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Deciphering the diversity of microbial 1-alkene producers and engineering of the production conditions

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Abstract

Volatile organic compounds are essential constituents of the plant microbiome and play an important role in inter- and intra-species communication. 1-Alkenes like 1-undecene are volatiles showing antimicrobial and antagonistic activity in plant-associated microbes such as *Pseudomonas* sp. Synthesis of 1-alkenes is often mediated by enzymes like the non-heme iron oxidases/decarboxylases UndA and UndB, which use medium-chain fatty acids as precursors. The pathways are highly conserved in three main genera: *Burkholderia*, *Pseudomonas*, and *Myxococcus*. Another important synthesis route is found in *Jeotgalicoccus* sp. ATCC 8456, via the fatty acid decarboxylase $OleT_{JE}$. However, there is still limited knowledge about the diversity of $OleT_{JE}$, UndA and UndB homologue enzymes in other microorganisms, especially in bacteria.

In previous research, the genome of *Jeotgalicoccus* sp. ATCC 8456 was sequenced and the information was employed for data mining. Furthermore, other 1-alkene producers belonging to the genera of *Pseudomonas* sp. and *Bacillus* sp. were identified. In this study, degenerate PCR-primers were designed binding to conserved regions of $OleT_{JE}$, UndA and UndB in order to explore the diversity of this type of enzymes in environmental samples. These primers allowed PCR-based screening of genomes and one metagenomic library to identify homologous 1-alkene biosynthetic genes. In doing so, five new UndA and UndB genes were discovered, with sequence identities ranging from 66.5 to 96.8%. By homology modelling and sequence analysis the affiliation of the genes to these enzyme families was confirmed.

Additionally, the conditions leading to 1-alkene production were evaluated *in vivo*. The production of certain 1-alkenes was successfully enhanced by targeted feeding with fatty acid precursors of a defined chain length. Feeding lauric acid to *Jeotgalicoccus* sp. ATCC 8456 led to a 1,000-fold higher 1-undecene production. In *Pseudomonas putida* 1T1, feeding lauric acid led to a 180% increased 1-undecene production and feeding tetradecanoic acid to a 600-fold increase of 1-tridecene.

The results of this study advance the current understanding of the microbial production of 1-alkenes and bring light onto the largely unexplored diversity of microbial biosynthetic routes for this type of biotechnologically promising bioactive molecules.

Kurzfassung

Flüchtige Stoffe sind essenzielle Bestandteile von Pflanzenmikrobiomen und spielen eine wichtige Rolle in der Kommunikation zwischen den Spezies. 1-Alkene wie 1-Undecen werden von pflanzenassoziierten Mikroben produziert und gehören zu den flüchtigen organischen Verbindungen. Sie sind bekannt für ihre antimikrobiellen und antagonistischen Aktivitäten. Die Synthese dieser 1-Alkene wird häufig von Enzymen, wie den nicht-Hämeisen Oxidasen/Decarboxylasen UndA und UndB, katalysiert, welche mittellange Fettsäuren als Substrat verwenden. Diese Stoffwechselwege sind in den folgenden drei Gattungen hoch-konserviert: *Burkholderia*, *Pseudomonas*, und *Myxococcus.* Eine weitere Syntheseroute, die Decarboxylase $OleT_{JE,}$ wurde in *Jeotgalicoccus* sp. ATCC 8456 gefunden. Trotz dieser Erkenntnisse ist bisher wenig über die Diversität homologer Enzyme von $OleT_{JE}$, UndA und UndB in anderen Mikroorganismen bekannt.

Das bereits annotierte Genom von *Jeotgalicoccus* sp. ATCC 8456 wurde für Data-Mining verwendet. Weiters wurden andere 1-Alkene-produzierende Bakterien aus den Gattungen *Pseudomonas* sp. und *Bacillus* sp. entdeckt. Degenerierte Primer wurden so konzipiert, dass sie an konservierte Bereiche von $OleT_{JE,}$ UndA und UndB binden. Damit konnte in einem PCR-basierten Screening nach homologen Genen in Genomen und einer metagenomischen Klonbibliothek gesucht werden. Dabei wurden fünf neue UndA- und UndB-Gene mit Sequenzidentitäten von 66.5 bis 96.8% entdeckt. Durch Homologie-basierte Modellierung und das Analysieren von konservierten Bereichen konnten die Sequenzen den UndA- und UndB-Enzymfamilien zugeordnet werden.

Zusätzlich wurden Bedingungen *in vivo* untersucht, welche zur Produktion von 1-Alkenen führen. Durch das Füttern von Fettsäuren mit bestimmter Länge konnte die Produktion der 1-Alkene gezielt beeinflusst werden. Das Füttern von Laurinsäure führte zu einer 1000-fach höheren Produktion von 1-Undecen durch *Jeotgalicoccus* sp. ATCC 8456. Bei *Pseudomonas putida* 1-T1*,* führte die Laurinsäure-Fütterung zu einer um 180% erhöhten Produktion von 1-Undecen und die Fütterung von Myristinsäure zu einer 600-fach erhöhten Produktion von 1-Tridecen.

Die Ergebnisse dieser Studie tragen zum Verständnis der mikrobiellen Produktion von 1-Alkenen bei. Außerdem bringen sie Licht in die noch größtenteils unerforschte Diversität dieser mikrobiellen Stoffwechselwege.

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1 Introduction

1.1 1-Alkenes and their industrial relevance

Alkenes are acyclic branched or unbranched aliphatic hydrocarbons having one carbon-carbon double bond and the general formula C_nH_{2n}. In case of 1-alkenes, also known as terminal olefins or α-olefins, the position of the double bond is at C_1 (C_α) (Moss et al., 1995). Oil refineries produce 1-alkenes on a scale of more than 100,000 t per year, as they are important platform chemicals (Kourist, 2015). Long chain 1-alkenes (C_{10}-C_{14}) are applied for production of surfactants and lubricants and short chain 1-alkenes (C_4–C_8) are applied as co-monomers in the production of high-density polyethylene and linear low-density polyethylene (van der Klis et al., 2012). 1-Alkenes also serve as chemical building blocks for fine chemicals like fragrances and pharmaceutical intermediates (Sun et al., 2018). In fact, 1-alkenes are not only platform chemicals. 1-Alkenes can directly be applied as drop-in compatible biofuels, meaning that terminal olefins are compatible with the existing petroleum-based infrastructure (Yan and Liao, 2009). Climate change and environmental concerns raised interest in bio-based chemicals and in biofuels produced with more sustainability and, therefore, a lower environmental footprint compared to the production derived from petroleum (Kang and Nielsen, 2017; Peralta-Yahya et al., 2012). Although microbial 1-alkene biosynthesis routes are known (Robinson and Wackett, 2019), 1-alkenes are almost exclusively derived from fossil resources nowadays (Rui et al., 2015). Especially in the past decade, novel biosynthetic routes have been discovered. $OleT_{JE}$ was discovered in *Jeotgalicoccus* sp. ATCC 8456 (Rude et al., 2011). UndA and UndB were recently identified for their ability of converting medium-chain fatty acids into terminal alkenes (Rui et al., 2015, 2014).

1.2 Biocatalysts for 1-alkene production

1.2.1 $OleT_{JE}$

$OleT_{JE}$ from *Jeotgalicoccus* sp. ATCC 8456 is able to catalyze the decarboxylation of medium-to-long chain fatty acids in the range of C_{12}-C_{20} converting them to 1-alkenes (C_{11}-C_{19}) using H_2O_2 as cofactor. Also, α- and β-hydroxylation of fatty acids are catalyzed by $OleT_{JE}$ as side reactions. Besides this single-step transformation, $OleT_{JE}$ can use a O_2 and a redox partner (NADPH) system (Fang et al., 2017). The fatty acid decarboxylation requires two electrons, coming either from H_2O_2 or NADPH (Kourist, 2015). The discovery of the H_2O_2-independent conversion opens more possibilities for

metabolic engineering in order to raise production of 1-alkenes using $OleT_{JE}$ (Liu et al., 2014).

1.2.2 UndA

UndA belongs to the Fe(II) enzyme family and catalyzes the conversion of fatty acids to 1-alkenes by oxidative decarboxylation. The reaction takes place in the presence of Fe(II) and oxygen, meaning the molecular oxygen is the only oxidant needed for the reaction (Rui et al., 2014).

1.2.3 UndB

The third protein employed in this project was the fatty acid desaturase UndB. To be more precise, UndB sequence homology analysis suggests that UndB might be a transmembrane protein, which is supported by predicted transmembrane domains. Also, three conserved His-boxes were described by Rui et al., which are suggested to include the ligands for the Fe(II) cluster. The hypothesized catalytic activity of UndB is compatible to the mechanism of other fatty acid desaturases. From the fatty acid substrate, two hydrogen atoms are removed to introduce a double bond, meaning that the fatty acid is converted to the corresponding 1-alkene via oxidative decarboxylation, similar to the reaction catalyzed by $OleT_{JE}$ and UndA (Rui et al., 2015).

1.3 Putative biological functions of 1-alkenes

The enormous biotechnological potential of bio-based 1-alkenes was already stated. However, the biological role of 1-alkenes is of special interest, but only limited knowledge is available so far. In general, hydrocarbons are known to fulfill cellular functions, like modulation of membrane fluidity as stress response, as well as interspecies interactions in microbial communities. Hydrocarbons even drive the global biogeochemical cycles (Robinson and Wackett, 2019). For example: the production of long chain olefins like hentriacontanonene (*n*-$C_{31:9}$), which was identified in nine out of 19 strains isolated from the arctic sea (Nichols et al., 1995), does increase at lower temperatures (Sukovich et al., 2010). That supports the assumption of bacteria being able to modulate cellular hydrocarbon content in response to temperature stress (Robinson and Wackett, 2019). Some 1-alkenes belong to the so-called microbial volatile organic compounds (mVOCs). These VOCs are typically small lipophilic compounds (up to C_{20}) characterized by a high vapor pressure and a low boiling point (Schmidt et al., 2015). Due to these properties, VOCs easily evaporate making them suitable for inter- and intraspecies bacterial communication over longer distances in

marine and terrestrial ecosystems (Schmidt et al., 2016). Especially 1-undecene got attention for its antagonistic and antibiotic properties, like inhibition of oomycetes, including the potato pathogen *Phytophthora infestans*. Hunziker et al. showed that *Pseudomonas* strains like *Pseudomonas putida* are able to produce 1-undecene and proved that 1-undecene significantly reduces mycelial growth of the fungal pathogen (Hunziker et al., 2015). Blom et al. investigated volatile mediated communication between bacteria and plants. 1-Undecene production was detected in species in the genera *Burkholderia*, *Bacillus* and *Serratia* (Blom et al., 2011; Robinson and Wackett, 2019). Further phylogenetic analysis showed that UndA is well conserved in all sequenced *Pseudomonas* species and in species belonging to the genera of *Acinetobacter*, *Burkholderia*, and *Myxococcus* (Rui et al., 2014). UndB is not as widely spread as UndA. Homologs of UndB were found in a few *Pseudomonas* and related species (Rui et al., 2015). $OleT_{JE}$ belongs to the cyp152 P450 enzyme family. Besides $OleT_{JE}$, enzymes of the same family were detected in *Kocuria rhizophila*, *Corynebacterium efficiens*, *Methylobacterium populi* and *Bacillus subtilis* using a homology model. These strains showed pentadecene production *in vitro* (Rude et al., 2011). Other than that, not much is known about the diversity of UndA, UndB and $OleT_{JE}$ homologous genes, especially in bacteria.

1.4 Bioprospecting - Exploring the diversity of 1-alkene biosynthesis

The need for new pharmaceuticals, antibiotics, agrochemicals, biocontrol products and food additives, or even compounds usable as biofuels, only to name a few, requires new strategies for prospection of the new sources. In times when the climate change is omnipresent (Lüthi et al., 2008), and the rise of multi resistance in human pathogens are responsible for an increasing number of deaths worldwide, bioprospecting bio-resources offers new ways for mining novel bio-products (Bérdy, 2012; Müller et al., 2016; Strobel and Daisy, 2003). Microorganisms are found in almost all known environments. This accomplishment requires adaptation and therefore highly divers and specific metabolites are produced by microorganisms (Coughlan et al., 2015). There are approximately 500,000 plant species and the associated microbial community was not studied for a striking majority (Berg et al., 2014). However, less than 1% of all bacteria isolated from environmental samples are cultivable and available for evaluating phenotypic properties (Torsvik et al., 1990). Therefore, to address bacteria and their metabolites, culture-independent methods are more promising. Metagenomics describes the analysis of DNA isolated directly from

environmental samples (Coughlan et al., 2015). Metagenomic sequencing allows to characterize the taxonomic composition and phylogenetic diversity from a microbial community. But also on a functional level microbial communities can be characterized by metagenomic sequencing and novel enzymes and metabolites can be found (Berg et al., 2014; Knight et al., 2012; Langille et al., 2013). Screening of metagenomes by degenerate primer-mediated PCR amplification was successfully used before to identify novel enzymes. (Karasev et al., 1994; Müller et al., 2015).

In this study, the diversity of UndA, UndB and $OleT_{JE}$ homologous genes in bacterial genomes and metagenomes was investigated using a similar PCR amplification screening approach.

1.5 Aim of this thesis

There is still a knowledge gap about the possible diversity of $OleT_{JE}$ homologous enzyme sequences in other microorganisms (especially in bacteria). Other biosynthetic routes for the microbial production of 1-alkenes, enabling bacterial production of the desired 1-alkenes by decarboxylation of fatty acids have been reported, like UndA and UndB in *Pseudomonas* sp., In previous studies conducted at the institute of environmental biotechnology, other 1-alkene producers (*Pseudomonas* sp. and *Bacillus* sp.) were identified in the institute's strains collection. Some of these strains have been reported in the literature, but only limited data is available regarding the responsible genes (enzymes) for 1-alkene biosynthesis. To shed light on the diversity of UndA, UndB and $OleT_{JE}$ homologous genes, a metagenomic fosmid library from the *Sphagnum* moss microbiome was screened by using degenerate primer-mediated PCR amplification.

Besides of the microbial diversity, it is important to understand under which conditions the *in vivo* production of 1-alkenes takes place. Previous analysis showed that the production of certain 1-alkenes by *Jeotgalicoccus* sp. ATCC 8456 can be influenced by the targeted feeding of fatty acids with a certain chain length as precursors for the reaction. More fatty acid experiments were carried out with *Jeotgalicoccus* sp. ATCC 8456 to reproduce and confirm previous results. Furthermore, fatty acid feeding experiments using *Pseudomonas* and *Bacillus* strains. were conducted as well. The findings of this study advance our knowledge about the conditions that lead to the *in vivo* 1-alkene production.

2 Materials and methods

All chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, Missouri, USA), Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) or Thermo Fisher Scientific (Massachusetts, USA) if not stated otherwise.

All liquids and/or materials were sterilized by autoclaving at 121 °C for 20 min. Non autoclavable additives were filtered using 0.2 µm disposable filters.

Further standard techniques such as sterile working, balancing or inoculation of media were performed according to general laboratory protocols operating with (micro-) organisms of the biohazard level 1.

2.1 Cultivation of bacteria and storage

All bacteria used in this project were taken from the culture collection of the Institute of Environmental Biotechnology, Graz University of Technology, Austria. Strains known to produce 1-alkenes are shown in Table 1. The metagenomic fosmid library (moss bib II, Institute of Environmental Biotechnology, Graz University of Technology, Austria) employed in this project was created using *Escherichia coli* EPI300-T1^{R} cells and was ready to use, stored at -70°C (Müller et al., 2015).

Table 1: Bacterial strains known to be able to produce 1-alkenes.

Species	**Strain**
Bacillus thuringiensis	3R2-29
Pseudomonas aeruginosa	QC14-3-8
Pseudomonas brassicacearum	L13-6-12
Pseudomonas brenneri	Baz30
Pseudomonas fluorescens	Baz53
Pseudomonas poae	RE*1-1-14
Pseudomonas putida	1T1
Jeotgalicoccus sp.	ATCC 8456

Bacteria grown on solid media for continuous use were kept at 4°C for few weeks, before renewing them again. Liquid bacterial cultures were always prepared freshly, either starting from a colony on solid media or from other liquid cultures. For long-term

storage, glycerol stocks were prepared with a glycerol concentration of 25% (v/v). The glycerol stocks were then stored at -70°C.

2.2 Growth media

For liquid *Jeotgalicoccus* sp. ATCC 8456 cultures, tryptic soy broth yeast extract (TSBYE, 30.0 g L^{-1} CASO broth and 0.5% (v/v) yeast extract (Rude et al., 2011)) was used. All other liquid bacterial cultures were prepared in NB (15.0 g L^{-1} nutrient broth II; Sifin diagnostics GmbH, Berlin, Germany).

In case of bacterial cultures on solid media, NB-agar (NB and 15.0 g L^{-1} agar) was chosen for all strains.

2.3 Gas chromatography-mass spectrometry for 1-alkene identification and quantification

Volatile organic compounds (VOCs) distinguish themselves due to the characteristic high vapor pressure and low boiling point. Short to medium-chain length 1-alkenes belong to the group of VOCs and the properties mentioned before making them suitable for gas chromatography-mass spectrometry (GC-MS) analysis. Headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC-MS) can be used to determine VOCs produced by bacterial cultures (Tait et al., 2014).

