Elucidation of a gene cluster involved in the biosynthesis of the volatile metabolite sodorifen emitted by *Serratia plymuthica* 4Rx13.

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Dedicated to my family, my sister Janine and Stephan

“Zwei Dinge sind zu unserer Arbeit nötig:
Unermüdliche Ausdauer und die Bereitschaft,
etwas, in das man viel Zeit und Arbeit gesteckt hat,
wieder wegzuwerfen.”

Albert Einstein

Plants and animals are known to emit volatile infochemicals for communication. However, for a long time microorganisms were overlooked as producers of these compounds. Recently, it has been demonstrated that microorganisms emit a plethora of volatile organic compounds (VOCs), with the rhizobacterium *Serratia plymuthica* 4Rx13, which is found in the rhizosphere of *Brassica napus*, producing a particularly rich volatile spectrum containing the unique compound sodorifen. Sodorifen is a polymethylated bicyclus that contains no heteroatoms ($C_{16}H_{26}$). The main aim of this thesis was to better understand the biosynthesis of this unusual compound. A comparative genome and transcriptome analysis of a sodorifen-producer (*S. plymuthica* 4Rx13) and a closely related non-producer isolate (*S. plymuthica* AS9) was performed, which highlighted four potential candidate genes for sodorifen biosynthesis. These genes are organized in a cluster localized on the antisense strand of the genome. This cluster consisted of genes annotated as follows: i) terpene cyclase, ii) methyl transferase, iii) 1-deoxy-D-xylulose-5-phosphate (DOXP)-synthase, and iv) isopentenyl diphosphate (IPP)-isomerase. Beside the DOXP-synthase knockout mutant, the genes of the cluster revealed a sodorifen-negative phenotype, providing evidence that these enzymes are involved in the biosynthesis of sodorifen. This is the first ever evidence that sodorifen can be classified as a microbial terpenoids. In contrast, the knockout mutant of DOXP-synthase (SOD_c20770) still emitted sodorifen. It was shown that at least two genes, a homolog of DOXP-synthase (SOD_c09180) and a farnesyl diphosphate synthase, outside the sodorifen cluster, which most likely complement the *dxs* gene within the sodorifen cluster. Genome mining revealed that the complete sodorifen cluster was only found in *S. plymuthica* with some related genes in *Pseudomonas chlororaphis* and *Streptomyces tsukubaensis*. Thus, it was concluded that the sodorifen cluster is unique in the bacterial world. Based on feeding experiments with isotope labeled precursors ([2.3-$^{13}C_2$]-succinate, [1.4-$^{13}C_2$]-succinate, L-[3-^{13}C]-alanine, and L- [S- $^{13}CH_3$]-methionine) and nuclear magnetic resonance (NMR) spectroscopy analysis, it was possible to propose a biosynthetic pathway for sodorifen. These experiments also showed that only one methyl group of sodorifen derived from methionine. The new alcohol of the terpene cyclase mutant is most likely not an intermediate of the sodorifen biosynthesis. Additionally, the product of the methyl transferase mutant was identified as farnesyl acetone. This research has significantly advanced our understanding of the biosynthesis of the unique microbial compound sodorifen.

Es ist seit langem bekannt, dass Pflanzen und Tiere flüchtige Verbindungen zur Kommunikation unter einander emittieren. Mikroorganismen wurden als Produzenten von flüchtigen Verbindungen lange Zeit übersehen. Erst kürzlich wurde nachgewiesen, dass auch sie eine Vielzahl an flüchtigen Verbindungen emittieren. Besonders das Rhizobakterium *Serratia plymuthica* 4Rx13, das aus der Rhizosphere von *Brassica napus* isoliert wurde, besitzt ein komplexes Spektrum von Volatilen mit der Hauptkomponente Sodorifen. Die Strukturaufklärung zeigte eine – für Naturstoffe ungewöhnliche – polymethylierte bizyklische Ringstruktur ohne Heteroatome ($C_{16}H_{26}$). Daher war das Ziel dieser Arbeit die Biosynthese von Sodorifen aufzuklären. Vergleichende Genom- und Transkriptomanalysen des Sodorifen produzierenden (*S. plymuthica* 4Rx13) und nicht-produzierenden Isolates (*S. plymuthica* AS9) wurden durchgeführt. Dies führte zur Identifizierung eines Clusters, bestehend aus den folgenden vier Genen: i) Terpenzyklase, ii) Methyltransferase, iii) 1-deoxy-D-xylulose-5-phosphate (DOXP)-synthase und iv) isopentenyl diphosphate (IPP)-isomerase. Bis auf die DOXP-Synthase Mutante, wiesen die Mutanten des Clusters einen Sodorifen negativen Phänotyp auf. Dies deutet auf eine Beteiligung dieser Enzyme bei der Sodorifen-Biosynthese hin und weist erstmalig daraufhin, dass Sodorifen in die Klasse der mikrobiellen Terpenoide einzuordnen ist. Da die *dxs* Mutante in der Lage war Sodorifen zu produzieren, komplementieren vermutlich mindestens zwei homologe *dxs* Gene, DOXP-Synthase (*SOD_c09180*) und Farnesylpyrophosphat-Synthase, die außerhalb des Sodorifen-Clusters im Genom vorhanden sind, die DOXP-Synthase des Clusters (*SOD_C20770*). Nachfolgend wurde mittels Genomvergleiche aufgezeigt, dass das komplette Sodorifen-Cluster nur in *S. plymuthica* Spezies und *Pseudomonas chlororaphis* und *Streptomyces tsukubaensis* vorhanden ist. Somit ist das Sodorifen-Cluster einzigartig unter den Bakterien. Auf der Grundlage von Fütterungsversuchen mit ^{13}C markierten Substraten ([2.3-$^{13}C_2$]-Succinat, [1.4-$^{13}C_2$]-Succinat, L-[3-^{13}C]-Alanin und L-[S-$^{13}CH_3$]-Methionin), sowie NMR-Analysen e einen potentiellen Sodorifen Biosyntheseweg aufzuzeigen. Interessanterweise zeigten diese Experimente auch, dass lediglich eine Methylgruppe in Sodorifen von Methionin stammt und dass der neue Alkohol der Terpenzyklase-Mutante sehr wahrscheinlich kein Intermediat der Sodorifen- Biosynthese ist. Zudem konnte die Verbindung der Methyltransferase-Mutante als Farnesylactone identifiziert werden. Insgesamt konnte diese Dissertation zu einen entscheidenden Fortschritt in der Aufklärung der Biosynthese dieses einzigartigen mikrobiellen Naturstoffs Sodorifen beitragen.

