AM1 (WP_003602931.1), *M. extorquens* DM4 (WP_015822811.1), *M. extorquens* PA1 (WP_012253791.1), *M. populi* BJ001 (WP_012454209.1) against putative 1-aminocyclopropane-1-carboxylate (ACC) deaminase (AcdS; ACCd) of *M. nodulans* ORS2060 (WP_015931930.1), *Methylobacterium* sp. WSM2598 (WP_012330260.1) and *M. radiotolerans* JCM2831 (WP_012318504.1). Red asterisks identify key differences in the amino acid residues of the active center of AcdS enzyme. Identical amino acids are highlighted in black.

Alignment of amino acid sequence of *M. extorquens* DSM13060 poly-3-hydroxybutyrate (PHB) depolymerase PhaZ1 and PhaZ2 with *R. eutropha* str. H16 PHB depolymerase PhaZ1 and PhaZ5. Dashed box identify the active sites for the *M. extorquens* intracellular depolymerase PhaZ1 (AVC166PQ) and PhaZ2 (AVC180PQ). Identical and similar amino acids are highlighted in black and grey, respectively.
Original publications


Reprinted with permission from The American Society for Microbiology (II) and Nature Publishing Group (III).

Original publications are not included in the electronic version of the dissertation.
663. Pakanen, Minna (2015) Visual design examples in the evaluation of anticipated user experience at the early phases of research and development
664. Hyry, Jaakko (2015) Designing projected user interfaces as assistive technology for the elderly
666. Luukkanen, Tero (2016) New adsorption and oxidation-based approaches for water and wastewater treatment: studies regarding organic peracids, boiler-water treatment, and geopolymers
667. Tolkkinen, Mari (2016) Multi-stressor effects in boreal streams: disentangling the roles of natural and land use disturbance to stream communities
668. Kaakinen, Juhani (2016) Öljyllä ja raskasmetalleilla pilantuneita maita koskevan ympäristöä ja lupamenettelyn edistäminen kemiallisella tutkimuksella
670. Rönkä, Nelli (2016) Phylogeography and conservation genetics of waders
671. Fucci, Davide (2016) The role of process conformance and developers’ skills in the context of test-driven development
672. Manninen, Outi (2016) The resilience of understory vegetation and soil to increasing nitrogen and disturbances in boreal forests and the subarctic ecosystem
673. Pentinsaari, Mikko (2016) Utility of DNA barcodes in identification and delimitation of beetle species, with insights into COI protein structure across the animal kingdom
674. Lassila, Toni (2016) In vitro methods in the study of reactive drug metabolites with liquid chromatography / mass spectrometry

Book orders:
Granum: Virtual book store
http://granum.uta.fi/granum/
Janne Koskimäki

THE INTERACTION BETWEEN THE INTRACELLULAR ENDOPHYTIC BACTERIUM, METHYLOBACTERIUM EXTORQUENS DSM13060, AND SCOTS PINE (PINUS SYLVESTRIS L.)