2.3.1 Gas chromatography-mass spectrometry method and analysis

Headspace sampling was applied, where a SPME fiber adsorbs volatiles from the gas phase. Before the extraction, the fiber was conditioned once for eight min at 40°C in the GC injector. All standards/samples were extracted with a PDMS-coated fiber (30 µm, for non-polar semi-volatiles [MW 80-500 g mol^{-1}]; Supelco, Pennsylvania, USA) for 15 min at 35°C. A splitless thermal desorption for 30 min at 270°C, 9.1473 psi, and a septum purge flow of 3 mL min^{-1} in the heated injection port of the gas chromatograph followed. An Agilent HP-5 column (30 m x 320 µm, 0.25 µm film) and the following temperature program was used for separation of analytes: 40°C/hold 2 min; 5°C min^{-1} to 110°C; 10°C min^{-1} to 280°C; hold 3 min with a flow of 1.2 mL min^{-1} under 9.1473 psi.

All samples were injected into air-tightly sealed 20 ml GC-MS vials (20 ml ND18 Headspace Screw Vial [clear], 75.5 x 22.5 mm, rounded bottom and ND18 Magnetic Screw Cap [eight mm hole] with Silicone/PTFE Septa [white/blue], 1.5 mm 55° shore

A; BGB Analytik Vertrieb GmbH, Rheinfelden, Germany). The samples were analyzed on an GC-MS system with He as carrier gas (Agilent 7890B/5977A Series Gas Chromatograph/Mass Selective Detector; and PAL RSI 85; CTC Analytics AG, Zwingen Switzerland). The eluents were analyzed in the mass range of 50 to 350 g mol^{-1} with a quadrupole mass spectrometer. The output was investigated with the associated software (Agilent MSD Productivity). The obtained mass spectra were analyzed using the NIST EI Mass Spectral Library (NIST MS Search Version 2.2, 2014).

2.3.2 1-Alkene standards

For the purpose of creating the 1-alkene mass spectra library and the determination of retention times of them, analytically pure 1-alkenes were analyzed by GC-MS. The terminal olefins were injected into air-tight sealed 20 ml GC-MS vials. For 1-C5 200 µL and for 1-C6, 1-C7 and 1-C8 150 µL of the pure gas were used. For 1-C9 and 1-C10, 5 mL of 1:2 million dilutions were prepared in the GC-vial, and 5 mL of 1:4 million dilutions of 1-C11, 1-C12, 1-C13, 1-C14, 1-C15, 1-C17, 1-C18 and 1-C19. The 1-alkenes were diluted in DMSO.

Furthermore, linear calibration curves were made using analytically pure terminal olefins as well. Therefore, five dilutions of 1-C9, 1-C10, 1-C11, 1-C13, 1-C15, 1-C17, 1-C18, 1-C19 were prepared in GC-MS vials and measured by GC-MS in a double approach. The peaks were integrated using the RTE integrator (Appendix, Table A1). The integrators variables are initial threshold, initial peak width, initial area reject and shoulders.

2.3.3 Fatty acid feeding of bacterial cultures

The conditions leading to 1-alkene production *in vivo* were observed by feeding fatty acids with a certain chain length as precursors for the reaction. The effect of providing a high (0.5% [v/v]) and low (200 µM) fatty acid concentration was analyzed by SPME GC-MS. The fatty acid stock solutions were prepared in DMSO. Therefore, precultures were prepared in TSBYE (in case of *Jeotgalicoccus* sp. ATCC 8456) or NB (for all other strains), which were incubated overnight. GC-MS vials were prepared containing 3 mL of the corresponding media mixed with fatty acids to provide the concentrations of 0.5% (v/v) (Table 2) and 200 µM before inoculation with 1% (v/v) of the precultures.

Table 2: Fatty acid concentrations of 0.5% (v/v) converted to mM.

Fatty acid	**Concentration [mM]**
Caproic acid (C6:0)	39.99
Caprylic acid (C8:0)	31.55
Pelargonic acid (C9:0)	28.44
Capric acid (C10:0)	25.92
Undecanoic acid (C11:0)	23.89
Lauric acid (C12:0)	25.13
Myristic acid (C14:0)	22.55
Palmitic acid (C16:0)	16.61
Arachidic acid (C20:0)	13.18

For *Jeotgalicoccus* the following fatty acids were fed: C6:0, C8:0, C9:0, C10:0, C11:0, C12:0, C14:0, C16:0 and C20:0. *Bacillus thuringiensis, Pseudomonas aeruginosa, Pseudomonas brassicacearum, Pseudomonas brenneri, Pseudomonas fluorescens, Pseudomonas poae* and *Pseudomonas putida* (Table 1) were fed with C12:0 and C14:0 in case of the 0.5% (v/v) concentration (Table 2), and in case of the 200 µM concentration the following fatty acids were used: C10:0, C11:0, C12:0, C14:0 and C16:0. For blank measurements (without addition of fatty acid) 1% (v/v) of pure DMSO was added to the media. The *Jeotgalicoccus* cultures were then incubated for 24 h at 26°C and all other cultures at 30°C for 24 h, before the samples were analyzed by SPME GC-MS.

2.3.4 Growth experiments using fatty acids

The effect of the fatty acids in the cultivation media at the concentrations of 0.5% (v/v) (13.18 to 39.99 mM) and 200 µM was determined as well. In 96-well plates (Sarstedt, Nümbrecht, Germany) 150 µL of TSBYE for cultivation of *Jeotgalicoccus* and 150 µL of NB for all other strains were mixed with C10:0, C12:0, C14:0 and C16:0 fatty acids. Additionally, the effect of DMSO was tested as well, by adding 1% (v/v) of pure DMSO to the media in one sample. As blank, only cultivation media was used. The fatty acid containing media were inoculated with 1% (v/v) of the corresponding precultures. The *Jeotgalicoccus* culture was then incubated at 26°C and all other bacterial cultures at 30°C and 250 rpm. The OD_{600} was measured at the following time points: 0 h, 1 h, 3 h, 5 h, 7 h, 24 h, 29 h and 48 h using a microtiter plate reader (Infinite® M200; Tecan,

Männedorf, Switzerland). The growth characterization in the 96-well plates was performed in four replicates.

For *Bacillus thuringiensis, Pseudomonas fluorescens* and *Jeotgalicoccus* sp. ATCC 8456, the growth experiment was repeated in higher volumes. Therefore, 100 mL flasks were filled with 20 mL of the corresponding cultivation media mixed with the fatty acids with concentrations of 0.5 % (v/v) and 200 µM. Again, one sample was prepared with 1% (v/v) DMSO instead of fatty acid solution and as reference, a cultivation in pure media was used. For *Bacillus thuringiensis, Pseudomonas fluorescens* the OD_{600} was determined after 0 h, 1 h, 3 h, 5 h, 7 h, 24 h and 27 h. For *Jeotgalicoccus* sp. ATCC 8456 the following time points were used: 0 h, 3 h, 7 h, 24 h, 30 h, 48 h, 53 h and 72 h. Here, the experiment was conducted in duplicate.

2.4 PCR-based screening

2.4.1 Design of degenerate primers

A nucleotide blastn and blastx analysis was performed to get highly similar sequences for primer design based on the following DNA sequences: *Jeotgalicoccus* sp. ATCC 8456 $OleT_{JE}$ (GenBank accession number HQ709266.1) (Rude et al., 2011), *Pseudomonas fluorescens* Pf-5 Chain A/UndA (GenBank accession number CP000076.1 [5024351-5025136]) (Rui et al., 2014), *Pseudomonas fluorescens* Pf-5 UndB (GenBank accession number CP000076.1 [238682-239755]) (Rui et al., 2015). These sequences were translated to their protein sequence. The protein sequences as well as the nucleotide sequences were aligned. In highly conserved regions found in the protein sequence alignment, degenerate primers were designed for UndA, UndB and $OleT_{JE}$ (Table 3).

Table 3: Degenerate primers designed binding to conserved regions of UndA, UndB and $OleT_{JE}$ homologous protein-coding genes.

Protein	**Primer name**	**Sequence (5'-3')**
UndA	UndA_Fwd_deg	BTA YCT SAT HGG BGG HTG
	UndA_Rev_deg	ATR ATY TCS ARB GCY TCC C
UndB	UndB_Fwd_deg	YGA YCT SAT YCA CWS SAT
	UndB_Rev_deg	GYT CSY TSA CSA CRA ART
$OleT_{JE}$	OleT_Fwd_deg	YRT NGA YMG HAA RGC DYT RT
	OleT_Fwd_deg	GNG GRA THA RRT CRA ABG G

2.4.2 Optimization of the PCR methodology

Before the actual screening could be performed, the PCR conditions and the lysis methods had to be optimized. Throughout the optimization, four different lysis methods were tested.

2.4.2.1 Homogenization using bead beater

The first cell lysis attempts were performed by homogenization (FastPrep™-24, MP Biomedicals™, California, USA). Therefore, some cell material was resuspended in 300 µL sterile 0.85% (w/v) NaCl solution and 200 µL of the cell suspension was then homogenized in tubes filled with 20% lysis matrix (0.1-0.25 mm glass beads) plus three 1.5 mm to 2.0 mm glass beads and the following settings: 6.0 m s^{-1}, 30 s. The tubes containing the lysis matrix were autoclaved prior use. After the first homogenization step, the suspension was heated at 95°C for 10 min in a thermocycler, followed by another homogenization round using the same settings, and another heating step at 95°C for 10 min. The lysed cells were centrifuged at 4,000 rpm and 4°C for 3 min. From the supernatant, 50 µL were transferred into new sterile 1.5 mL reaction tubes and kept on ice until use as template for PCR.

2.4.2.2 Microwave cell lysis

In order to address the different cell morphology of the gram positive *Jeotgalicoccus* sp. ATCC 8456, which might cause trouble during the cell lysis, a lysis method was tested using a microwave. Some cell material was transferred into a 1.5 mL reaction tube and put into the microwave for 3 min. The lysed cells were then resuspended using sterile 0.85% NaCl solution and this cell suspension was directly used as template. If not stated otherwise 0.9 µl of cell suspension was used in a standard PCR reaction in a final volume of 15 µl.

2.4.2.3 Denaturation at 98°C

Some cell material was suspended in 50 µL sterile 0.85% (w/v) NaCl solution and heated to 98°C for 15 min in a heat block. The denaturated cells were then centrifuged at 4,000 rpm and 4°C for 2 min.

2.4.2.4 Denaturation at 98°C combined with lysozyme

Here, some cell material was suspended in 50 µL nuclease free ddH_2O mixed with 20 mg mL^{-1} lysozyme. The cell suspension was then incubated at 37°C for 60 min,

before denaturation at 98°C for 15 min in the PCR machine and the centrifugation at 4,000 rpm and 4°C for 2 min.

During the optimization of the PCR conditions, two different polymerases were used. The OneTaq® Quick-Load® 2X master mix with standard buffer (New England Biolabs, Ipswich, USA) and the Taq-&GO™ ready-to-use 5X master mix (MP Biomedicals™, California, USA). Based on the manufacturer's manuals, different reaction mixtures with different total volumes were tested, using different template concentrations, primer concentrations, and sometimes filter sterilized DMSO was added.

Additionally, to test if the lysis method or the primers were the source of missing amplification during PCRs, BASYS00026_Flav primers (Table 4) were used, which are known to work with the *Jeotgalicoccus* sp. ATCC 8456 template DNA.

Table 4: BASYS00026_Flav primers obtained from the Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Austria

Primer name	**Sequence (5'-3')**
Fwd_BASYS00026_Flav	CGTCCACATATGAATGAAGTCATTAATTTGATTAATAATCACCGTTCGTTAAG
Rev_BASYS00026_Flav	CTCAGCGAATTCTTATTGACCTAAAAATCCTTGTTCTTTTAAGAACGCATC

Furthermore, throughout the optimization, new primer pairs were designed for $OleT_{JE}$ and UndB (Table 5).

Table 5: Degenerate primers designed binding to conserved regions of UndB and $OleT_{JE}$ homologous genes.

Protein	**Primer name**	**Sequence (5'-3')**
UndB	UndB_Fwd_deg2	WSC ATG TAY TTY CGC AAG CA
	UndB_Rev_deg2	CDS YTC BAY RTC GCC GTA
$OleT_{JE}$	OleT_Fwd_deg2	BGT NAA BAC NYT DTT YGG TAA AGG
	OleT_Fwd_deg2	GCN AAR WAY TTM ATH GWY TCT TCC AT

For the actual optimization of the PCR conditions using degenerate primers, different temperature gradients, touchdown PCRs and 2-step PCRs were performed. More details are shown in Table 6.

Table 6: Every PCR program (A-H) tested throughout the optimization process summarized in one table.

Program	Protein	Step	Temperature	Time	Note
A		Initial denaturation	94°C	5 min	
		Denaturation	94°C	30 s	25 cycles
		Annealing	70°C	30 s	
	UndA	Elongation	72°C	30 s	
	UndB			40 s	
	$OleT_{JE}$			48 s	
		Final elongation	72°C	10 min	
B		Initial denaturation	94°C	5 min	
		Denaturation	94°C	30 s	30 cycles
		Annealing	49-69.7°C	30 s	
	UndA	Elongation	72°C	40 s	
	UndB			45 s	
	$OleT_{JE}$			60 s	
		Final elongation	72°C	10 min	
C	UndB	Initial denaturation	94°C	5 min	
		Denaturation	94°C	30 s	30 cycles
		Annealing	45-72°C	30 s	
		Elongation	72°C	40 s	
		Final elongation	72°C	10 min	
D		Initial denaturation	94°C	5 min	30 cycles
		Denaturation	94°C	30 s	
		Annealing	51-45°C	30 s	
	UndA	Elongation	72°C	30 s	
	UndB			40 s	
		Final elongation	72°C	10 min	

E		Initial denaturation	94°C	5 min	
		Denaturation	94°C	30 s	30 cycles
		Annealing	45-68°C	30 s	
	UndA	Elongation	68°C	30 s	
	UndB			40 s	
	$OleT_{JE}$			48 s	
		Final elongation	68°C	10 min	
F	UndB	Initial denaturation	94°C	5 min	
		Denaturation	94°C	30 s	30 cycles
		Annealing	55-46°C	30 s	
	UndB	Elongation	68°C	40 s	
	$OleT_{JE}$			48 s	
		Final elongation	68°C	10 min	
G	UndB	Initial denaturation	94°C	5 min	
		Denaturation	94°C	30 s	5 cycles
		Annealing	50°C	30 s	
		Elongation	68°C	40 s	
		Denaturation	94°C	30 s	30 cycles
		Annealing	55-46°C	30 s	
		Elongation	68°C	40 s	
		Final elongation	68°C	10 min	
H		Initial denaturation	94°C	5 min	
		Denaturation	94°C	30 s	30 cycles
		Annealing	45-68°C	30 s	
	UndB	Elongation	68°C	32 s	
	$OleT_{JE}$			56 s	
		Final elongation	68°C	10 min	

2.4.3 Cell lysis and template preparation

A metagenomic clone library (moss bib II, Institute of Environmental Biotechnology, Graz University of Technology, Austria) containing DNA isolated from the *Sphagnum magellanicum* associated microbiota was used to screen for new UndA, UndB and $OleT_{JE}$ homologs. Each of the clones taken from the metagenomic library was

cultivated in one slot of a 96-well plate and frozen at -70°C as glycerol stocks (Müller et al., 2015). For the screening performed during this project, 100 of the 96-well plates were thawed. Pools of ten plates each were prepared by combining cell material of ten plates into one new plate. Therefore, slots of a sterile 96-well plates were filled with 100 µL of NB medium mixed with 12.5 µg mL^{-1} chloramphenicol and inoculated with cells of ten plates of the metagenomic library. The 96-well "master-plates" containing the pools of ten clones in each slot were incubated overnight at 37°C and 110 rpm. Then, "master-plates" were replicated in fresh medium and incubated overnight at 37°C and 110 rpm as well.

For the cell lysis, 50 µL of the overnight culture were transferred into sterile PCR 96-well plates (Sarstedt AG & Co. KG, Nümbrecht, Deutschland) and covered with an optically clear sealing tape (Sarstedt AG & Co. KG, Nümbrecht, Deutschland). The cells were then denaturated at 98°C for 15 min in a PCR machine, followed by a centrifugation at 4000 rpm for 3 min. The supernatant was directly used as template for PCR.

In case solid cultures were used, some cell material was resuspended in sterile 0.85 % (w/v) NaCl solution before denaturation and centrifugation.

2.4.4 PCR programs and mixture

For the PCR based screening, a reaction mixture was used with a total volume of 15 µL, containing 7.5 µL of OneTaq® Quick-Load® 2X master mix with standard buffer (New England Biolabs, Ipswich, USA), 0.75 µL DMSO, 2 µL of the template, 0.6 µL of a 20 µM forward and reverse primer solution and 3.55 µL of ddH_2O.

The PCR program started with initial denaturation at 94°C for five min followed by 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 51.2°C for UndA, 55.3°C for UndB and 46°C for $OleT_{JE}$, and elongation at 68 °C for 30 s for UndA, 32 s for UndB and 70 s for $OleT_{JE}$. The program finished with a final elongation at 68°C for ten min.