1. Introduction

1.1 The rhizosphere, a hotspot of microbial activity

The soil provides a habitat for a plethora of organisms living in diverse communities. In particular, the rhizosphere (the area directly surrounding the roots of a plant) is a hotspot of microbial activity. The complex network of interactions between microorganisms and plant roots (**Figure 1.1**), and the exchange of nutrients and lysates between them (Foy and Carson, 1974; Lynch and Whipps, 1991) are of particular interest, leading to many research groups focusing on the rhizosphere in recent years (Kloepper et al., 1980; Van der Putten et al., 2001; Wenke et al., 2010). This work has demonstrated the diversity of highly dynamic interactions between plant roots and beneficial, pathogenic soil microbes, invertebrates, and root system competitors (Bais et al., 2006). Furthermore, it has been shown that the density of bacteria within the rhizosphere is approximately 10–1000 fold higher than that in the surrounding soil (Hiltner, 1904; Lugtenberg and Kamilova, 2009). The plant is able to distinguish between wanted and unwanted organisms and influences the settlement of organisms through the release of specific metabolites and chemical compounds via root exudates (Bais et al., 2006). This phenomenon, which is known as the “rhizosphere effect,” was first described by Hiltner (1904), who stated that many microorganisms are attracted by root exudates and observed that the number of microorganisms increased toward the roots.

The release of certain nutrients leads to a distinct crosstalk between the plant and the surrounding microorganisms, which further drives the colonization of the roots. The organisms within the rhizosphere communicate, inhibit or promote the growth of other organisms, or are attracted or repelled by the molecules that are released by the plant or other microorganisms. Thus, the interactions between plant roots and microorganisms can have a positive or negative effect on the community occupying a particular habitat.

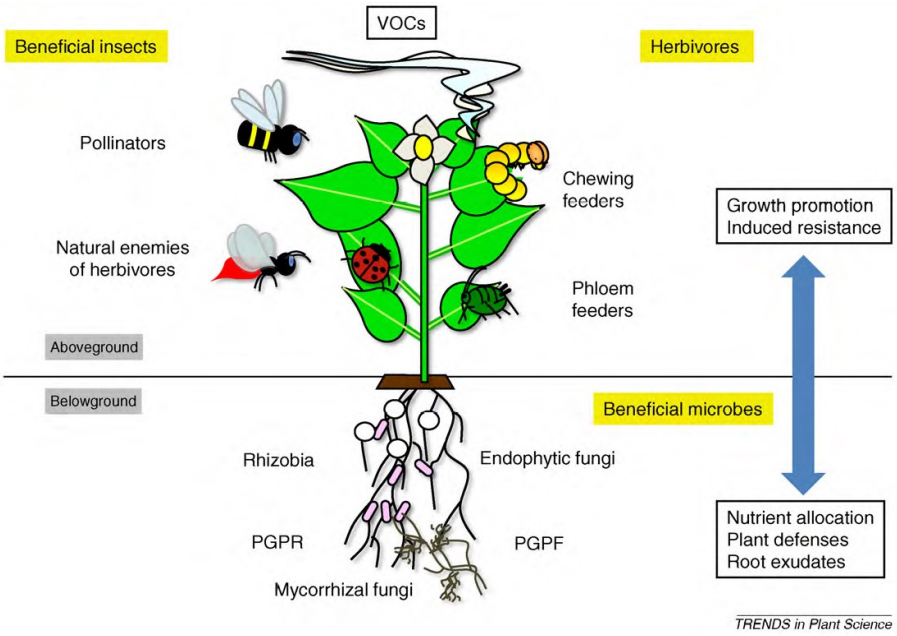


Figure 1.1: Multitrophic interactions between a plant and belowground and aboveground organisms. PGPR: plant growth-promoting bacteria, PGPF: plant growth-promoting fungi (Pineda et al., 2010).

1.2 Rhizobacteria

The term “rhizobacteria” describes bacteria that colonize the roots and form symbiotic relationships with plants. Although parasitic rhizobacteria do exist, a mutualistic association is more common. Rhizobacteria are often plant growth-promoting bacteria (PGPR), a term that was coined by Kloepper (1978). The growth-promoting effect on the plant can result from the absence of pathogens or protection of the plant against soil-borne diseases (Lugtenberg and Kamilova, 2009). Several aspects of such interactions are illustrated in **Figure 1.2**. In a symbiotic relationship, the plant provides the necessary exudates, while the bacteria fix nitrogen, solubilize phosphor, or produce phytohormones (e.g., auxin). Plant-pathogenic bacteria and fungi are dispelled by the plant through the release of certain compounds, e.g., volatile oils such as carvacrol or thymol (Dorman and Deans, 2000). In addition, several bacterial strains such as *Bacillus subtilis*, *B. amyloliquefaciens*, *Enterobacter cloacae*, and *Serratia plymuthica* emit volatile compounds, which also operate as plant growth-promoting compounds (Lugtenberg and Kamilova, 2009; Kai et al., 2010).

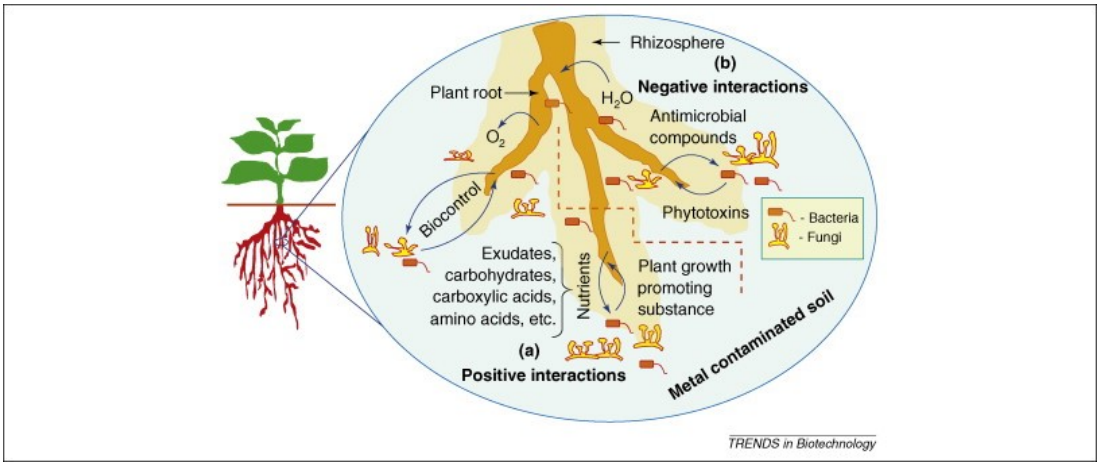


Figure 1.2: Positive and negative plant-microbe interactions within the rhizosphere. (Rajkumar et al., 2010)

1.3 Interactions between plant roots and microbes

The interactions between plants and microbes can be classified as i) negative, ii) positive, or iii) neutral for the plant. Negative interactions include competition, parasitism, pathogenesis, and herbivory; while positive interactions occur as a result of symbiotic associations with epiphytes and mycorrhizal fungi and root colonization by PGPR (Bais et al., 2006). Neutral interactions are not further discussed within this thesis.

1.3.1 Negative interactions

Plants that are under attack from pathogenic microorganisms secrete phytoalexines or defense proteins to physically protect their vulnerable root cells (Bais et al., 2006). For example, sweet basil (*Ocimum basilicum*) produces rosmarinic acid, which has an antimicrobial activity against *Pseudomonas aeruginosa* (Bais et al., 2002); and *Arabidopsis thaliana* increases the amount of indolic and phenyl propanoid in its root exudates during infection by the root-pathogenic oomycete *Phythium sylavaticum* (Bednarek et al., 2005).

When infesting plants, many pathogenic bacteria produce virulence factors, the production of which is often controlled by quorum sensing (QS). This regulatory mechanism controls cell density and also has a communication function (Kaiser and Losick, 1993; Bassler, 1999). QS activation is mediated by autoinducer molecules such as N-acyl homoserine lactones (AHLs) (von Bodman et al., 2003), lactones, or

diketopiperazines. AHLs mostly function by activating QS-controlled genes (Bais et al., 2006).

Parasitism is another negative interaction that can occur. This may involve the formation of a haustorium, a specialized root structure that allows the parasite to connect with the host vascular tissue. The connection is developed by the parasite responding to chemical compounds that are released by the host. Among others, a phenolic derivative has been identified, which triggers the change from vegetative to parasitic growth within the host (Albrecht et al., 1999). Phenolic compounds induce the formation of haustoria in *Striga asiatica* and *Agalinis purpurea* (MacQueen, 1984; Riopel and Timko, 1995), allowing the parasite to withdraw water and nutrients from the host (Kuijt, 1969).

1.3.2 Positive interactions

During positive interactions, bacteria provide the plant with nutrients that allow it to repel pathogens, leading to increased plant growth. The most well-known positive interaction is the symbiotic association between bacteria and leguminous plants, whereby fixed atmospheric nitrogen is shared. This interaction is very specific, with only *Rhizobium leguminosarum* bv. *viciae* able to induce nodules in plants belonging to the *Pisum*, *Vicia*, and *Lens* genera. The signal compounds that achieve this specific interaction are classified as isoflavonoids. For example, the isoflavonoids daidzein and genistein, which are produced by soybeans (*Glycine max*), are responsible for driving the association with *Bradyrhizobium japonicum* through the induction of specific genes (Bais et al., 2006).

In contrast, the interaction between arbuscular mycorrhizal fungi and plant roots is less selective. In this symbiotic association, the plant benefits from increased nutrient uptake and improved fitness, while the fungi are able to extract lipids and carbohydrates from the plant. The fungi invade the host root tissue by detecting a chemical response in the plant (Bais et al., 2006), and the growth of the fungi is then stimulated by flavonoids (Harrison, 2005).

As explained in section 1.2, PGPR colonize the plant roots and stimulate their growth (Beneduzi et al., 2012). Due to their positive effect on plant growth, certain bacteria are used in agriculture as biofertilizers, which are substances that contain living microorganisms that are applied to seeds, plant surfaces, or soil in order to colonize

the rhizosphere or the interior of the plant to achieve higher yields and plant weights, and increased seed production, as well as to control diseases in crops (Vessey, 2003). For example, it has been found that the growth of canola can be increased by up to 10–40% when coated with PGPR prior to planting and agricultural crop yield can be increased by 10–20% (Kloepper et al., 1980, 1991).

Colonization of the plant roots by certain microorganism is, in many cases, advantageous for the plant. For example, *Burkholderia cepacia* is able to control the growth of *Fusarium* spp. and also stimulates the growth of maize (*Zea mays*) under iron-poor conditions through the production of siderophores (Bevivino et al., 1998). Similarly, studies have shown that *Serratia plymuthica* HRO-C48 (Berg, 2000) can significantly promote growth in strawberries (*Fragaria ananassa*), and biologically control the fungal pathogens *Verticilium dahliae* and *Phyotophthera cactorum* (Kurze et al., 2001), likely due to the release of chitinase, which acts as an antifungal agent (Müller et al., 2009). Consequently, this bacterium is currently commercially used in agriculture (RhizoStar R®, E-nema GmbH Raisdorf, Germany).

1.4 Microbial volatile organic compounds

Microbial volatile organic compounds (mVOCs) are produced by a wide range of microorganisms, but were overlooked for a long time. Recently, a database was set up that currently includes approximately 1200 VOCs that are released by 350 bacterial and 100 fungal species (Lemfack et al., 2014), highlighting the plethora of VOCs that are emitted by microorganisms. These VOCs are considered specialized metabolites and have distinct functions, e.g., antibiotics, waste products, or signaling compounds within a given organism (Lou et al., 1999), Williamson et al., 2006). They have a low molecular mass, are lipophilic, have a low boiling point, and are usually emitted mixed together (**Figure 1.3**). They are also often characteristic of particular microorganisms (Lee et al., 2006; Thorn et al., 2011). These characteristics make VOCs suitable as infochemicals, because they are able to pass through porous soil, and can act over both short and long distances (Maffei et al., 2011). Some mVOCs and their functions are well known. For example, geosmin, a compound with an earthy odor that can often be recognized in forests, is the main compound in the profile of *Streptomyces* species (Gerber and Lechevalier, 1965); and acetoin is typically released by *Bacillus* species during the growth phase but not during sporulation (Kominek and Halvorson, 1965).

Indole is a typical volatile compound that is found in the spectrum of *Escherichia coli*, which has an unpleasant odor in high concentrations, and is involved in diverse microbial processes such as plasmid stability, and biofilm and spore formation (Lee and Lee, 2010; Li and Young, 2013). Benzaldehyde, pyrazine, and dimethyl disulfide are other frequently emitted VOCs (Smith and Kelly, 1988; Blumer and Haas, 2000; Dickschat et al., 2005; Van Immerseel et al., 2010). VOCs have diverse functions. For example, benzaldehyde can inhibit spore formation (Kai et al., 2009), pyrazine is used as an energy source by different microorganisms (Müller and Rappert, 2010), and dimethyl disulfide is able to inhibit mycelium growth (Kai et al., 2009). In addition to the effect of individual compounds, previous studies have also clearly demonstrated that the mixture of mVOCs released can be advantageous or detrimental for other organisms, including bacteria, fungi, invertebrates, and plants living in the same habitat (Effmert et al., 2012; Kanchiswamy et al., 2015).

In addition, mVOCs are of commercial interest. The aromas of foods such as cheeses and beverages such as beer and wine result from the metabolic activity of microbes, and are important features for selling the products; and the perfume industry is constantly searching for new compounds, which the emerging field of mVOCs will likely contribute to. Another interesting aspect is that mVOCs such as monoterpenes and sesquiterpenes have potential as biofuels; e.g., the Patagonian fungal endophyte NRRL 50072 produces diverse medium-chain and highly branched VOCs that are referred to as “myco-diesel” (Griffin et al., 2010).