2.4.5 Evaluation of PCR results

The PCR products were loaded onto 1.5% (w/v) agarose gels. Either a 100 bp or a 1 kb ladder (GeneRuler™, 0.1 µg $µL^{-1}$, 50 µg; Thermo Scientific, Massachusetts, USA) were used as marker. The samples were mixed with loading dye (0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA in 10 mM Tris-HCl, pH 7.6) before loading them on the gel. For the gel and the electrophoresis, 1x TAE buffer was

used. The gels were run at 110 V, 300 mA, 50 W for 50 min. Staining was done in an 0.0001% ethidium bromide solution for 30-40 min. Afterwards, the gel was observed under UV-light and using the GelCompar II software.

2.5 Sequencing

2.5.1 PCR product purification

For sequencing, positive PCR results were reproduced using the Q5® High-Fidelity DNA polymerase (New England Biolabs, Ipswich, USA) with the following reaction mixture: 10 µL Q5® reaction buffer, 1 µL dNTP mix (10mM), 2 µL of each 20 µM primer solution, 8 µL template, 0.5 µL Q5® polymerase, 2.5 µL DMSO and 24 µL PCR grade H_2O. The PCR programs were adjusted to the Q5® polymerase in comparison to the conditions described in Chapter 2.4.4 and therefore the elongation time was halved, and the elongation temperature was increased to 72°C. The PCR products were purified in order to sequence them. Some PCRs led to more than one specific product. In these cases, the bands containing the wanted product were cut from the gel before purification. For the specific products, where only one band was determined, the PCR product was purified directly. The purification was performed with the Wizard® SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA) following the producer's manual.

2.5.2 Sequencing

The purified PCR products were sent for Sanger sequencing to Microsynth AG (Balgach, Switzerland). Sample preparation was performed according to the company's manual.

Using the degenerate primers designed in conserved regions of the proteins, only partial sequences were obtained. In order to gain the whole sequence, new primers were designed, binding in the middle of the known sequence, directing outwards. The detected fosmids of the metagenomic library, showing positive amplification of UndA and UndB homologous DNA sequences, were isolated using the GeneJet Plasmid Miniprep Kit from Thermo Scientific (Massachusetts, USA) according to the producer's manual and sent for sequencing with the newly designed primers (Table 7).

Table 7: Primers designed for sequencing binding in the middle of the sequence pointing outwards.

Protein	Primer name	Sequence (5'-3')
UndA	A11_2_1_A_Fwd	AGCTCTGATGCTGTTCAA
	A11_2_1_A_Rev	ACCAGGTCTGTAAAAGCAA
	E5_7_4_A_Fwd	GGCCTATCCAGCTCTGAT
	E5_7_4_A_Rev	AGCAAAGTCTTGAACAGC
UndB	A6_1_6_B_Fwd	AATGTACGGCATCAACCT
	A6_1_6_B_Rev	AGAACCAATCATCGCGCA
	E7_1_6_B_Fwd	GATGAAACCTGAGTAGGGA
	E7_1_6_B_Rev	TTTCTCCAAAACTGCACC

The degenerate primers were not only applied for screening the clones of the metagenomic library, but also on the strains identified as producers of 1-alkenes by GC-MS analysis (Table 1). These PCR products were sent for sequencing as well, except from the product obtained with *Jeotgalicoccus* sp. ATCC 8456, because the genome of *Jeotgalicoccus* sp. ATCC 8456 was already sequenced and the exact sequence of $OleT_{JE}$ is already published. The genomes of *Pseudomonas brassicacearum* L13-6-12 and *Pseudomonas poae* RE*1-1-14 were sequenced and annotated previously by the Institute of Environmental Biotechnology, Graz University of Technology as well. Therefore, the sequence obtained by sanger-sequencing could be applied in this study directly on the annotated genome to determine the open reading frames (ORFs) of UndA and UndB in the genomic sequences of *Pseudomonas brassicacearum* L13-6-12 and *Pseudomonas poae* RE*1-1-14.

For the remaining strains, the sequences of the PCR products were used for a blast analysis. Per strain and gene, four sequences showing the highest similarity were aligned and the ORFs were determined in the resulting consensus sequence upon alignment. The sequences obtained that way were applied for designing primers binding to the start and stop codons of the ORFs to amplify the whole gene (Table 8).

Table 8: Primers binding to the beginning (start) and the end (stop codon) of UndA and UndB homologous genes in the genomes of 1-alkene producing strains (Table 1) in order to amplify the whole gene.

Protein	Strain	Primer name	Sequence (5'-3')
UndA	L13-6-12	BRA_Fwd_A	ATGTCGGCCGGCCCGGCC
		BRA_Rev_A	TCAGCTTTCCGCCAGTGCCAGG
	Baz30	BRE_Fwd_A	ATGGAAGCCTCGAGTTACCC
		BRE_Rev_A	TCASGCYTCGCTGGCRACSM
	Baz53	FLU_Fwd_A	ATGGAAGCCTCVAGTTACCCYG
		FLU_Rev_A	TCAYGCCTCGCTGGCGAC
	RE*1-1-14	POAE_Fwd_A	ATGGAAGCCTCAAGTTACCC
		POAE_Rev_A	TCAGGCCTGACTGGCCAC
	1T1	PUT_Fwd_A	ATGGAAGCCTCRAGTTACCCHG
		PUT_Rev_A	TCAYGCCTCGCTGGCGAC
UndB	L13-6-12	BRA_Fwd_B	ATGCACGGCACTTGCGCA
		BRA_Rev_B	TCAGACCCGAACCGTATCCCCT
	Baz30	BRE_Fwd_B	ATGGACGGTACTTCTGCAAG
		BRE_Rev_B	TCAGCKCGYYGYCSTTTC
	Baz53	FLU_Fwd_B	ATGGACGGTACTTSTGCAAGTC
		FLU_Rev_B	TCAGCGCRTCGCCGTTTC
	RE*1-1-14	POAE_Fwd_B	ATGTACGCATCACAGTTCTCAG
		POAE_Rev_B	TCAAGCGTGCGCAGGCGG
	1T1	PUT_Fwd_B	ATGGACGGTACTTSTGCAAG
		PUT_Rev_B	TCAGCGCRTCGCCGTTTC

This way, the whole sequence could be obtained by PCR. The following reaction mixture was used: 10 µL Q5® reaction buffer, 1 µL dNTP mix (10mM), 2 µL of each 20 µM primer solution, 8 µL template, 0.5 µL Q5®, 2.5 µL DMSO and 24 µL PCR grade H2O. The PCR program was optimized for each primer pair individually, using a temperature gradient ranging from 45°C to 72°C. The PCR programs for each primer pair and strain are summarized in Table 9 for UndA and in Table 10 for UndB.

Table 9: Optimized PCR programs for every primer pair from Table 8 designed to amplify UndA homologous genes.

Strain	Step	Temperature	Time	Note
	Initial denaturation	94°C	5 min	
	Denaturation	94°C	30 sec	
L13-6-12		70°C		
Baz30		64°C		
Baz53	Annealing	69°C	30 sec	30 cycles
RE*1-1-14		70°C		
1T1		70°C		
	Elongation	72°C	20 sec	
	Final elongation	72°C	10 min	

Table 10: Optimized PCR programs for every primer pair from Table 8 designed to amplify UndB homologous genes.

Strain code	Step	Temperature	Time	Note
	Initial denaturation	94°C	5 min	
	Denaturation	94°C	30 sec	
L13-6-12		67.5°C		
Baz30		66.5°C		
Baz53	Annealing	67.5°C	30 sec	30 cycles
RE*1-1-14		67.5°C		
1T1		67.5°C		
	Elongation	72°C	30 sec	
	Final elongation	72°C	10 min	

The PCR products were then purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA) according to the producer's manual, and sent to Microsynth AG for sequencing using the primers shown in Table 8. Due to the sequencing methodology, only part of the UndA and UndB homologue sequences were obtained. Again, new primers were designed binding in the middle of the known sequences pointing outwards for a second sequencing step, in order to obtain the whole sequence for each strain (Table 11). As mentioned before, there was no need

for a second sequencing step for *Pseudomonas brassicacearum* L13-6-12 and *Pseudomonas poae* RE*1-1-14, because the genome sequence is already known and together with the partial sequence, the ORFs for UndA and UndB were determined.

Table 11: Primers designed for sequencing binding in the middle of the sequence pointing outwards.

Gene	Strain	Primer name	Sequence (5'-3')
UndA	Baz30	BRE_Fwd_A_LC	CGATGAAGTGGCTGAAGA
		BRE_Rev_A_LC	CCCAGTGCACCCAATAATC
	Baz53	FLU_Fwd_A_LC	AAGAACTGCAAGCGCAAAA
		FLU_Rev_A_LC	TTGGTCTTGGTGAGGTTT
	1T1	PUT_Fwd_A_LC	GGCCGATTCGTTGATTGT
		PUT_Rev_A_LC	AATAGTCGGCATGGTTGAG
UndB	Baz30	BRE_Fwd_B_LC	GCTCCAACATGCACTACT
		BRE_Rev_B_LC	TTGTGGTGATTGAGGTGA
	Baz53	FLU_Fwd_B_LC	GTGGTATGTGTTTTTGGG
		FLU_Rev_B_LC	CTTGTGGTGATTGAGGTG
	1T1	PUT_Fwd_B_LC	GCTCCAACATGCACTACT
		PUT_Rev_B_LC	TGTGGCCGGTGATGTAGA

3 Results

3.1 Quantification of 1-alkenes

For the identification of the 1-alkenes produced by bacterial cultures, the retention times of analytically pure 1-alkenes were determined by SPME GC-MS. Table 12 shows the retention times.

Table 12: Retention times of analytically pure 1-alkene standards.

1-Alkene	**Retention time (min)**
1-C5	1.601
1-C6	1.951
1-C7	2.842
1-C8	4.549
1-C9	7.222
1-C10	10.126
1-C11	13.202
1-C12	16.139
1-C13	18.471
1-C14	20.264
1-C15	21.754
1-C17	24.275
1-C18	25.419
1-C19	26.598

Furthermore, to compare the concentration of the 1-alkenes produced by different bacterial cultures, calibration curves were prepared and the linear equations were determined (Appendix, Table A2).

3.2 Fatty acid feeding

Engineering the conditions of microbial 1-alkene production was one of the main goals of this study. Therefore, fatty acids with a certain chain length were fed to influence the production of the corresponding 1-alkenes *in vivo*. The effect of feeding fatty acids in concentrations of 0.5% (v/v) (13.18 to 39.99 mM depending on the 1-alkene) and of 200 µM was investigated.

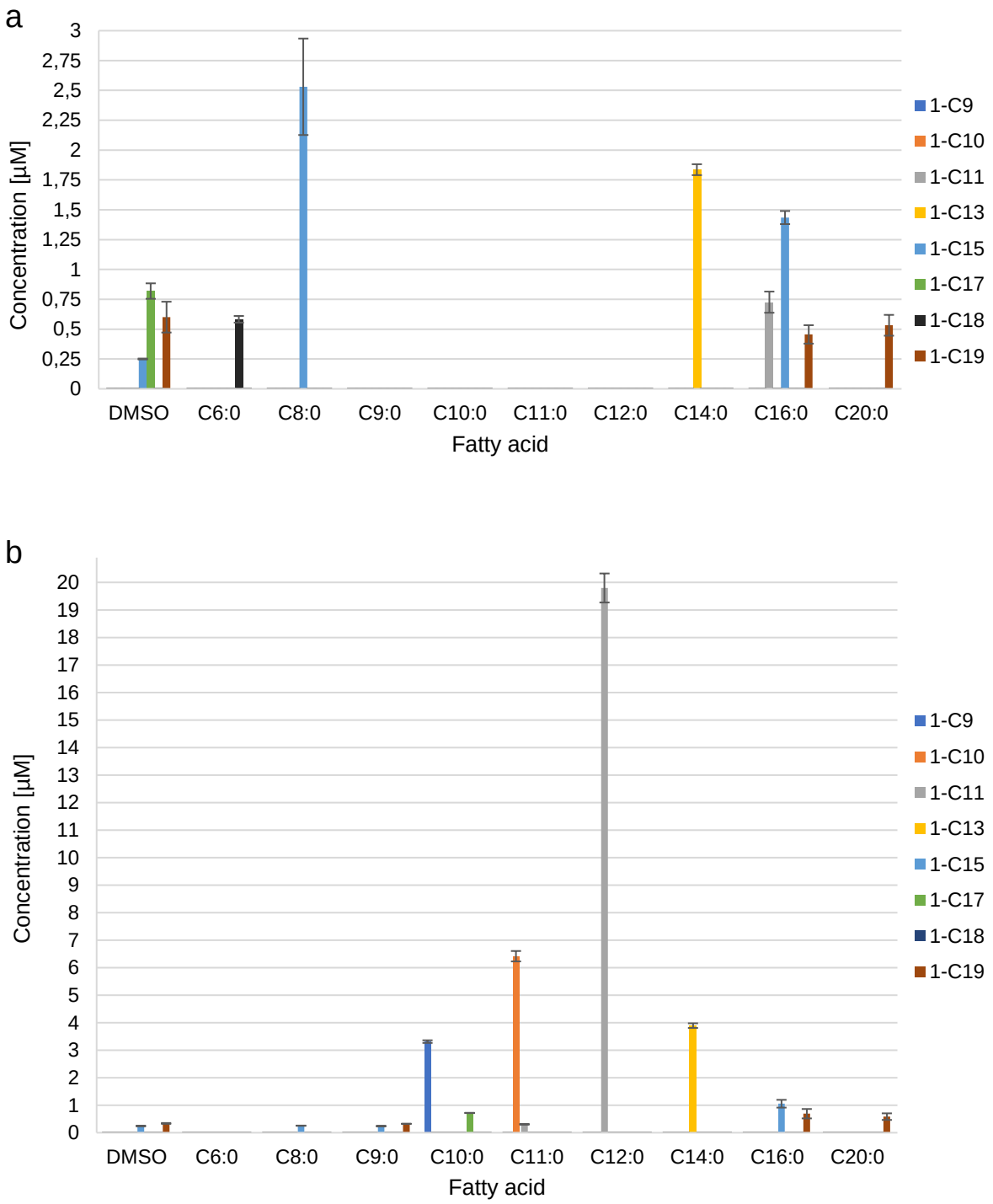


Figure 1: Feeding fatty acids in the concentration of (a) 0.5% (v/v) (13.18 to 39.99 mM) and (b) 200 µM led to the production of 1-alkenes in *Jeotgalicoccus* sp. ATCC 8456 cultures. The concentrations of the produced 1-alkenes are shown in this diagram.

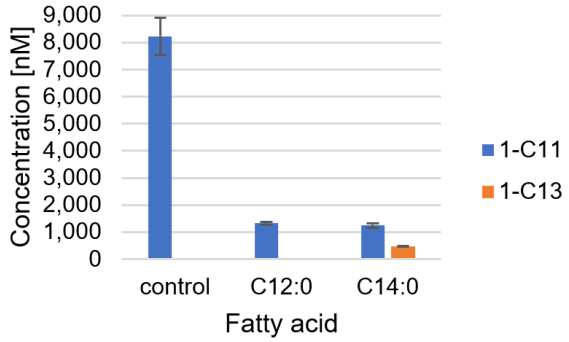
The results of the two different fatty acid concentrations differ significantly in the employed model organism *Jeotgalicoccus* sp. ATCC 8456 as shown in Figure 1. Feeding 0.5% fatty acids, the highest 1-alkene concentration was achieved with octanoic acid leading to 2530.21 nM 1-pentadecene (1-C15) which was 10-fold higher

compared to the control. The concentration of 1-pentadecene was also comparably high using hexadecenoic acid, which would be the expected product considering the decarboxylation of the fatty acid. This is also true for feeding tetradecanoic acid and conversion to 1-tridecene, which could be detected only upon feeding and not in the control.

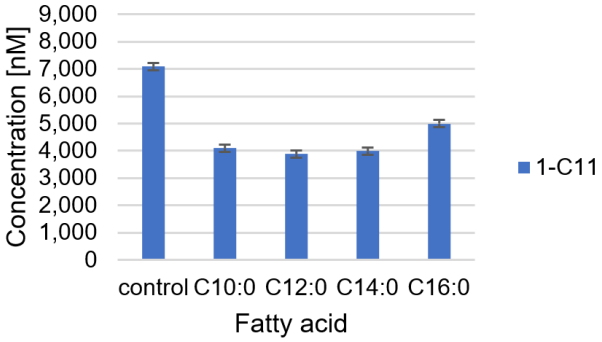
In the 200 µM fatty acid feeding experiment using *Jeotgalicoccus* cultures, the highest product concentration was obtained by feeding dodecanoic acid. In this case, the produced 1-undecene was 1,000-fold higher than in the control. In significantly higher concentrations compared to the control, the expected 1-alkene products were obtained when feeding decanoic acid, undecanoic acid, dodecanoic acid, tetradecanoic acid and hexadecenoic acid (Figure 1).