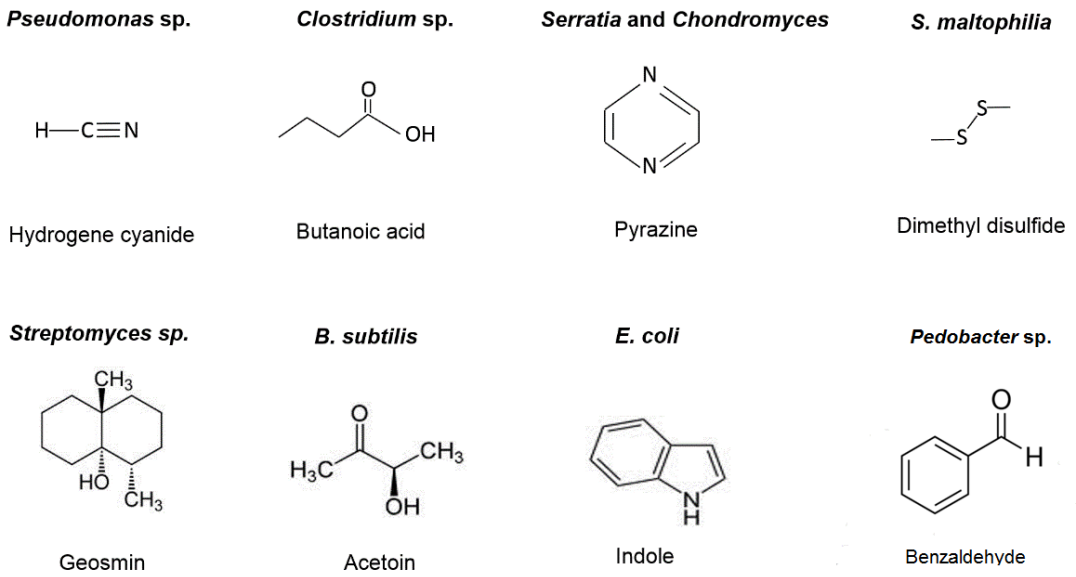


Figure 1.3: A selection of volatile compounds emitted by different microorganisms.

1.5 Genus *Serratia*

Bacteria of the genus *Serratia* are ubiquitously found in all habitats: in water, on plants, on animals, and they are quite prominent in the soil. These are opportunistic, Gram- negative bacteria belonging to the family Enterobacteriaceae. They are typically rod shaped, facultative anaerobes, and motile. For a long time, the taxonomy of this genus was not fully clear and so the bacteria belonging to it were renamed from time to time (Grimont et al., 1977; Mahlen, 2011; Manzano-Marín et al., 2012). As of 2014, the genus *Serratia* contains 15 species: *S. entomophila* (Grimont et al., 1988), *S. ficaria* (Grimont et al., 1981), *S. fonticola* (Gavini et al., 1979), *S. glossinae* (Geiger et al., 2010), *S. grimesii* (Grimont et al., 1982), *S. liquefaciens* (Grimes and Hennerty, 1931), *S. marcescens,* which was assigned by Bizio (1823) (Hejazi and Falkiner, 1997), *S. nematodiphila* (Zhang et al., 2009), *S. odorifera* (Grimont et al., 1978), *S. proteamaculans* (Grimont et al., 1919), *S. plymuthica* (Lehmann and Neumann, 1896), *S. quinivorans* by Grimont (1983, newly designated by Ashelford et al., 2002), *S. rubidaea* (Stapp, 1940), *S. symbiotica* (Sabri et al., 2011), and *S. ureilytica* (Bhadra et al., 2005).

The colonies of some strains have coloration ranging from white, through pink to red. *S. marcescens* is particularly well-studied due to its production of the red pigment prodigiosin and its pathogenic features. Pyrimine is another pigment that is frequently observed among *Serratia* species, which is a water-soluble pink pigment containing iron (Fe^{2+}) (Grimont and Grimont, 2006). *Serratia* species are also a good source of antibiotics, such as carbapenem, the polyketide oocydin A (Matilla et al., 2012), or indole-3-acetic acid (Kalbe et al., 1996). *S. plymuthica* 4Rx13 (formerly *S. odorifera*), *S. odorifera*, *S. marcescens*, and *S. proteamaculans* also emit many VOCs (Kai et al., 2010; Weise et. al., 2014).

Out of 98 VOCs, that were found to be produced by *S. plymuthica* 4Rx13, only 16 could be identified and only three were found in the volatile spectrum of all four named *Serratia* species; the other compounds were found in several species or appeared as a unique feature. For example, 2-decanone was only present in *S. marcescens*, indole was only present in *S. odorifera* DSM4582, and sodorifen was only present in *S. plymuthica* 4Rx13. This observation indicates that some volatiles belong to the repertoire of common VOCs, but others are species specific and unique.

The effects of certain VOCs have been tested in co-cultivation experiments, which have shown that they can have a positive or negative effect on organisms depending

on the individual compound or mixture. For example, various studies have shown that some bacterial volatiles promote growth in *Arabidopsis thaliana* (Ryu et al., 2003, 2004; Ping and Boland, 2004; Gutiérrez-Luna et al., 2010), while dimethyl disulfide, ammonia, and probably some other additive compounds inhibit it. It has also been shown that the CO_2 released by *S. plymuthica* 4Rx13 has a growth-promoting effect on *A. thaliana* (Kai et al., 2010).

1.5.1 The VOC profile of S*erratia plymuthica* 4Rx13

The bacterium *S. plymuthica* 4Rx13 occurs in the rhizosphere of *Brassica napus* (Berg et al., 2002). Investigations concerning the VOC profile of this bacterium were first conducted by Dr. Marco Kai (2007), who analyzed the emitted compounds quantitatively and qualitatively and determined the timing of their release (Kai et al., 2010).

The combination of gas chromatography/mass spectrometry (GC/MS) with proton- transfer-reaction mass spectrometry (PTR-MS) resulted in a comprehensive VOC profile for *S. plymuthica* 4Rx13 (Kai et al., 2010). The highest levels of VOCs (both quantitatively and qualitatively) were emitted at the start of the stationary phase (24–48 h), which included dimethyl disulfide, dimethyl trisulfide, methanethiol, terpenoids, 2-phenylethanol, and other aromatic compounds (Kai et al., 2010). The VOC spectra also highlighted that the new and unique compound sodorifen was the dominant compound present (approximately 50%).

The mass spectrum of sodorifen showed a unique fragmentation pattern at m/z 134, 135, and 136 (**Figure 1.4B**), and the structure of sodorifen (**Figure 1.4A**) was elucidated ($C_{16}H_{26}$) and verified by synthesis (Reuss et al., 2010). The structure of sodorifen is extraordinary due to its lack of heteroatoms and its high degree of methylation.

The first investigations into the biosynthesis of sodorifen in *S. plymuthica* 4Rx13 were performed by Dr. Teresa Weise (dissertation, 2013). These investigations included numerous feeding experiments using different amino acid combinations and carbon sources. Large amounts of sodorifen were detected using a minimal medium supplemented with succinate, which resulted in a tenfold increase in emissions compared with growth on the complex medium NB. When glucose was used as the sole carbon source, there were only low levels or an absence of sodorifen emission.