The influence of feeding fatty acids was also tested in the *Pseudomonas* strains and the *Bacillus thuringiensis* species identified to be able to produce 1-alkenes. Here, the same fatty acid concentrations were tested as with *Jeotgalicoccus*. Looking on the 1-alkene concentrations achieved with *Jeotgalicoccus*, higher output was achieved using 200 µM fatty acid, especially considering the dodecanoic acid, but also the tetradecanoic acid feeding. In the *Pseudomonas* and the *Bacillus* cultures, the results of the 0.5% feeding showed that feeding tetradecanoic acid led to the expected 1-tridecene production, whilst no 1-tridecene was detected in the control. For *Pseudomonas fluorescens* and *Pseudomonas putida*, the production of 1-undecene was also increased if any fatty acid was fed. Feeding 200 µM fatty acids to the *Pseudomonas* and the *Bacillus* cultures showed similar results for 1-undecene, but other than in the *Jeotgalicoccus* cultures, the production was not significantly increased. Also, feeding decanoic acid, tetradecanoic acid and hexadecanoic acid did not lead to the production of the expected 1-alkenes, like it did in the 0.5% feeding. Nevertheless, the 1-undecene production was successfully increased when feeding fatty acids in both concentrations to *Pseudomonas fluorescens* (up to 180%) and *Pseudomonas putida* (up to 220%). The results of the fatty acid feeding with the *Pseudomonas* and the *Bacillus* cultures are summarized in Figure 2.

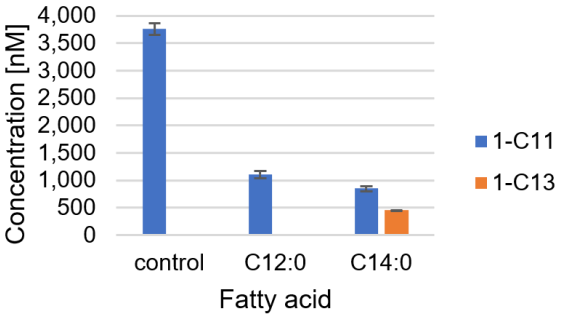
1a *Bacillus thuringiensis* (0.5% fatty acid)



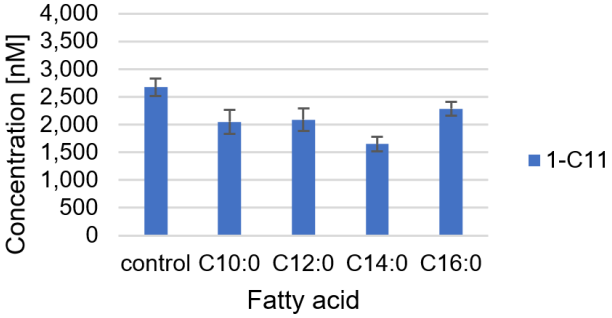
1b *Bacillus thuringiensis* (200 µM fatty acid)



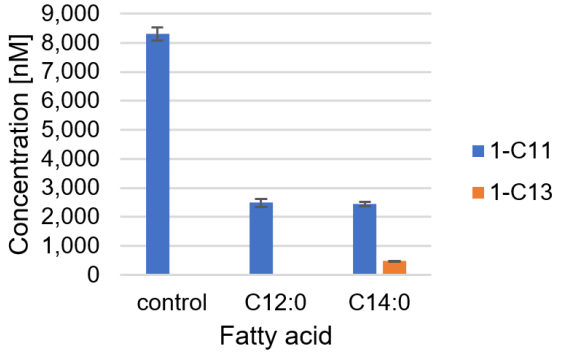
2a *Pseudomonas aeruginosa* (0.5% fatty acid)



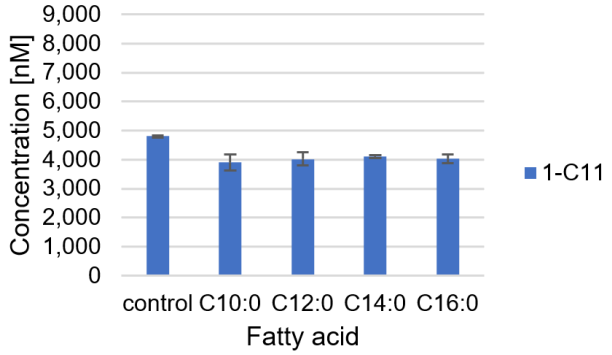
2b *Pseudomonas aeruginosa* (200 µM fatty acid)



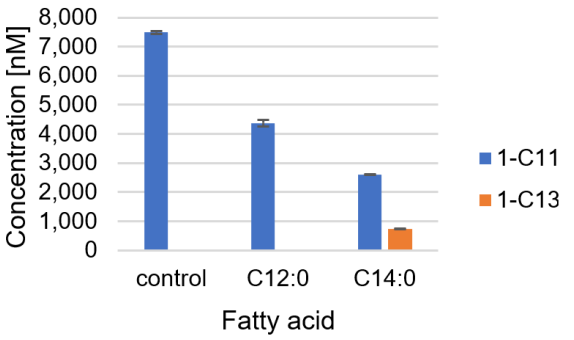
3a *Pseudomonas brassicacearum* (0.5% fatty acid)



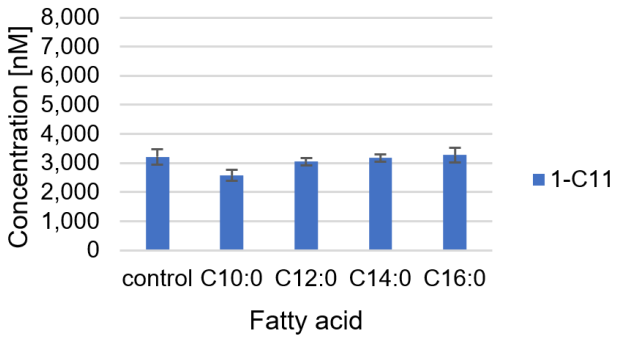
3b *Pseudomonas brassicacearum* (200 µM fatty acid)



4a *Pseudomonas brenneri* (0.5% fatty acid)



4b *Pseudomonas brenneri* (200 µM fatty acid)



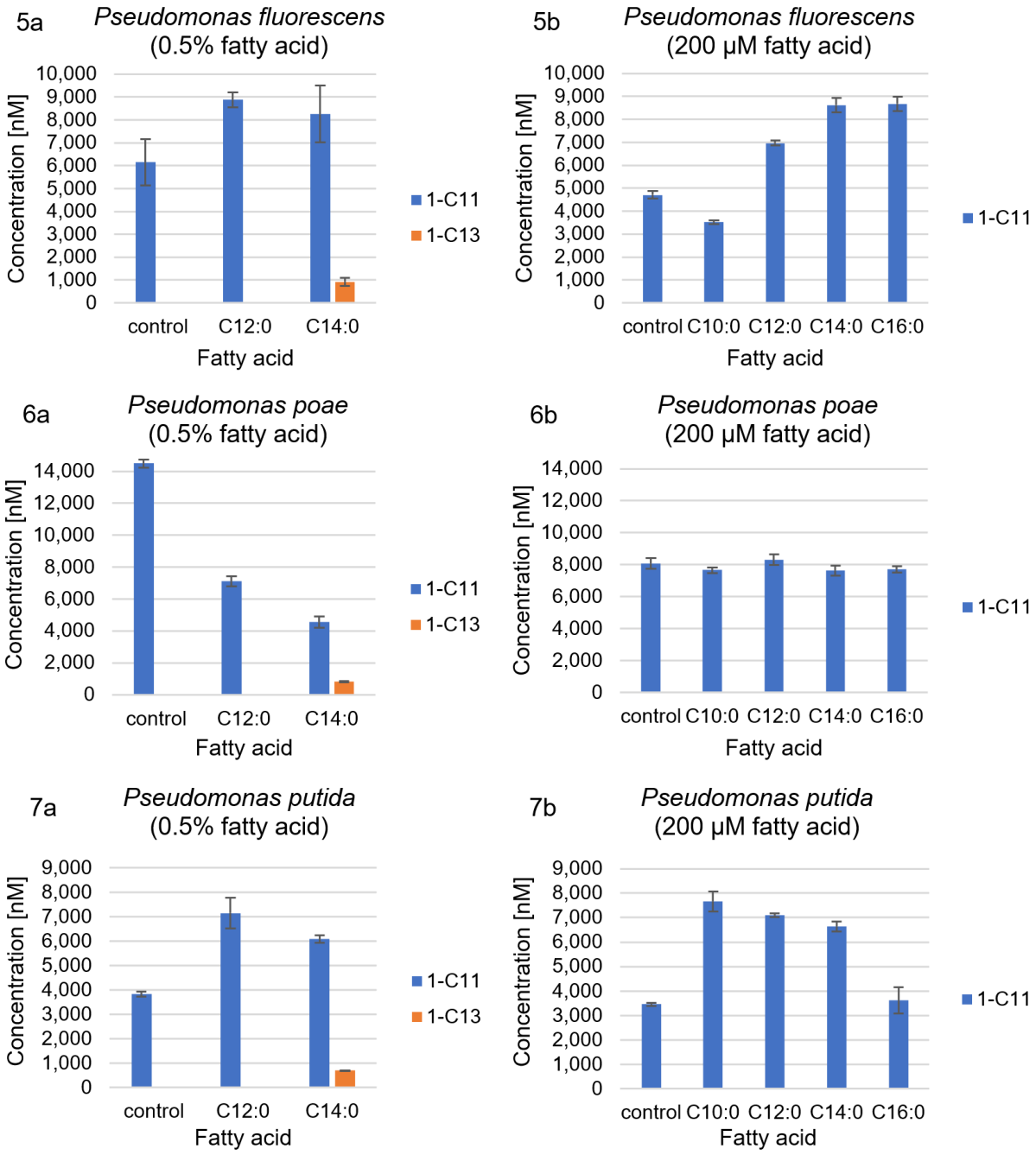
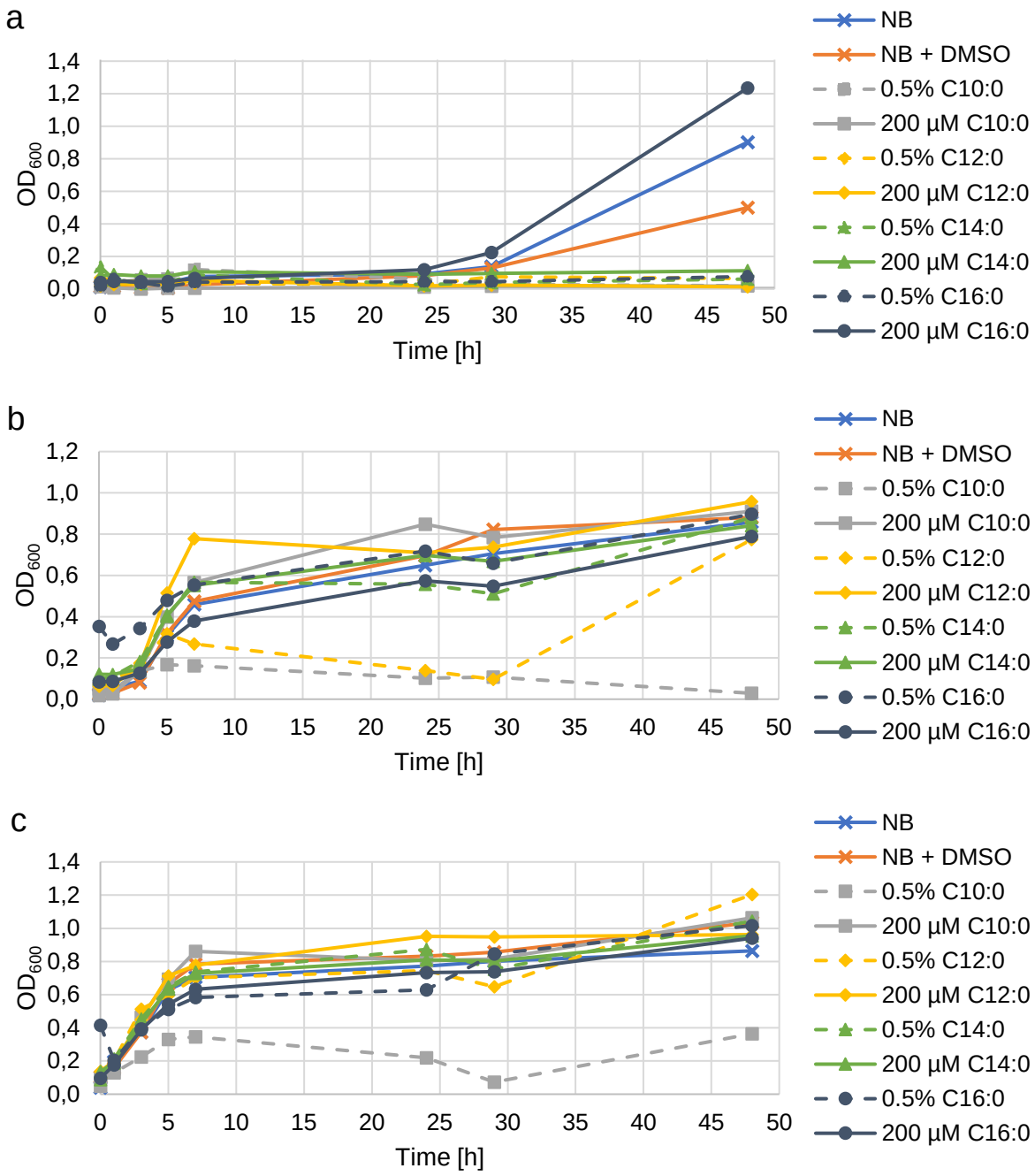


Figure 2: Feeding fatty acids to different bacterial strains (1: *Bacillus thuringiensis*; 2: *Pseudomonas aeruginosa*; 3: *Pseudomonas brassicacearum*; 4: *Pseudomonas brenneri*; 5: *Pseudomonas fluorescens*; 6: *Pseudomonas poae*; 7: *Pseudomonas putida*) in concentrations of (a) 0.5% (v/v; 13.18 to 39.99 mM) and (b) 200 µM led to *in vivo* production of certain 1-alkenes. The determined 1-alkene concentration are shown in this figure.

3.3 Growth experiments

The effect of adding fatty acids to the cultivation media on the bacterial growth was evaluated. At first, the growth experiments were performed in very small scale, using 150 µL growth medium mixed with 0.5% (Table 2) or 200 µM fatty acids and inoculated

with 1-alkene producing bacteria (Table 1). At certain time points, the OD_{600} was determined to analyze the influence of fatty acids on the bacterial growth (Figure 3).



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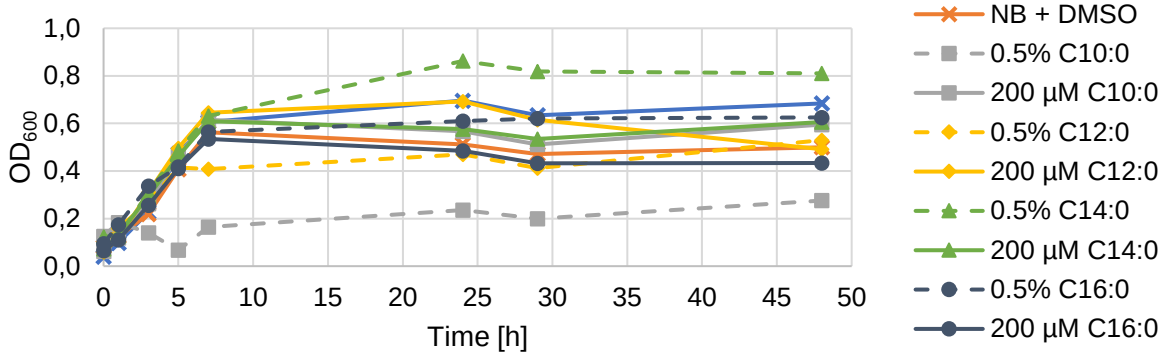
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0,4
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OD600
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30
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Time [h]
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NB + DMSO
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200 µM C10:0
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200 µM C12:0
0.5% C14:0
200 µM C14:0
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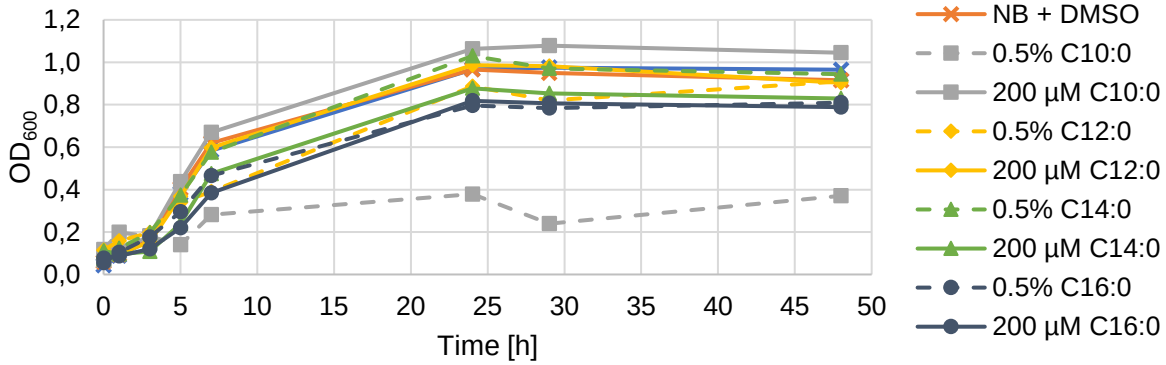
200 µM C16:0