Analysis of the VOC profiles of various *Serratia* species also revealed that only certain *S. plymuthica* isolates were sodorifen-producers (Weise, dissertation, 2013). These isolates (4Rx13, HRO-C49, and 3Re-4-18) originate from plants, whereas sodorifen non-producers were mostly derived from humans.

Due to its rare appearance in other species and unique structure, it was speculated that sodorifen is synthesized via a new metabolic pathway. Subsequently, Teresa Weise (dissertation, 2013) compared the genomes of a sodorifen-producer and non-producer and assembled a list of 312 unique genes for the isolate 4Rx13. These candidate genes were potentially interesting for further investigations to elucidate the biosynthesis of this compound.

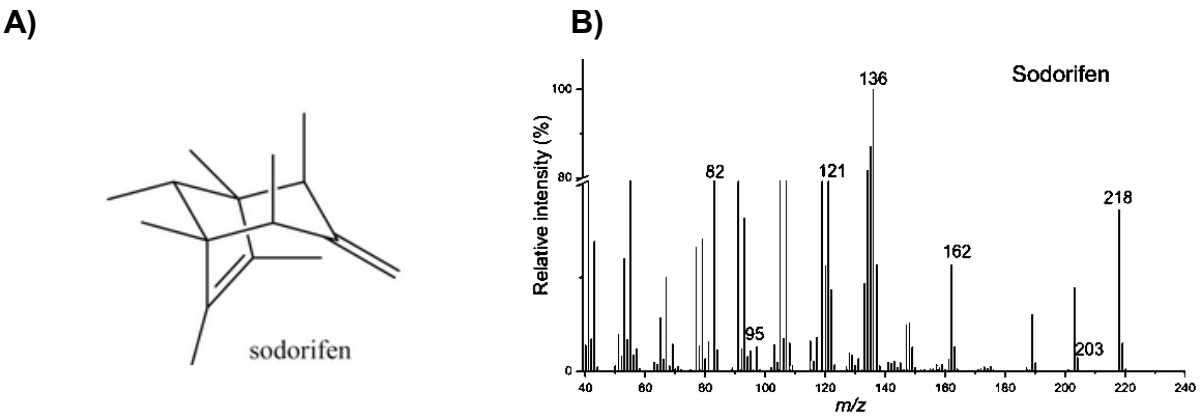


Figure 1.4: (A) Structure and (B) mass spectra of sodorifen.

1.6 Aim of this thesis

Due to the unique structure of sodorifen, it has not been possible to assign its synthesis to any known metabolic pathway. Therefore, it was hypothesized that new proteins and enzymes are probably involved. The aim of this thesis was to determine the genes that are responsible for the biosynthesis of sodorifen in *S. plymuthica* 4Rx13.

A comparative approach was used to identify candidate genes, which involved i) an extended comparative genome analysis of a sodorifen-producer and non-producer, ii) comparison of transcription levels in the isolate *S. plymuthica* 4Rx13 with the non-producer isolate *S. plymuthica* AS9, and iii) feeding experiments with isotope-labeled substrates coupled with nuclear magnetic resonance (NMR)-spectroscopy analysis of the products.

2. Materials and Methods

Table 2.1: Wild type bacteria. SCAM (strain collection of antagonistic microorganisms, Department of Microbiology, University of Rostock, Germany), DSMZ (Deutsche Stammsammlung von Mikroorganismen und Zellkulturen GmbH; Leibniz Institute, Braunschweig, Germany).

Genus	Species	Isolate	Origin
Serratia	*plymuthica*	4Rx13	SCAM
		AS9	Swedish University of Agricultural Sciences, Uppsala, Sweden
		AS12	Swedish University of Agricultural Sciences, Uppsala, Sweden
		V4	Harvard Medical School, Boston, USA
		HRO-C48	SCAM
		3Re-4-18	SCAM
	marcescens	Db11	Institute of Medical Microbiology, University of Rostock, Germany
	odorifera	DSM 4582	DSMZ
	protemacculans	568	Brookhaven National Laboratory, New York, USA

2.1 Bacterial strains, media, and growth conditions

All investigations were conducted using the bacterial species *Serratia plymuthica* (hereafter *S. ply.*). Of particular interest was the *S. ply.* 4Rx13 isolate, which was segregated from the rhizosphere of *Brassica napus* (Prof. Gabriele Berg, Institute of Environmental Biotechnology, University of Graz, Austria). Other closely related strains that were analyzed included *S. ply.* V4, which was supplied by the research group of R. Kolter (Boston, Harvard Medical School, USA) and was isolated from a biofilm that was formed on pasteurizer plates during sanitized milk processing (Cleto et al., 2014); and *S. ply.* AS12, which was supplied by the research group of Sadhna Alström

(Neupane et al., 2012) and was isolated from rapeseed roots in Sweden. In addition, *S. ply.* HRO-C48, which was isolated from the rhizosphere of *Brassica napus*, and *S. ply.* 3Re-4-18, which was isolated from the endorhiza of *Solanum tuberosum* in Rostock, Germany, were sequenced and investigated.

All bacteria were cultivated at 30°C in a complex medium (NB II; Carl Roth, Karlsruhe, Germany; 3.5 g/l peptone from casein, 2.5 g/l peptone from meat, 2.5 g/l peptone from gelatin, 1.5 g/l yeast extract, 5 g/l NaCl, 15 g/l agar-agar, pH 7.2) or in liquid nutrient broth [NB II without agar-agar; Carl Roth, Karlsruhe, Germany)]. Some experiments were also conducted in Davis Minimal Medium (DMM) [Davis and Mingioli, 1950; 7 g/l K_2HPO_4 (Carl Roth, Karlsruhe Germany), 3 g/l KH_2PO_4 (Carl Roth), 0.5 g/l trisodium citrate (Carl Roth), 0.1 g/l $MgSO_4$ (VK Labor-und Feinchemikalien, Germany), 1 g/l $(NH_4)2SO_4$ (VK Labor-und Feinchemikalien)] + a specific carbon source or amino acid combinations. Unless otherwise stated, the DMM was always used in combination with 55 mM of single amino acids (Carl Roth, Karlsruhe, Germany) or 20 mM of amino acids in combinations; and with 55 mM of organic acids, glucose, or pyruvate (Carl Roth). The pH was measured with pH filter paper (Macherey-Nagel, Düren, Germany), the OD_{600} was determined with a photometer (UV/Visible Spectrometer, Ultrospec 2000; Pharmacia Biotech), and the number of colony forming units (CFUs) was calculated.

For further analysis of the emitted compounds, the bacteria were cultivated with isotope-labeled compounds using the following carbon sources: [1.4-$^{13}C_2$]-succinate, [2.3-$^{13}C_2$]-succinate, L-[3-^{13}C]-alanine, L-[2.3-$^{13}C_2$]-alanine, and L-[*S*-$^{13}CH_3$]-methionine. To incorporate ^{13}C, the cells were grown in a minimal medium supplemented with the labeled carbon source at a ratio of 80:20 ^{12}C. An exception to this was L-methionine, which was provided as a mixture containing 20 mM L-alanine, 20 mM L-threonine, and 20 mM L-[*S*-$^{13}CH_3$]-methionine. Bacteria were grown for 96 h. Every 24 h, the column of the VOC-collection system was eluted with 300 µl and 100 µl D6-benzene.

Table 2.2: Plasmids and vectors used.

Plasmid	Orgin	Inducer	Selection marker	Expression host
pJET	Thermo Fisher Scientific, Waltham, USA	-	amp	*E.coli* XL-1-Blue
pUC19	Own propagate, University of Rostock, Germany	1 mM IPTG	amp	*S. plymuthica* 4Rx13
pRED/ET	Gene Bridges, Heidelberg, Germany	10 % Arabinose	kan	*S. plymuthica* 4Rx13
708-FLPe	Gene Bridges, Heidelberg, Germany	Thermosensitive	cm	*S. plymuthica* 4Rx13
pFRT	Gene Bridges, Heidelberg, Germany	-	kan	*E.coli* HC 100

2.2 Trapping of volatiles

The volatile compounds within the headspace of a liquid bacterial culture were trapped on the absorbent SuperQ (50 mg; Alltech, Deerfield, USA). Bacteria were cultivated in a modified Erlenmeyer flask. The VOC-collection system, which was established by our research group (Kai et al., 2010; Weise, dissertation, 2013), was used to collect volatile compounds within the headspace of the bacterial culture. The column containing the trapped volatiles was then eluted with the solvent dichloromethane (Carl Roth, Karlsruhe, Germany) at 24-h intervals over a 72-h period. Nonyl acetate (5 ng/10 µl; Carl Roth, Karlsruhe, Germany) was always added as an internal standard. A detailed description of the system is provided in the supplementary section.

2.2.1 VOC-collection system

Adsorption of the bacterial organic compounds was conducted using either the VOC-collection system (**supplementary Figure S1**) or solid-phase microextraction (SPME). Bacteria were cultivated in 6 ml NB II overnight at 30°C and 170 rpm, and were then transferred into a 100-ml main culture once OD_{600} = 0.5–1. A detailed description of the process used to trap volatile compounds on the absorbent SuperQ and the VOC-collection system apparatus is provided in Kai et al. (2010). However, some modifications were made in the present study to achieve greater sensitivity and accumulate single volatile compounds. This involved placing two openings in the side of a 500-ml Erlenmeyer flask and adding a grind at the bottle neck, along with a lid for

closing the system. The elution of volatiles with dichloromethane (Carl Roth, Karlsruhe, Germany) was performed at 24-h intervals over 3 days, and nonyl acetate (5 ng/10 µl; Carl Roth, Karlsruhe, Germany) was always carried along as an internal standard. The absorbent SuperQ was used to trap the volatile compounds, which were measured using GC/MS (**supplement, parameter analysis, Table S2**).

2.2.2 Headspace measurement using solid-phase microextraction

Another technique for trapping bacterial VOCs is SPME, whereby the volatile compounds are absorbed onto a fiber and then analyzed by thermal desorption from the fiber. The fiber is coated with the absorbent compound polydimethylsiloxane (100 µm, PDMS, SPME Fiber Assortment Kit 4; Supelco, USA), which is appropriate for organic volatiles. Nonyl acetate (10 ng/5 µl) was also carried along as an internal standard by injecting it into the headspace of the bacterial culture. An overnight culture with OD_{600} = 0.5–1 was transferred into a 100-ml Erlenmeyer flask with a final value of OD_{600} = 0.005 and cultivated at 30°C and 170 rpm. The fiber was prepared by first heating it to remove unwanted particles, and then running a conditioning and checking program on the GC/MS.

The fiber was introduced into the headspace of 5 ml of a main culture and incubated for 1 h. Measurements were made at specific time points over 3 days or more (24 h, 48 h, and 72 h). The fiber was then transported to the GC/MS, and the sample was transmitted to the capillary by thermal desorption and analyzed (**supplement, parameters for analysis, Table S3**).

2.3 Analysis of volatile compounds by gas chromatography mass spectrometry

Samples were analyzed with the Shimadzu GC/MS-QP500 system (Kyoto, Japan), equipped with a DB5-MS column (60 m × 0.25 mm × 0.25 µm; J&W Scientific, Folsom, California, USA). Samples of 1 µl were injected splitless or with a split ratio of 1:25 at 200°C with a solvent delay of 2 min using a CTC autosampler (CTC Analytics, Zwingen, Switzerland). Helium was used as a carrier gas at a flow rate of 1.1 ml/min. The capillary was equipped with diphenyl (dimethyl) polysiloxan with 5% phenyl groups (DB05) as the stationary phase. The generated mass fragments were selected based on their mass to charge ratio using a quadrupole mass analyzer, in which mass spectra were recorded using scan mode (70 eV, *m/z* = 40–280). The selected masses were monitored and analyzed with the software Lab Solution (Shimadzu, Duisburg,

Germany). To determine the structure of the volatile compounds, the detected mass spectra were compared with data held by the Mass Spectral Library of the National Institute of Standards and Technology (NIST147).

2.4 Analysis by NMR spectroscopy

The NMR spectra of unfractionated crude volatile blends were recorded by Dr. Stephan von Reuss at the Max Planck Institute for Chemical Ecology (MPI CE), Jena, Germany, using a Bruker AMX-400 instrument (Bruker). Samples were prepared by eluting the volatiles trapped on SuperQ with C_6D_6 (or CD_2Cl_2) and concentrating to a total volume of 550 µl in a stream of nitrogen. Standard one-dimensional ^1H NMR (400 MHz) spectra were recorded by accumulating 128 scans, using an acquisition time of 5 s. Standard one-dimensional broadband decoupled ^{13}C {^1H} NMR (100 MHz) spectra were recorded by accumulating 1024–10 k scans using an acquisition time of 1.4 s. Two-dimensional phase cycled double-quantum-filtered (*dqf*)-COSY spectra were recorded using 8192 data points in F2 and 512 increments in F1 by accumulating eight scans each, using an acquisition time of 1.5 s. Two-dimensional heteronuclear HSQC spectra were recorded using 2048 data points in F2 and 256 increments in F1 by accumulating 64 scans. Two-dimensional heteronuclear HMBC spectra were recorded using 4096 data points in F2 and 128 increments in F1 by accumulating 128 scans. Two-dimensional homonuclear H,H-NOESY spectra of samples that were degassed by bubbling argon gas through the solution for 5 min were recorded using 2048 data points in F2 and 256 increments in F1 by accumulating 64 scans, using mixing times of 500 or 750 ms. Two-dimensional spectra were zero filled to 8k × 4k and phased using the topspin software (Bruker).

2.5 Molecular techniques

2.5.1 Polymerase chain reaction

The amplification of DNA fragments was conducted by polymerase chain reaction (PCR) in a thermocycler (Biometra, Göttingen, Germany). A standard PCR was performed using Taq-polymerase I (overexpressed; Department of Biochemistry, University of Rostock, Germany). Specific DNA (NucleoSpin Tissue Kit; Macherey-Nagel, Düren, Germany) or plasmid DNA (NucleoSpin Plasmid; Macherey-Nagel) was isolated and used as a template. For longer amplification products (> 3 kb), the High-

Fidelity Polymerase *Pfu* or Phusion® (Fermentas, St. Leon-Rot, Germany) was utilized.