e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OD600
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Time [h]
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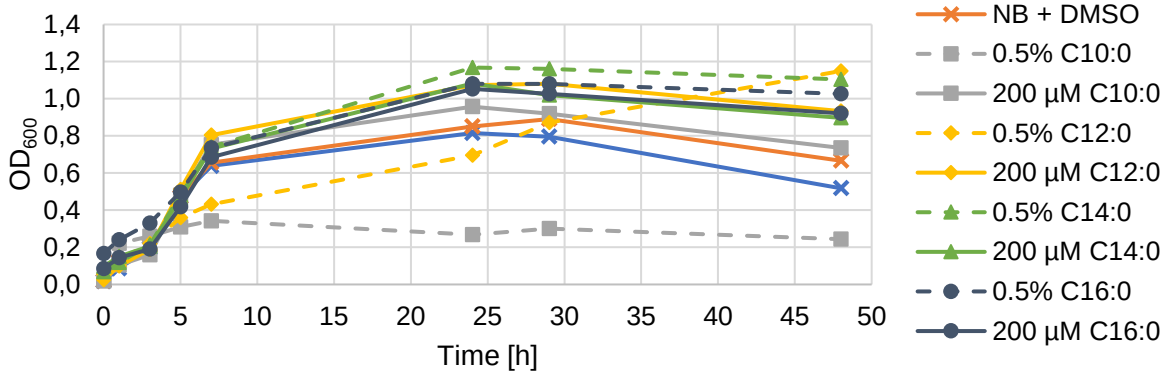
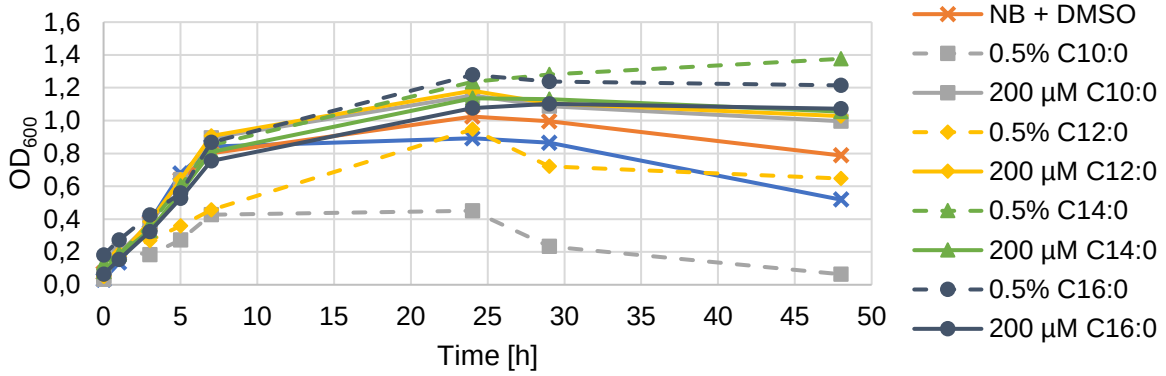
NB + DMSO
0.5% C10:0
200 µM C10:0
0.5% C12:0
200 µM C12:0
0.5% C14:0
200 µM C14:0
0.5% C16:0
200 µM C16:0

f


1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
OD600
0
5
10
15
20
25
30
35
40
45
50
Time [h]
NB
NB + DMSO
0.5% C10:0
200 µM C10:0
0.5% C12:0
200 µM C12:0
0.5% C14:0
200 µM C14:0
0.5% C16:0
200 µM C16:0

g


1,6
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OD600
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25
30
35
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45
50
Time [h]
NB
NB + DMSO
0.5% C10:0
200 µM C10:0
0.5% C12:0
200 µM C12:0
0.5% C14:0
200 µM C14:0
0.5% C16:0
200 µM C16:0

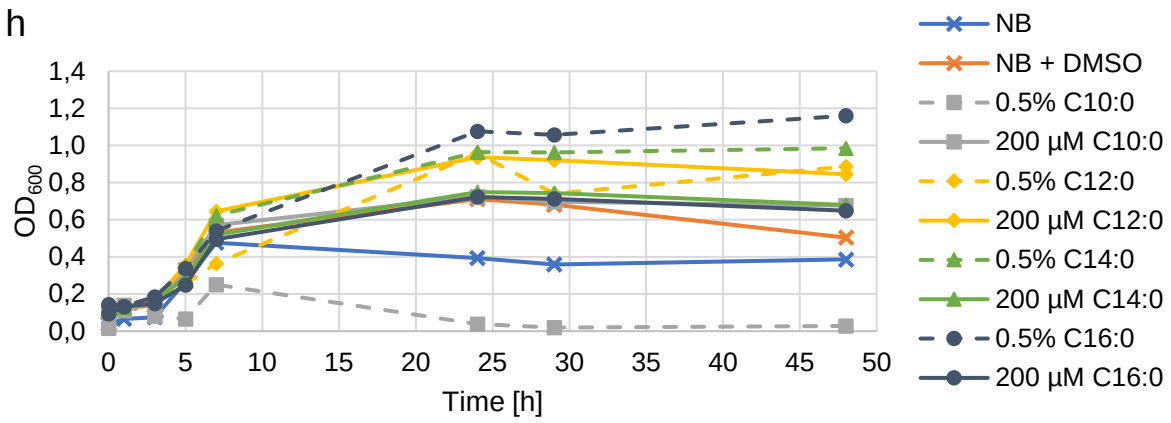

Figure 3: Small scale growth experiments in 96-well plates using cultivation media mixed with fatty acids in the concentrations of 0.5% (v/v; 13.18 to 39.99 mM) and 200 µM. Evaluated strains: a) *Jeotgalicoccus* sp. ATCC 8456, b) *Bacillus thuringiensis*, c) *Pseudomonas aeruginosa*, d) *Pseudomonas brassicacearum*, e) *Pseudomonas brenneri*, f) *Pseudomonas fluorescens*, g) *Pseudomonas poae*, h) *Pseudomonas putida* (Table 1);

For *Jeotgalicoccus*, the time frame was too short in this experiment to show the influence on the growth completely. Nevertheless, there was a tendency of better growth in media without fatty acid supplementation. The addition of DMSO as cosolvent seemed to slow down growth. Interestingly, the highest OD_{600} was measured in TSBYE mixed with 200 µM hexadecanoic acid. For the rest of the cultures, only little growth of *Jeotgalicoccus* was observed.

For all other cultures the presence of 0.5% decanoic acid showed a negative effect: the growth was much lower compared to the control and to the other fatty acids. Even the sample with 200 µM decanoic acid did not show the same effect on bacterial growth. For the rest of the samples, an interpretation of the results is difficult.

In general, there were some issues with the growth experiment in the 96-well plates. The plates were incubated at 30°C and although they were wrapped up with parafilm, evaporation of media took place. Also, the fatty acids did not fully dissolve in the media, especially in higher concentrations like in case of the 0.5%, which might have influenced the measurement of the optical density, meaning that the results should be interpreted with care and the values obtained for the 0.5% samples could be too overestimated. Therefore, a second growth experiment was performed in bigger scale using 20 mL cultures in 100 mL flasks. This experiment was done with *Bacillus thuringiensis, Pseudomonas fluorescens* and *Jeotgalicoccus* sp. ATCC 8456 (Table ***1***). The experiment in bigger scale showed clear differences compared to the experiments performed in 96-well plates (Figure 4, Figure 5, Figure 6). Furthermore, a

negative effect of DMSO, which was used to dissolve and dilute the fatty acids, could be excluded.

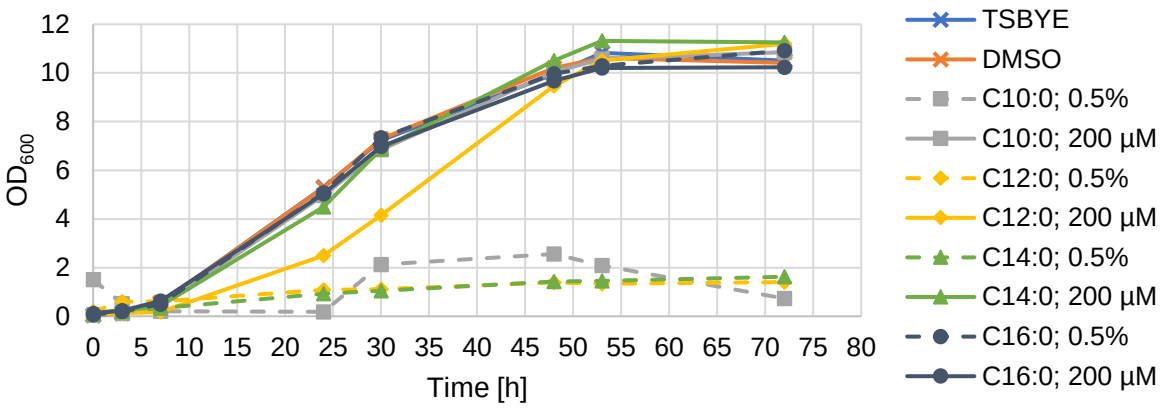

Figure 4: Growth experiments with *Jeotgalicoccus* sp. ATCC 8456 in 100 mL flasks using 20 mL cultivation media mixed with fatty acids in the concentrations of 0.5% and 200 µM.

The growth experiment in the 100 mL flasks was prolonged for *Jeotgalicoccus* sp. ATCC 8456 compared to the first experiment (Figure 4). Except from 0.5% hexadecanoic acid, providing fatty acids in higher concentration (0.5%, Table 2) led to significant decrease in growth. It should be highlighted that 200 µM dodecanoic acid led also to a slowdown in growth. In the fatty acid feeding experiments, it was shown that feeding dodecanoic acid at this very same concentration led to highly increased (1,000-fold) 1-undecene production. This finding, together with the results of the growth experiments, suggest that the metabolism of *Jeotgalicoccus* sp. ATCC 8456 is triggered for production of 1-alkenes instead of biomass when feeding dodecanoic acid.

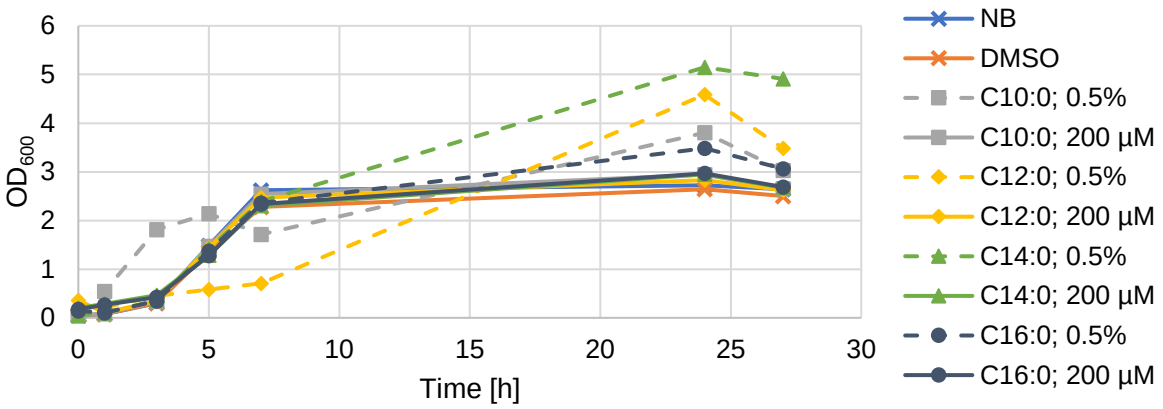

Figure 5: Growth experiments with *Bacillus thuringiensis* in 100 mL flasks using 20 mL cultivation media mixed with fatty acids in the concentrations of 0.5% and 200 µM.

As shown in Figure 5, the growth of *Bacillus thuringiensis* was only lower for decanoic acid compared to the control, but only in the beginning. Instead, higher OD_{600} was measured for all fatty acids provided in the concentration of 0.5%. However, it might be possible that turbidity was increased due to the presence of undissolved fatty acids, disturbing the OD_{600} measurements.

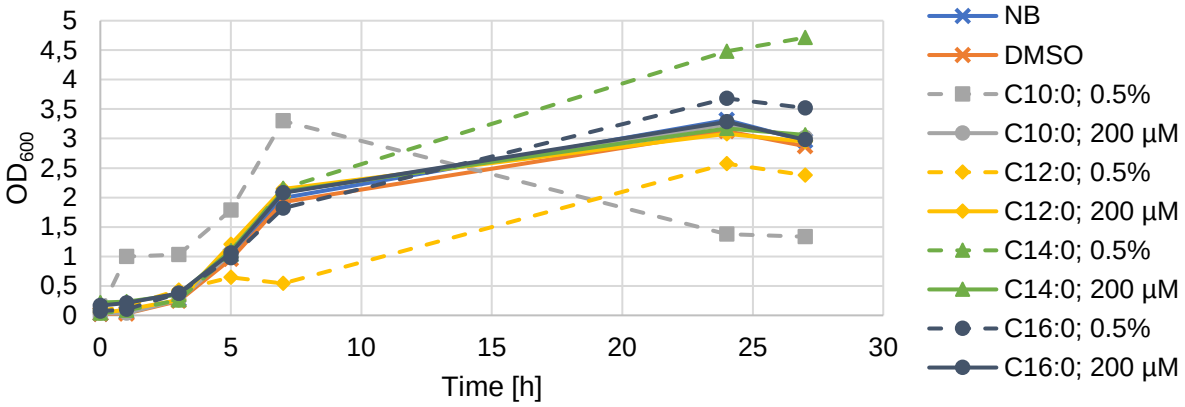

Figure 6: Growth experiments with *Pseudomonas fluorescens* in 100 mL flasks using 20 mL cultivation media mixed with fatty acids in the concentrations of 0.5% and 200 µM.

For *Pseudomonas fluorescens*, the growth was decreased with decanoic acid and dodecanoic acid in a concentration of 0.5%. The measured OD_{600} was for the 0.5% decanoic acid culture higher in the beginning, compared to the rest. But again, this might be an artefact of the OD measurement due to the low solubility of the decanoic acid, leading to higher turbidity; over time, the concentration of the fatty acid might decrease, with concomitant increase in solubility. Like for the *Bacillus thuringiensis*

cultures, the highest OD_{600} was measured in the 0.5% tetradecanoic acid culture. Also, the 0.5% hexadecanoic sample reached higher OD_{600} values than the rest Figure 6.

3.4 PCR-based screening

In order to do a proper PCR based screening, cell lysis, reaction content and PCR conditions for the UndA, UndB and $OleT_{JE}$ primer pairs had to be optimized, because degenerate primers are designed based on sequence homology, it is expected that with these primers not only the known UndA, UndB and $OleT_{JE}$ gene sequences can be amplified, but also similar variants. Since certain strains are known or expected to contain these genes, they could be employed as positive controls (*Jeotgalicoccus* sp. ATCC 8456 carries the $OleT_{JE}$ gene; *Pseudomonas* sp. are expected to carry UndA and UndB gene homologs).

3.4.1 Optimization of the PCR conditions and cell lysis method

The first attempts to amplify UndA, UndB and $OleT_{JE}$ genes using degenerate primers were partially successful only for UndA. Here a standard PCR program was employed (Table 6, A). For the reactions (Figure 7) containing DNA from *P. brenneri* (4), *P. fluorescens* (5) and *P. poae* (6) PCR products with the expected size were obtained.

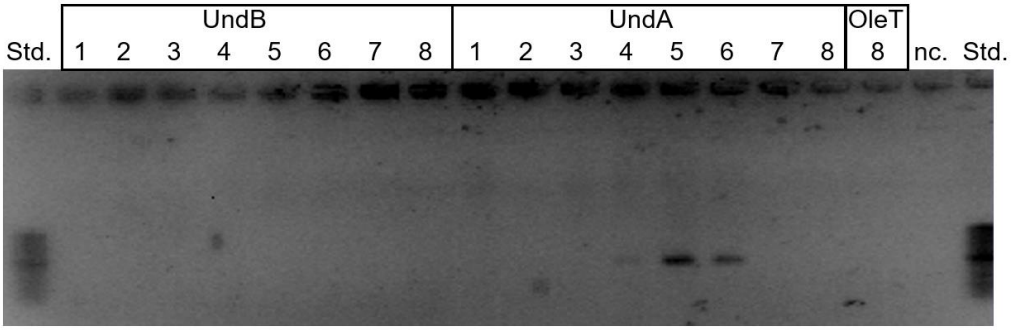

Figure 7: Results of a standard PCR using degenerate primers (Table 3). Lysed cell material from solid cultures of the following bacterial strains was used as template: *Bacillus thuringiensis* (1), *Pseudomonas aeruginosa* (2), *Pseudomonas brassicacearum* (3), *Pseudomonas brenneri* (4), *Pseudomonas fluorescens* (5), *Pseudomonas poae* (6), *Pseudomonas putida* (7) and *Jeotgalicoccus* sp. (8) (Table 1); nc.: negative control (without template). As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

In order to address the different cell morphology of *Jeotgalicoccus* sp. ATCC 8456, which might have caused trouble during the cell lysis, the next PCR was done with a template obtained by microwave cell lysis (Chapter 2.4.2.2). Simultaneously, a temperature gradient was applied to optimize primer annealing (Table 6, B). For all other 1-alkene producing strains a temperature gradient PCR was tested as well,

adapting the elongation times (45 and 40 s; Table 6, B) for UndA and UndB primers, respectively. The same standard reaction mix was used except from the template, which was gained in this case using the homogenization method. For none of the genes a PCR product was obtained using this temperature gradient PCR program (Figure A1, Figure A2). Another temperature PCR using UndB primers was performed with a broader temperature gradient (Table 6, C). A contamination occurred and the bands obtained occurred not at the expected height (Figure A3).