Amplification reactions were conducted in a total volume of 50 µl, consisting of 1 µl of 10 mM DNTP-Mix, 1 µl of each primer, 1 µl template × (where × = bacterial DNA or plasmid), 0.5 µl polymerase with or without the proofreading function, 10 µl buffer, and 35 µl nuclease-free water. Primers were designed according to the gene properties (temperature, GC content, and secondary structures) and were usually 21 nucleotides long, with the exception of the oligonucleotides for mutagenesis, which were 72 nucleotides long. A complete list of primers can be found in **supplementary Table S1**. The annealing temperature for the amplification of DNA was dependent on the temperature of the primers (x), and the elongation time was selected according to the size of the gene or DNA (1 kb/1 min). All primers were ordered as standard products and shipped by Sigma-Aldrich (St. Louis, USA) or Invitrogen (Carlsbad, USA).

The PCR was performed in a Biometra PCR-cycler (Göttingen, Germany) or a Hybaid PCR-cycler (Ulm, Germany), with an annealing temperature (x) depending on the set of oligonucleotides (a list of primers is provided in **supplementary Table S1**).

Colony-PCR composition scheme		
Template	1	µl
$MgCl_2$ (25 mM)	3	µl
10 × buffer without $MgCl_2$	5	µl
dNTPs (10 mM)	1	µl
Forward primer	1	µl
Reverse primer	1	µl
Taq polymerase	1	µl
Water	12	µl

Buffer, MgCl2, dNTPs, and polymerase were obtained from Thermo Fisher Scientific, Waltham, USA. The designed primers were either supplied by Sigma-Aldrich (St. Louis, USA) or Invitrogen (Carlsbad, USA).

PCR program for Taq polymerase			
Denaturing	2 min	94°C	
Denaturing	30 s	94°C	
Annealing	x	y	30 cycles
Elongation	1 min	72°C	
Elongation	10 min	72°C	
	∞	10°C	

x = time, dependent on size of amplified DNA–usually 1 kb/min

y = temperature, dependent on primer pair

Phusion Polymerase-PCR composition scheme		
Template	1	µl
HF or GC buffer with $MgCl_2$ (1.5 mM)	10	µl
dNTPs (10 mM)	1	µl
Forward primer	1	µl
Reverse primer	1	µl
Phusion Polymerase F-530S (2 U/µl)	0.5	µl
Water	35	µl

PCR program for Phusion Polymerase			
Denaturing	30 s	98°C	
Denaturing	10 s	98°C	
Annealing	30 s	y	30 cycles
Elongation	90 s	72°C	
Elongation	10 min	72°C	
	∞	10°C	

2.5.2 Mutagenesis

Mutagenesis was performed with the “Quick and Easy Gene Deletion Kit” by Red/ET Recombination (Gene Bridges, Heidelberg, Germany), which allows the product of a gene to be changed by specifically introducing an antibiotic cassette by homologous recombination.

The manufacturer's protocol was used as a guideline and had previously been established for *Serratia plymuthica* 4Rx13 (Weise, dissertation, 2013). The kit is constructed on a Red/ET system (Zhang et al., 2000) using two helper plasmids (pRed/ET and pFRT) for single mutants, and an additional plasmid (Flpe-708) can be used to generate double mutants.

For the gene knockout, primers of 71–73 nucleotides were used, which consisted of 21–22 nucleotides that were homologous to the gene and 51–53 nucleotides that were homologous to the helper plasmid pFRT of the cassette. The 21–23 nucleotides flanked the FRT-PGK-gb2-neo-FRT cassette at each site that coded for neomycin resistance, with a kanamycin cross-resistance. This 1700-bp DNA construct was derived with PCR (High-Fidelity PCR), eluted from the gel (NucleoSpin Gel and PCR Clean-up; Macherey-Nagel, Düren, Germany), and then integrated into the target gene to cause its dysfunction. The helper plasmid pRed/ET was required to integrate the functional cassette by homologous recombination. The plasmid DNA was isolated with the functional cassette, which were flanked by homology arms, and the plasmid pRed/ET was introduced through electroporation.

The transformation of bacterial cells was accomplished by making them competent using Untergasser's protocol for electrocompetent cells (Untergasser, 2008; Weise, dissertation, 2013) but with some modifications. The mutants were verified by using specific primer combinations that showed the presence of the functional cassette (FRT-primer), as well the exact integration (FRT-primer + gene-specific primer) into the gene (**Figure 2.1**). A list of all of the primers used in this research is compiled in **supplement S1**.

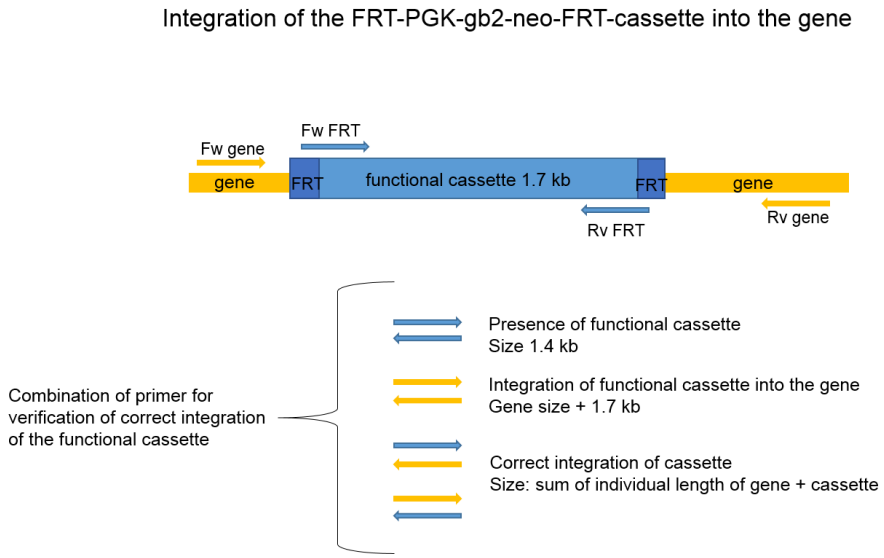


Figure 2.1: Schematic diagram to illustrate the position of applied primer combinations for the verification of mutagenesis.