Further PCRs utilizing different polymerases (One Taq®, Taq-&GO™) did not work as well (Figure A4). Since amplification with the same primers worked for some of the strains before (*P. brenneri, P. fluorescens, P. poae*), it was assumed that further optimization of the conditions could lead to successful amplification. This prompted the idea to apply a touchdown PCR using UndA and UndB primers (Table 6, D). Here, the annealing temperature was set to decrease 0.2°C every cycle until 45°C were reached at cycle 30. On the gel, a slight band with expected size was obtained for *Pseudomonas fluorescens* in case of the PCR performed using degenerate UndA primers (Figure 8). All other samples for UndA and for UndB as well did not work out with the touchdown approach.

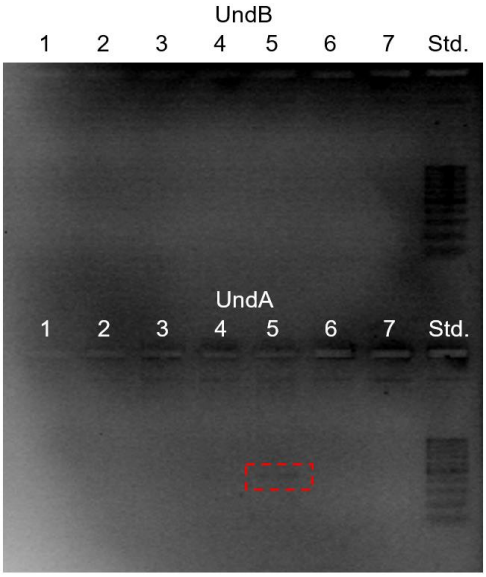

Figure 8: Result of a touchdown PCR using degenerate UndA and UndB primers (Table 3). The red rectangle highlights the slight band obtained with the *Pseudomonas fluorescens* template. Lysed cell material from solid cultures of the following bacterial strains was used as template: *Bacillus thuringiensis* (1), *Pseudomonas aeruginosa* (2), *Pseudomonas brassicacearum* (3), *Pseudomonas brenneri* (4), *Pseudomonas fluorescens* (5), *Pseudomonas poae* (6) and *Pseudomonas putida* (7) (Table 1). As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

The results from another temperature gradient PCR with UndA primers and *P. fluorescens* as template shed some light on the inconsistency of the applied cell lysis methodology. In this experiment, the cell lysis was done twice, in separate PCR tubes. One of the supernatants of the duplicates was used as template in the temperature gradient PCR (Table 6, C). The other supernatant was then used in a separate reaction mixture only amplified at 50.5°C. Interestingly, no PCR product was obtained throughout the whole gradient, but the expected band was determined in the PCR performed separately with no temperature gradient (Figure 9). This is exemplary for the inconsistency of the lysis method and using the supernatant as template for PCR.

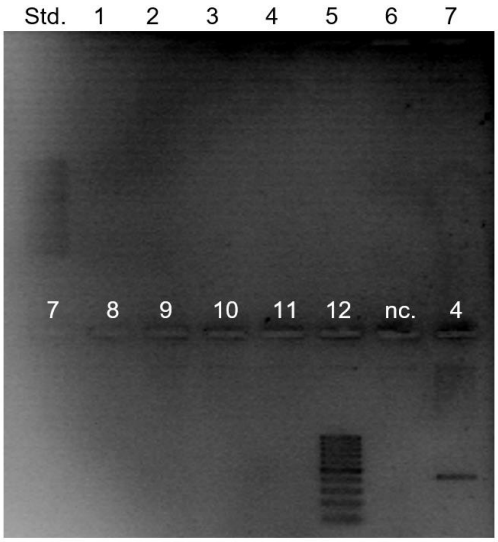

Figure 9: Gel picture obtained after separating the products of a temperature gradient PCR. Temperature gradient 45°C-72°C: 1: 45.0°C; 2: 45.6°C; 3: 47.5°C; 4: 50.5°C; 5: 53.7°C; 6: 56.9°C; 7: 60.1°C; 8: 63.3°C; 9: 66.5°C; 10: 69.5°C; 11: 71.4°C; 12: 72.0°C; nc.: negative control at 45.0°C (without template). As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

In order to evaluate the effect of the template concentration on the amplification, different template volumes were tested next (1 to 3 µL of cell lysate). This was tested in standard PCRs for UndA and UndB with template DNA obtained from *Pseudomonas fluorescens* using the homogenization method. The expected band was obtained for UndA and using only 1 µL of template (Figure 10).

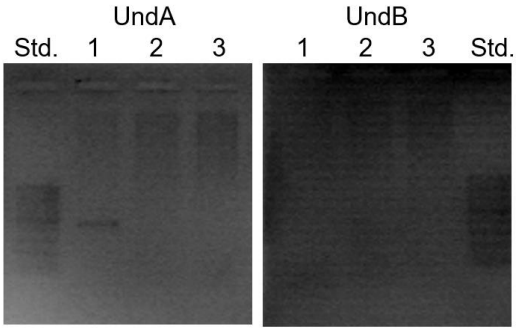

Figure 10: Gel-picture from a standard PCR using degenerate UndA and UndB primers (Table 3). Different amounts of template were used in this case: 1: 1µL template; 2: 2µL template; 3: 3µL template; As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

Again, to optimize the annealing temperature for the degenerate UndA primers, a temperature gradient (45°C-68°C) PCR with template DNA from *P. fluorescens* was performed (Table 6, E). For the degenerate UndA primers, an optimal annealing temperature of 51.2°C and the optimal PCR program could be defined (Figure 11).

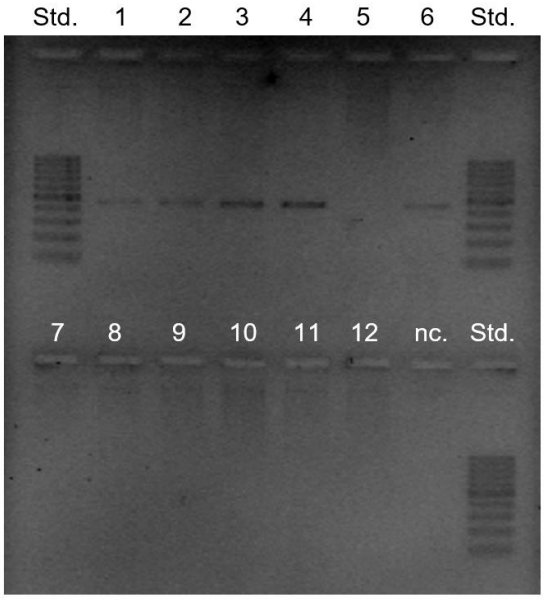

Figure 11: Temperature gradient PCR products separated by gel-electrophoresis. Degenerate UndA primers (Table 3) were used for this PCR. Temperature gradient 45°C-68°C: 1: 45.0°C; 2: 46.1°C; 3: 48.7°C; 4: 51.2°C; 5: 53.5°C; 6: 55.3°C; 7: 57.0°C; 8: 58.8°C; 9: 60.8°C; 10: 63.7°C; 11: 66.2°C; 12: 67.8°C; nc.: negative control at 45.0°C (without template). As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

Using the PCR reaction mix which was successfully working with the UndA primers, a temperature gradient PCR and a touchdown PCR were performed with degenerate UndB primers (Table 6, E and F, respectively). Again, the template PCR was obtained

from a solid *P. fluorescens* and *P. poae* culture. Unluckily, no bands but only smear was obtained in the temperature gradient PCR (Figure 12).

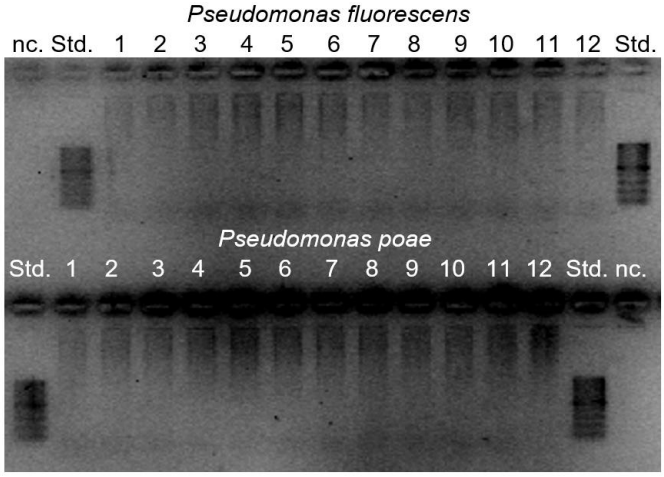

Figure 12: Temperature gradient PCR products separated by gel-electrophoresis. Degenerate UndB primers (Table 3) were used for this case. Temperature gradient 45°C-68°C: 1: 45.0°C; 2: 46.1°C; 3: 48.7°C; 4: 51.2°C; 5: 53.5°C; 6: 55.3°C; 7: 57.0°C; 8: 58.8°C; 9: 60.8°C; 10: 63.7°C; 11: 66.2°C; 12: 67.8°C; nc.: negative control at 45.0°C (without template). As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

Figure 13 shows the result of the touchdown PCR. Again, only smear and no bands are shown on the gel.

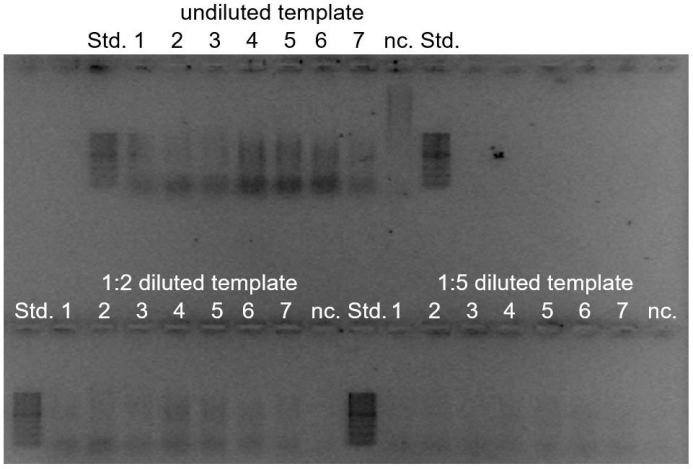

Figure 13: Gel picture obtained after separating the products of a touchdown PCR with degenerate UndB primers (Table 3). The template was used undiluted, diluted 1:2 and diluted 1:5. Lysed cell material from solid cultures of the following bacterial strains was used as template: *Bacillus thuringiensis* (1), *Pseudomonas aeruginosa* (2), *Pseudomonas brassicacearum* (3), *Pseudomonas brenneri* (4), *Pseudomonas fluorescens* (5), *Pseudomonas poae* (6) and *Pseudomonas putida* (7) (Table 1); nc.: negative control (without template). As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

Furthermore, a 2-step PCR was done with the UndB primers with a touchdown PCR (55-46°C) as second step (Table 6, G). The template was obtained by denaturation at 98°C, like described in 2.4.2.3. Undiluted and 1:2 diluted supernatant was used as template DNA. The 2-step PCR approach did not work out as well, as no bands were obtained (Figure A5).

Next, a temperature gradient PCR was done with the OleT primers (Table 6, E). The template DNA was obtained from *Jeotgalicoccus* sp. ATCC 8456 using the homogenization method. No bands could be determined on the gel after separating the PCR products by electrophoresis (Figure A6).

Then, two lysis methods were tested in a touchdown PCR (Table 6, F). The denaturation at 98°C (2.4.2.3) and the denaturation at 98°C combined with lysozyme treatment (2.4.2.4). Additionally, to test if the lysis method or the primers were the source of the problems during the PCRs, BASYS00026_Flav primers (Table 4) were used, which are known to work with the *Jeotgalicoccus* sp. ATCC 8456 template DNA. And indeed, bands were only obtained for the BASYS00026_Flav primers (Figure 14) meaning that the degenerate OleT primers used in this PCR did not work most probably. Also, the denaturation with lysozyme did not show any products. Therefore, lysozyme was no used again in following cell denaturation reactions.

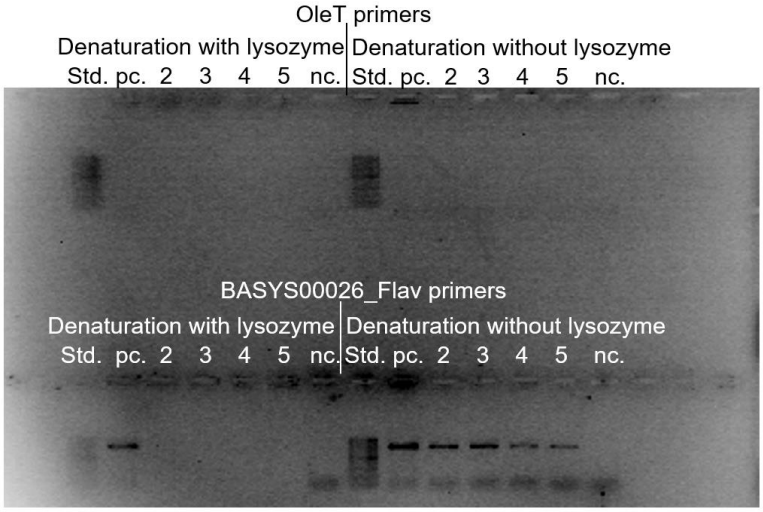

Figure 14: Gel picture obtained after separating the products of a touchdown PCR using degenerate OleT primers (Table 3) and BASY00026_Flav primers (Table 4). The template was used undiluted (2), diluted 1:2 (3), diluted 1:5 (4) and diluted 1:10 (5). Lysed cell material from a solid culture of *Jeotgalicoccus* (Table 1) was used as template. For the positive control (pc.), isolated genomic DNA from *Jeotgalicoccus* (Table 1) was used. nc.: negative control (without template). As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

Due to no positive results with the UndB and the OleT primers, new ones were designed (Table 5). Using these new primers, two temperature gradient PCRs were performed (Table 6, H). The evaluation of the PCR results showed, that the optimal annealing temperature for the new OleT primers was 46.1 °C and for the new UndB primers, the optimal annealing temperature was between 55.3°C and 57.0°C (Figure 15). In case of the OleT primers however, the cycle number was increased to 35 and the elongation time was prolonged to 1 min 10 s for future PCRs, because the bands were weak and for the screening stronger bands are required to detect possible $OleT_{JE}$ homologous genes.

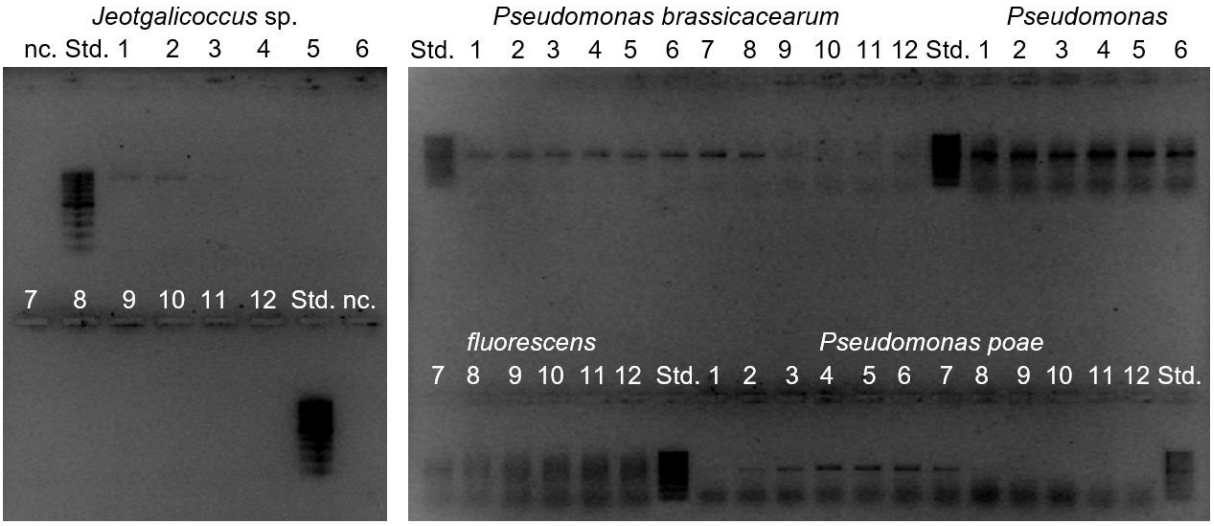

Figure 15: Temperature gradient PCR products separated by gel-electrophoresis. Degenerate OleT and UndB primers (Table 5) were used for this case. Temperature gradient 45°C-68°C: 1: 45.0°C; 2: 46.1°C; 3: 48.7°C; 4: 51.2°C; 5: 53.5°C; 6: 55.3°C; 7: 57.0°C; 8: 58.8°C; 9: 60.8°C; 10: 63.7°C; 11: 66.2°C; 12: 67.8°C; nc.: negative control at 45.0°C (without template). As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

3.4.2 Screening of a metagenomic fosmid clone library from Sphagnum moss

Over 9,000 clones of a metagenomic clone library originated from *Sphagnum* moss were screened for UndA, UndB and $OleT_{JE}$ homologous genes using the following degenerate primers: UndA_Fwd_deg & UndA_Rev_deg (Table 3), UndB_Fwd_deg2 & UndB_Rev_deg2 and OleT_Fwd_deg2 & OleT_Fwd_deg2 (Table 5). No positive hits were obtained for OleT, but for UndA and UndB two positive hits each were found in the metagenomic library: E5-119 and A11-112 for UndA; A6-156 and E7-156 for UndB. The positive library hits and all other 1-alkene producing strains (Table 1) except from *Jeotgalicoccus* were screened again for UndA and UndB homologous genes to confirm previous PCR results. Except from the UndB homologous gene in *Bacillus*

thuringiensis, every other PCR result could be reproduced (Figure 16). In case of *Pseudomonas brassicacearum*, the PCR was repeated with newly streaked out cell material to reproduce positive PCR results.

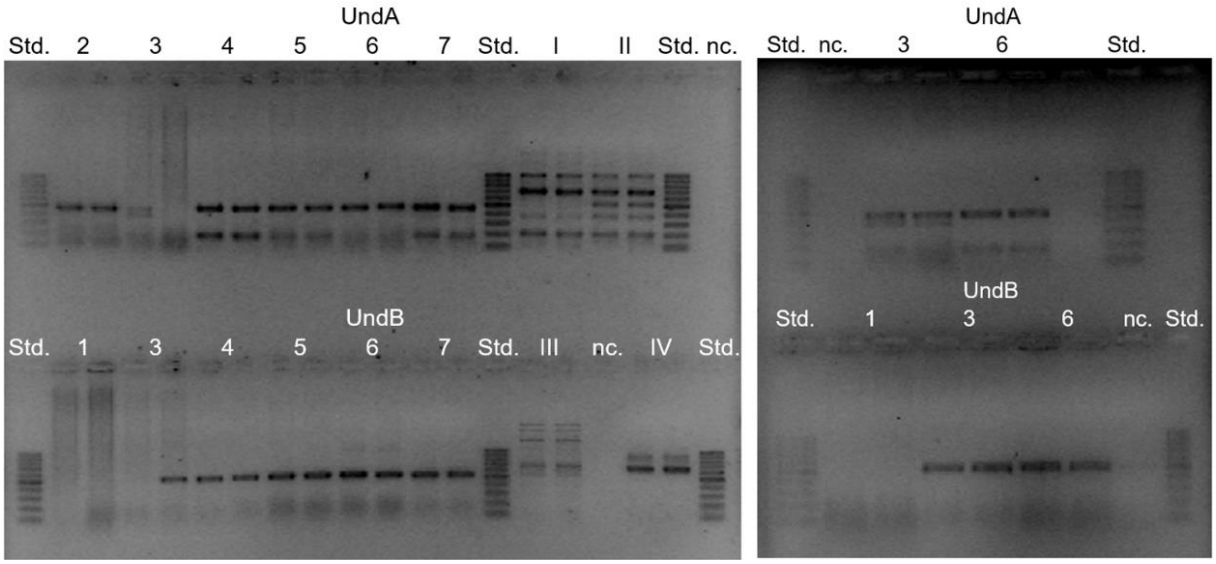

Figure 16: Result of a standard PCR using degenerate UndA (Table 3) and UndB primers (Table 5). The templates were obtained from the following bacterial strains: *Bacillus thuringiensis* (1), *Pseudomonas aeruginosa* (2), *Pseudomonas brassicacearum* (3), *Pseudomonas brenneri* (4), *Pseudomonas fluorescens* (5), *Pseudomonas poae* (6), *Pseudomonas putida* (7) (Table 1), and also from the following metagenomic library clones: E5-119 (I), A11-112 (II), E5-119 (III) and A11-112 (IV); nc.: negative control (without template). As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

3.5 Sequencing

All PCR products shown in Figure 16 were cut from the gel and purified before sending them for sequencing. The same primers as for the PCRs were used for sequencing: UndA_Fwd_deg/UndA_Rev_deg (Table 3), UndB_Fwd_deg2/UndB_Rev_deg2 and OleT_Fwd_deg2/OleT_Fwd_deg2 (Table 5). These degenerate primers were designed binding to conserved regions embedded in the sequence and therefore, using them only part of the protein-coding sequence (ORF) was obtained. Using the obtained, partial sequences, new primers were designed pointing outwards to obtain the whole ORFs (Table 7). Doing so, sequences of different sizes were obtained, which were aligned, and the consensus sequences were then used for bioinformatic analysis.

For clone A11-112, the first three hits in a blastn and blastx search (NCBI) were all designated as “DNA topoisomerase IV subunit A” or “topoisomerase IV subunit A” with the following GenBank accession numbers: ATQ42752.1, ACG78276.1 and

ATC31599.1. The same results were also obtained for blast analysis of the clone E5-119 sequence, showing that the same sequences were obtained in A11-112 and E5-119. This was also proven by sequence alignment. The screening for UndA homologous genes in the fosmid clone library using the highly degenerate primers did not work out, instead other sequences were amplified in the PCR screening approach, most likely due to the high number of degenerate bases. To prove this, the A11-112 sequence was also used for homology modeling, where also the “topoisomerase IV subunit A” was the top hit with only 27% identity. Screening for UndB homologs, the result looked similar and no UndB homologous gene could be found in the metagenomic library using the degenerate primers. For the clone E7-156, the first three hits doing a blastx were “nucleoside-diphosphate kinase” with the GenBank accession numbers: PAW76678.1, WP_142525840.1 and WP_024806956.1. For A6-156, the first two hits were “trehalose-6-phosphate hydrolase” and “PTS trehalose transporter subunit IIBC” with the following GenBank accession numbers: STF28441.1 and WP_089705548.1.

Furthermore, partial sequences of UndA and UndB homologous genes were also obtained for some *Pseudomonas* strains. The purified PCR products were sequenced as well. These partial sequences were analyzed using blastn (NCBI) and for each partial sequence, the four sequences showing the highest identity were aligned. Based on the resulting consensus sequence from alignment, primers were designed binding to the beginning and end of the sequences (Table 8). Using them for sequencing followed by another sequencing step with the primers shown in Table 11 the whole sequence was obtained. The sequences were then aligned and the ORFs of the UndA/UndB homologous genes were determined. These ORFs were applied again using blast analysis. Applying the UndA sequences in blastx searches showed that the sequences are not annotated as UndA or some kind of fatty acid decarboxylase, but as “TenA family transcriptional regulator” (WP_057008085.1) or as “Pyrroloquinoline quinone (PQQ) biosynthesis protein C [Coenzyme transport and metabolism]“ (WP_003236680.1) for example. For UndB the situation was different. Applying the obtained sequences for blastx, the sequences found were annotated as “fatty acid desaturase”. UndB was described as fatty acid desaturase for the mechanism of fatty acid decarboxylation and therefore the catalysis of 1-alkene production (Rui et al., 2015). UndB homologs are therefore annotated correctly for their function, contrary to UndA homologs.

Fortunately, there is a crystal structure of *Pseudomonas fluorescens* Pf-5 UndA (PDB accession number 6P5Q) available (Zhang et al., 2019) found by HHBlits (Remmert et al., 2012). Based on this structure, homology modelling was performed to support the assumption that the sequences obtained are homologous to annotated UndA sequences. The homology modelling showed high sequence identity ranging from 89.7 to 90.72% to the UndA sequence from *Pseudomonas fluorescens* Pf-5 for all UndA homologous sequences found in the *Pseudomonas* strains (Table 13). Unfortunately, no crystal structure is annotated for UndB and therefore no homology modeling could be done.

Table 13: Homology modelling results obtained using SWISS-MODEL. The template was a *Pseudomonas fluorescens* Pf-5 UndA (PDB accession number 6P5Q) (Benkert et al., 2011; Bertoni et al., 2017; Bienert et al., 2017; Guex et al., 2009; Waterhouse et al., 2018; Zhang et al., 2019)

Protein	Seq Identity	Coverage	Quality information	
			QSQE	QMEAN
BRA-A	89.66	0.87	0.70	-0.88
BRE-A	90.28	0.98	0.74	-0.78
FLU-A	90.30	0.92	0.72	-0.45
POAE-A	89.87	0.93	0.75	-0.64
PUT-A	90.72	0.92	0.71	-0.52

The UndA and UndB sequences obtained were aligned to look for differences in the DNA sequences. The alignments were then analyzed by pairwise comparison using CLC workbench version 8.1.2. The percentage of identity is shown in Figure 17. The sequence identity between the identified UndA gene homologs from the different *Pseudomonas* strains varied between 66.5 and 96.8%. For UndB the identity varied between 74.7 and 96.6%, meaning that the UndB homologs seemed to be more similar to each other, compared to the UndA homologs. However, the UndA sequence found in *P. brassicacearum* was about 130 bp longer than the rest, explaining the lower identity in this case. This sequence comparisons show that the identified genes differ from each other depending on the bacterial species.

a

		1	2	3	4	5
BRA-A-ORF	1	100,00	66,89	66,48	67,07	66,70
BRE-A-ORF	2	66,89	100,00	85,92	87,76	86,30
FLU-A-ORF	3	66,48	85,92	100,00	87,34	96,77
POAE-A-ORF	4	67,07	87,76	87,34	100,00	87,73
PUT-A-ORF	5	66,70	86,30	96,77	87,73	100,00

b

		1	2	3	4	5
BRA-B-ORF	1	100,00	77,96	78,61	74,66	77,87
BRE-B-ORF	2	77,96	100,00	87,13	80,32	87,04
FLU-B-ORF	3	78,61	87,13	100,00	81,67	96,57
POAE-B-ORF	4	74,66	80,32	81,67	100,00	80,68
PUT-B-ORF	5	77,87	87,04	96,57	80,68	100,00

Figure 17: Percentage identity of the UndA (a) and UndB (b) sequences of *Pseudomonas brassicacearum, Pseudomonas brenneri, Pseudomonas fluorescens, Pseudomonas poae* and *Pseudomonas putida*, determined by alignment pairwise comparison.

4 Discussion

One major goal of this thesis was the investigation of the bacterial 1-alkene production. For a better understanding of the conditions leading to *in vivo* 1-alkene production, reaction engineering was applied.

Previous experiments already showed that it is possible to trigger the production of certain 1-alkenes by targeted fatty acid feeding. In this thesis, fatty acids were fed in lower and higher concentrations. For comparing the product yields of 1-alkenes under different reaction conditions, reliable quantification of the partially medium to highly volatile products is necessary. All measurements of the produced 1-alkenes were done using SPME GC-MS. Although this method shows comparable results to other chromatography methods (De Jager et al., 2008), the stated 1-alkene concentrations should be considered with caution. High standard deviations and variances occurred in the measurements due to the fiber used for enrichment and micro-extraction (alteration of the extraction performance over time). Furthermore, high variability on the cultivation must be considered as well since a complex medium was employed. However, for comparison between samples in the fatty acid feeding and to get an idea of the effect induced by the feeding, this quantification method was proven highly valuable for a comparative study.

Interestingly, *Jeotgalicoccus* sp. ATCC 8456 behaved differently than all other tested strains, as the highest measured 1-alkene production was recorded upon feeding lauric acid in the concentration of 200 µM. Feeding lauric acid in the concentration of 200 µM resulted in a 1000-fold increase of 1-undecene compared to the control, while no 1-undecene was produced in any of the 0.5% fatty acid feeding samples. Besides the expected 1-undecene production with the 200 µM lauric acid feeding, the feeding experiments with the same concentration of arachidonic-, palmitic-, tetradecanoic-, undecanoic- and decanoic acid led to the production of the corresponding and targeted 1-alkenes. This is interesting because $OleT_{JE}$ is known for catalyzing the decarboxylation of medium- and long-chain fatty acids in the range of C_{12}-C_{20} (Belcher et al., 2014; Robinson and Wackett, 2019). In this study however, providing C11:0 and C10:0 as precursor also led to the production of the corresponding (C_{n-1}) 1-alkene *in vivo*, meaning that in its original organism $OleT_{JE}$ has a broader substrate range than known from previous studies. In the 0.5% feeding, only the longer chained fatty acids showed the expected results. The corresponding 1-alkenes 1-nonadecen and

1-pentadecene were produced in similar amount compared to the 200 µM feeding. For 1-tridecene, the measured concentration was lower compared to feeding of 200 µM tetradecanoic acid. In case of feeding 0.5% octanoic acid, production of 1-pentadecanoic acid was induced significantly. In a previous study, the genome of *Jeotgalicoccus* sp. ATCC 8456 was sequenced and the information was applied in bioinformatic analysis. The genome of *Jeotgalicoccus* sp. ATCC 8456 contains genetic information coding for the whole fatty acid metabolism (Friedrich, 2019). Therefore, it can be hypothesized that two molecules of octanoic acid might be combined to hexadecanoic acid before decarboxylation and production of 1-pentadecene.

For all other strains used in the fatty acid feeding, the results looked different. In general, feeding fatty acids did not increase 1-alkene production compared to the control for the tested bacteria, except from *Pseudomonas fluorescens* and *Pseudomonas putida,* where the measured 1-alkene production was up to 180 and 220% higher for both tested concentrations of fatty acids (0.5% and 200 µM). However, the strong increase in production of 1-alkenes achieved with *Jeotgalicoccus* sp. ATCC 8456 at the lower concentration of 200 µM fatty acid feeding could not be measured for all other strains. Although four different fatty acids were used in the 200 µM feeding, only 1-undecene was detected by GC-MS, including the control. In contrary, feeding 0.5% tetradecanoic acid resulted in induction of 1-tridecene production in every strain tested. The fatty acid feeding experiments showed that *Jeotgalicoccus* sp. ATCC 8456 reacted differently compared to the *Pseudomonas* strains and *Bacillus thuringiensis*. Considering also the growth experiments, *J*. sp. ATCC 8456 was more sensitive towards fatty acids in higher concentrations, as 0.5% of C10:0, C12:0 and C14:0 slowed down growth significantly by 650 to 1400%. That was not the case for *Bacillus thuringiensis*, where delayed growth could be determined only after feeding 0.5% of C10:0 and C12:0 (growth decrease by 130 and 320%, respectively after 7 h). *Pseudomonas fluorescens* showed a decreased growth (210 and 120%) when feeding 0.5% C10:0 and C12:0, but it was not as decisive as observed for *J*. sp. ATCC 8456. Although at a much lower level, feeding fatty acids triggered also the production of the corresponding (C_{n-1}) 1-alkenes in *Pseudomonas* strains and *Bacillus thuringiensis* in the end. In conclusion, the question arises why these bacteria can produce 1-alkenes and why fatty acids are inducing their production. Saturated and unbranched fatty acids ranging from eight to 18 carbon atoms have shown antibacterial activity under certain conditions in previous studies (Bergsson et al., 2001; Desbois and Smith, 2010). In this

regard, as already mentioned, the growth experiments with higher concentrations of fatty acids showed reduced bacterial growth. This rises the idea, that 1-alkene production induced by fatty acids might be even a detoxification reaction. However, 1-alkenes are known to have further beneficial and antagonistic effects for bacteria as well.

Comparing only the control samples of the fatty acid experiment shows another difference between the 1-alkene production systems. In case of *Jeotgalicoccus* sp. ATCC 8456, longer chained 1-alkenes were detected, namely 1-pentadecene, 1-hepadecene and 1-nonadecene. In all other strains only 1-undecene was detected when no fatty acid substrate was provided. The difference of the product and substrate range for 1-alkene production might indicate a different biological role for the corresponding organism. *Jeotgalicoccus* sp. ATCC 8456 was first isolated from a Korean fish paste named Jeotgal (Robinson and Wackett, 2019; Yoon, 2003). The production of Jeotgal includes fermentation of fish at high salt concentrations up to 40% (Koo et al., 2016). Consequently, only halotolerant and halophilic organisms can survive in such an environment. Typically, the membrane phospholipid composition changes in response to increased salt concentrations. It is known for halotolerant bacteria, that medium- to long chain hydrocarbons accumulate in the membrane in salty environments (Kates, 1986). In general, two strategies of coping with high salt concentrations are used by bacteria. One strategy is characterized by the production of organic osmotic solutes, which accumulate in the cytoplasm to exclude salts from the cells. The second strategy is the so-called salt-in strategy. Usually, microorganisms using a salt-in strategy cannot survive in low salt media. However, there are also mixed forms of these two strategies (Oren, 2008). *Jeotgalicoccus* sp. ATCC 8456 seems to use a mixed strategy. On the one hand it can definitely grow in low salt media, but on the other hand, enzymes from organisms using the salt-in strategy are known for their increased productivity in higher salt concentrations. This phenomenon was shown for $OleT_{JE}$, since its highest activity was measured with NaCl concentrations ranging from 0.5 to 2.0 M (Jiang et al., 2019). The hydrocarbon metabolism of *Jeotgalicoccus* sp. ATCC 8456, including the production of certain 1-alkenes might be the consequence of the halotolerant properties of *Jeotgalicoccus* sp. ATCC 8456. The biological role of shorter chain 1-alkenes like 1-undecene might be a different one. 1-Undecene belongs to the volatile organic compounds well-known for inter- and intraspecies communication (Schmidt et al., 2016) and is produced by antagonistic bacteria.

Antifungal activity of 1-undecene was already shown in previous studies (Hunziker et al., 2015; Zhou et al., 2014).

Different results of the fatty acids feeding, and the growth experiments might of course be a consequence of different biosynthesis routes as well. The enzyme $OleT_{JE}$ isolated from *Jeotgalicoccus* sp. ATCC 8456 is capable of fatty acid decarboxylation (Rude et al., 2011). Using the degenerate primers designed for the screening, no UndA or UndB was detected in *Jeotgalicoccus* sp. ATCC 8456. $OleT_{JE}$ could not be detected in any other strain than *Jeotgalicoccus* sp. ATCC 8456. UndA was found in every other strain except from *Bacillus thuringiensis*. The abundance of UndA in the genus *Pseudomonas* matched the literature reports (Robinson and Wackett, 2019; Rui et al., 2014). UndB was detected in all strains tested, except from *Jeotgalicoccus* sp. ATCC 8456, *Bacillus thuringiensis* and *Pseudomonas aeruginosa*. Rui et al. mentioned that UndB homologous genes occur in some *Pseudomonas* species, but explicitly wrote that UndB is absent in *Pseudomonas aeruginosa* and *Pseudomonas putida* (Rui et al., 2015). In this project however, an UndB homologous gene was found in *Pseudomonas putida* 1-T1. For every bacterium tested throughout this project, a possible 1-alkene production system was detected, except from *Bacillus thuringiensis*. Neither the degenerate primers for $OleT_{JE}$, nor the degenerate primers for UndA or UndB did work in the PCR amplification with *Bacillus thuringiensis*. Using a homology model of $OleT_{JE}$, Rude et al. identified other 1-alkene producing organisms than *Jeotgalicoccus* sp. ATCC 8456, carrying cyp152 P450s as well. Among them was *Bacillus subtilis.* A fatty acid hydroxylase from *B. subtilis* (GenBank accession number NP_388092) with an identity of 41% to the $OleT_{JE}$ homology model was identified. The authors purified the enzyme and cloned it into *E. coli* for heterologous expression. Applying palmitic acid as substrate, 1-pentadecene was measured *in vivo* and *in vitro*, as expected (Rude et al., 2011). That means, that there are cyp152 P450 genes homologous to $OleT_{JE}$ present in the *Bacillus* genus. The low percentage identity of only 41% between $OleT_{JE}$ and the fatty acid hydroxylase found in *Bacillus subtilis* might explain that the degenerate primers designed based on the $OleT_{JE}$ sequence did not work out in this case. This is a general disadvantage of using sequence- and PCR-based approaches to search for enzymes. During this study only two out of 9,000 screened clones were identified for UndA and UndB each (although false positives), meaning that a very low hit-rate of 0.022% was achieved. Low hit-rates in metagenomic clone library screening are common (Müller et al., 2015). High number of degenerate bases in primers allow

to amplify a broader spectrum of targeted structures. On the other hand, the risk of getting false positives increases, as happened in this study. Like all PCR-based methods, primer bias must be considered as well, and such a screening strategy can never reflect actual quantification (Iwai et al., 2010). Nevertheless, the functionality of the PCR amplification-based screening method using the same degenerate primers was proven by successfully amplifying $OleT_{JE}$, UndA and UndB genes using genomic DNA from strains already known for their ability of 1-alkene production. Furthermore, due to the possibility of getting larger fragments and even total ORFs, this kind of screening gives much more information and insight compared to other screening methods, like sequence diversity for example (Iwai et al., 2010).

The newly identified UndA sequences were further analyzed using homology modelling to confirm their association to this enzyme family. High sequence identities were obtained for each of them, meaning that the sequences found in the *Pseudomonas* strains are homologous to the annotated *Pseudomonas fluorescens* Pf-5 UndA crystal structure (PDB accession number 6P5Q). Unfortunately, there is no UndB crystal structure available making homology modeling impossible. Rui et al. described UndB as a membrane-bound fatty acid desaturase. Like all other membrane-bound desaturases, UndB is predicted to have the following three conserved histidine-rich motives, the so called His-boxes: $H_{89}DLIH_{93}$, $H_{128}(L/F)(N/H)H_{131}H_{132}$ and $H_{297}(G/S/A)IH_{300}H_{301}$ (Rui et al., 2015). All three of these His-boxes could be found in each single of the six novel UndB sequence obtained in this study.

5 Conclusion and outlook

During the study, two different 1-alkene biosynthesis routes were investigated. On the one hand, the $OleT_{JE}$ system present in *Jeotgalicoccus* sp. ATCC 8456, and on the other hand, the UndA/UndB system present in *Pseudomonas* strains. The two systems behaved differently during the fatty acid feeding and the growth experiments. In *J.* sp. ATCC 8456 fatty acids did induce and increase targeted 1-alkene production significantly, as a 1,000-fold increase in 1-undecene production was observed feeding lauric acid as precursor, compared to the control. For the *Pseudomonas* strains, an increased 1-alkene production was observed only in *Pseudomonas putida* and *Pseudomonas fluorescens*. Nevertheless, the induced decarboxylation of fatty acids and production of the targeted corresponding 1-alkene was successfully induced in every strain tested. To shed light on the diversity and to identify new bacterial 1-alkene producers, a degenerate primer-mediated PCR amplification screening of a metagenomic fosmid library was performed. Using this approach, only a low hit-rate was achieved with two false positive clones per primer pair were obtained. However, using the very same degenerate primers, five homologous sequences of UndA and UndB, respectively, were successfully amplified from *P. brassicacearum* L13-6-12*, P. brenneri* Baz30*, P. fluorescens* Baz53*, P. poae* RE*1-1-14 and *P. putida* 1T1. The whole gene sequences could be determined in the end, showing all to be new members of this enzyme families with sequence identities ranging between 66.5 to 96.8% for UndA und UndB, respectively. Homology modelling of UndA was displaying high sequence identity to the sole available UndA crystal structure. For the new UndB sequences, three conserved His-domains present in all membrane-bound desaturases were identified. These results support the claim that novel UndA and UndB homologous genes were found in this study. Heterologous expression of the newly obtained UndA and UndB sequences, followed by activity validation would be advisable in future studies to complete the functional analysis of the discovered 1-alkene biosynthesis genes.

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Appendix

Table A1: Dilutions of 1-alkenes prepared and measured by GC-MS. The peaks were integrated using the RTE integrator to determine the area.

1-Alkene	Concentration [nM]	Area
1-Nonene	2890	16394
	3853.33	111130 ± 72515
	5780	596863 ± 130019
	11560	1642645.5 ± 102768.5
	28900	5525624 ± 901668
1-Decene	2640	132447.5 ± 120196.5
	3520	300625.5 ± 9312.5
	5280	1273662 ± 300178
	10560	3837469.5 ± 55636.5
	26400	13743163 ± 1062568
1-Undecene	607.5	343626.5 ± 117808.5
	810	795913 ± 225966
	1215	1569668 ± 362764.5
	2430	2798330 ± 305030.5
1-Tridecene	525	494115.5 ± 308001.5
	700	1760905 ± 53884
	1050	2434611 ± 793045
	2100	8881710 ± 2956708
1-Pentadecene	460	873487.5 ± 18493.5
	613.33	1519883 ± 149985
	920	5446166.5 ± 815888.5
	1840	8863017 ± 1253467
1-Heptadecene	460	34398.5 ± 2949.5
	613.33	46940.5 ± 18176.5
	920	123233 ± 953
	1840	775965 ± 236275
	3680	3348966 ± 154818
1-Octadecene	391.25	54484.5 ± 5820.5
	521.67	1152879 ± 1108672
	782.5	624714 ± 100390
	1565	3054263.5 ± 574376.5
	3130	12149597 ± 32744
1-Nonadecene	493.33	257548.5 ± 116303.5
	740	526986.5 ± 400066.5
	1480	1628145.5 ± 77694.5
	2960	3559702.5 ± 428802.5

Table A2: Linear equations from calibration curves obtained from 1-alkene dilutions measured by GC-MS.

1-Alkene	**Linear equation (y = kx + d)**
	y = [ ], x = [nM], k = [nM^{-1}]
1-Nonene	y = 213.3x - 681663
1-Decene	y = 580.5x - 1,761,883.1
1-Undecene	y = 1,295.6x - 262,909.1
1-Tridecene	y = 5,262.2x - 2,362,711.8
1-Pentadecene	y = 5,798.3x - 1,381,058.3
1-Heptadecene	y = 1046.9x - 707312
1-Octadecene	y = 4,328.7x - 2,125,269.2
1-Nonadecene	y = 1350.7x - 422639

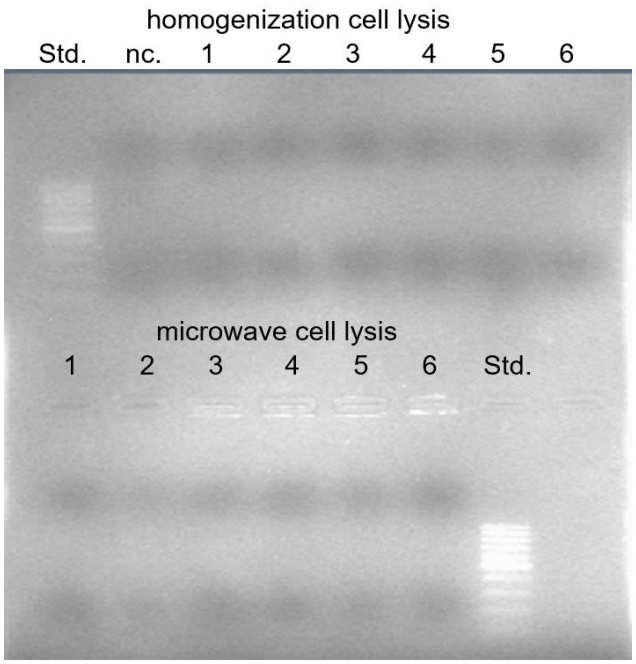

Figure A1: Temperature gradient PCR using degenerate OleT primer (Table 3). Two cell lysis methods were tested, homogenizing the cells and cell lysis using a microwave. Temperature gradient 49°C-70°C: 1: 50.2°C; 2: 54.8°C; 3: 58.4°C; 4: 61.6°C; 5: 66.0°C; 6: 69.7°C; nc.: negative control at 50.2°C (without template). As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

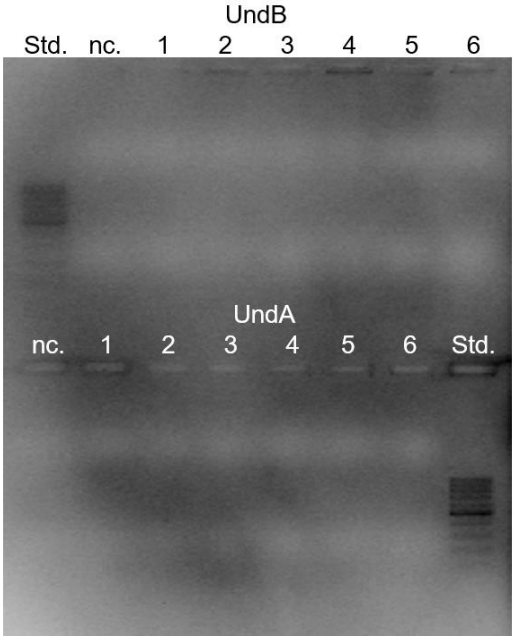

Figure A2: Temperature gradient PCR using degenerate UndA and UndB primers (Table 3). Temperature gradient 50°C-69.5°C: 1: 50.0°C; 2: 51.9°C; 3: 56.4°C; 4: 61.2°C; 5: 65.9°C; 6: 69.5°C; nc.: negative control at 50.0°C (without template). As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

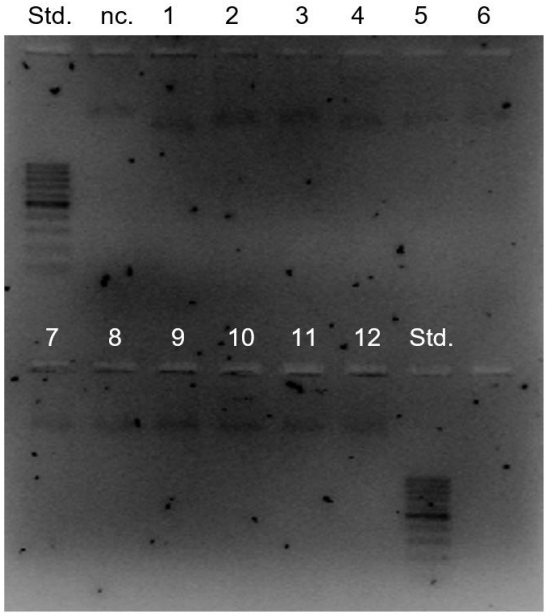

Figure A3: Temperature gradient PCR using degenerate UndB primers (Table 3). Temperature gradient 45°C-72°C: 1: 45.0°C; 2: 45.6°C; 3: 47.5°C; 4: 50.5°C; 5: 53.7°C; 6: 56.9°C; 7: 60.1°C; 8: 63.3°C; 9: 66.5°C; 10: 69.5°C; 11: 71.4°C; 12: 72.0°C; nc.: negative control at 45.0°C (without template). As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

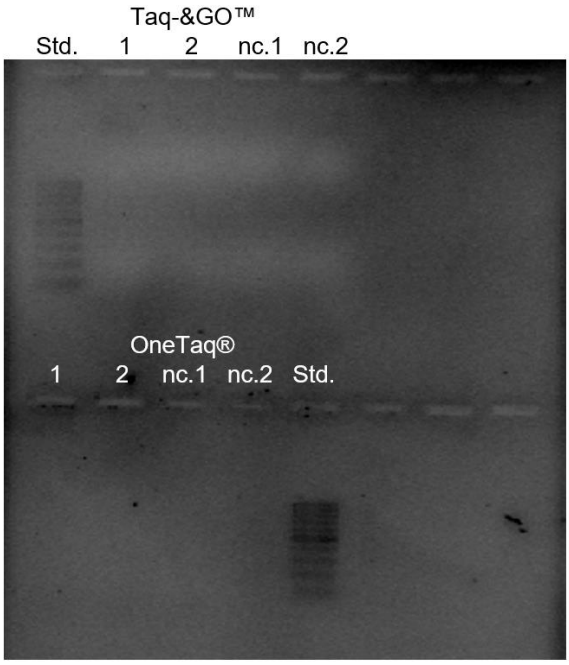

Figure A4: Gel-picture from a standard PCR using degenerate UndB primers (Table 3) and two different polymerases: the Taq-&GO™ and the OneTaq®. The homogenization cell lysis method was used to create the templates with cell material taken form solid *Pseudomonas fluorescens* (2) and *Pseudomonas poae* (1) cultures. nc.1: negative control without template; nc.2: negative control with template (1) but without primers. As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

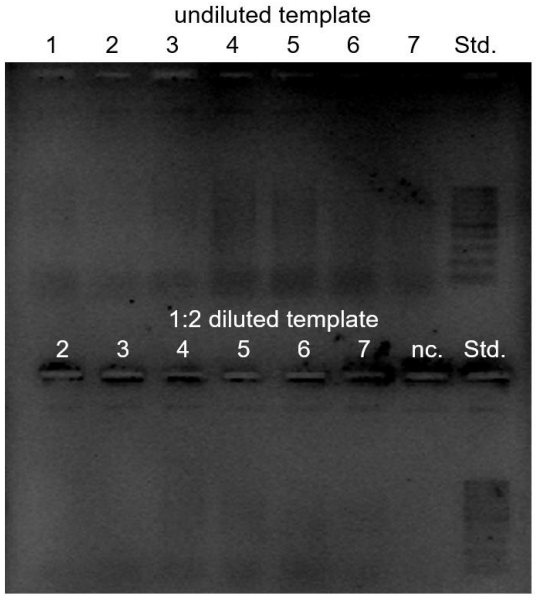

Figure A5: Result of a 2-step PCR using degenerate UndB primers (Table 3). The templates were used undiluted and diluted 1:2 and were obtained from the following bacterial strains: *Bacillus thuringiensis* (1), *Pseudomonas aeruginosa* (2), *Pseudomonas brassicacearum* (3), *Pseudomonas brenneri* (4), *Pseudomonas fluorescens* (5), *Pseudomonas poae* (6) and *Pseudomonas putida* (7) (Table 1); nc.: negative control (without template). As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.

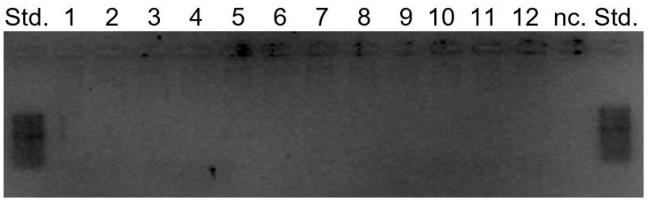

Figure A6: Temperature gradient PCR products separated by gel-electrophoresis. Degenerate OleT primers (Table 3) were used for this case. Temperature gradient 45°C-68°C: 1: 45.0°C; 2: 46.1°C; 3: 48.7°C; 4: 51.2°C; 5: 53.5°C; 6: 55.3°C; 7: 57.0°C; 8: 58.8°C; 9: 60.8°C; 10: 63.7°C; 11: 66.2°C; 12: 67.8°C; nc.: negative control at 45.0°C (without template). As Standard (Std.) a 100bp GeneRuler (Thermo Scientific) was used.