2.5.3 Plasmid-assisted complementation of mutants

The gene function of the mutants was restored through the introduction of a plasmid containing the wild type gene. This gene was amplified by PCR (Phusion Polymerase; Thermo Fisher Scientific, Waltham, USA), and restriction sites of *Eco*RI and *Bam*HI were added, following which the construct was subcloned into the vector pJET (CloneJET PCR Cloning Kit; Thermo Fisher Scientific). The plasmid was introduced into chemically competent cells of the *E. coli* strain XL-1 Blue. Cells were plated on agar supplemented with the antibiotic ampicillin, and were then verified using gene-specific primers and sequenced (GATC Biotech, Konstanz, Germany). Following this, the plasmid and the vector pUC19 were extracted and digested with the FastDigest restriction enzymes *Eco*RI and *Bam*HI (Thermo Fisher Scientific, Waltham, USA), allowing the fragment to be ligated into the inducible pUC19 vector (own stock; Department of Biochemistry, University of Rostock, Germany). An exception to this was for the gene *SOD_c20760*, which was treated with *Eco*RI and *Bgl*II. The pUC19 vector was then inserted into electrocompetent cells of the mutant, and positive clones could be selected on solid medium supplemented with ampicillin. The electroporation conditions followed the established transformation protocol (Weise, dissertation, 2013).

The mutant containing the complementary plasmid was cultivated in complex liquid media (NB II) at 30°C and 170 rpm until it reached OD_{600} = 0.5. It was then induced with isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM final concentration) after 24 h to initiate gene expression in the plasmid. The VOC profile was monitored over the next 48 h to measure the quantity of sodorifen emitted. However, no sodorifen emission was observed, likely due to the lac promoter of the pUC19 vector not being appropriate for *S. plymuthica* 4Rx13, resulting in the proteins not being expressed. Therefore, a native gapDH promoter region (500 bp in frame with the gene) was cloned into the pUC19 vector and introduced into the mutants to translate the protein. However, this approach also did not produce the desired effect, possibly due to the weak expression of the gene. Thus, a strong promoter was selected and cloned in front of the gene, which resulted in the VOC profile showing a small emission of sodorifen after only 24 h.

2.6. Nucleic acid analysis

2.6.1 Plasmid DNA

Due to difficulties in distinguishing the plasmid sequences from the draft genomes, the presence of plasmid DNA in *S. ply.* V4 was checked using the Plasmid DNA Purification Kit NucleoBond® Xtra Midi/Maxi (Macherey-Nagel, Düren, Germany). The isolate V4 was cultivated in a 5-ml cultivation tube overnight in a complex medium at 30°C and 170 rpm. A 300-ml culture was then inoculated until OD_{600} = 4–5 was reached. This was performed according to the manufacturer's instructions for low-copy plasmid purification and was then checked on a 1% agarose gel (45 min, 70 V, 400 A).

2.6.2 RNA isolation

Colonies of the isolates 4Rx13 and AS9 were inoculated for 24 h in 5–6 ml minimal medium supplemented with 55 mM succinate (Carl Roth, Karlsruhe, Germany) at 30°C and 170 rpm. The cells were then introduced into 100 ml fresh minimal medium with a final OD_{600} = 0.005 and cultivated. One culture was harvested after 24 h and another after 48 h. The culture was centrifuged at 5000 ×*g* and 4°C for 20 min, following which the supernatant was discarded and the sediment was treated with the NucleoSpin RNA II Kit (Machery-Nagel, Düren, Germany).

2.6.3 Separation of nucleic acids by gel electrophoresis

The separation of the DNA was conducted by horizontal gel electrophoresis in a mini chamber (Biostep, Jahnstorf, Germany). This involved a 1% agarose gel (Carl Roth, Karlsruhe, Germany) in 1 × TAE buffer (50 × stock solution: 2 M Tris-HCl, 1 M NaAc, 0.005 M EDTA, pH = 8.0; Carl Roth, Karlsruhe, Germany). Once the gel had cooled, 0.005% [v/v] ethidium bromide was added and the sample was mixed with 6× loading dye (2.4 ml 50 × TAE buffer, 12 ml glycerol, 5.6 ml water, two pinches of bromophenol blue, a pinch of xylencyanol, and Orange G), together with a marker for estimating the size of the nucleic acid molecules (GeneRuler, 1 kb DNA ladder, 0.5 µg/µl; Thermo Fisher Scientific, Waltham, USA). The samples were separated at 70 V, 400 A for 60 min in 1 × TAE buffer, following which the DNA could be visualized under UV light.

2.6.4 Southern blot

The Southern blot technique was used to show that the correct protocol had been used for mutagenesis and that the functional knockout had been achieved by integrating the functional cassette once into the genome. For this analysis, the genes of the terpene cluster were screened. The DNA of these genes was isolated according to a manual protocol (Cheng and Jiang, 2006) and treated with the restriction enzymes *Not*I and *Xho*I (Fermentas, St. Leon-Rot, Germany) at 37°C for 15 h, following which the enzymes were inactivated (20 min, 80°C). The 1% (w/v) agarose gels with 0.005% (v/v) ethidium bromide (Carl Roth, Karlsruhe, Germany) were placed into a minichamber (Biostep, Jahnstorf, Germany) and loaded with a DNA concentration of 15 µg. DNA fragments were then separated (45 min, 70 V, 400 mA), following which they were passed through various washing steps with different solutions. The gel was first shaken for 30 min with a depurination solution (680 mM HCl, v/v). It was then treated twice for 20 min each with a denaturing solution (1.5 M NaCl, 0.5 M NaOH) and subsequently washed twice for 30 min each with a neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl, 1 mM EDTA, pH 7.2), with distilled water rinses between each of these steps. A capillary blot was then set up for an overnight transfer of DNA onto a positively charged nylon membrane (Roche, Basel, Switzerland).

The DNA on the membrane was immobilized with a Crosslinker (Techne, Minneapolis, USA) by UV light (60 s, 0.120 J/cm^2), following which it was prehybridized for 50 min at 68°C. It was then placed in hybridization tubes (Biometra, Göttingen, Germany) and hybridized in a hybridization oven (Peqlab Biotechnologie GmbH, Erlangen, Germany)

at 68°C overnight, with 20 ng of probe per ml of hybridization solution. The detection probe was derived from the functional cassette and control DNA was carried along with it. The probe was labeled by integrating DIG-dUTP (Roche, Basel, Switzerland) and the concentration was checked with testing strips (Roche), which showed a concentration of 12 ng/µl.

Following hybridization, the membrane was passed through a series of washing steps. The membrane was first incubated twice for 5 min each with 25 ml of the first washing solution (2 × SSC, 0.1% SDS) at room temperature, and this was then repeated with the second washing solution (0.2 × SSC, 0.1% SDS) for 15 min at 42°C. The membrane was then incubated twice for 15 min each with the third washing solution (0.1 × SSC, 0.1% SDS) at 68°C. Following this, the membrane was rinsed twice for 5 min each with maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) at room temperature and then with 1 × blocking solution (1% blocking solution (Roche, Basel, Switzerland), dissolved in maleic acid buffer) for 30 min at room temperature. The membrane was then incubated with an antibody (Anti-DIG-AP-AK, 150 mU/ml; Roche, Basel, Switzerland). It was then washed twice more for 15 min each with maleic acid buffer at room temperature, and then incubated with detection buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5, 50 mM $MgCl_2$ solution) followed by CDP-Star (0.25 mM; Roche, Basel, Switzerland) for 5 min. Chemiluminescence was detected using a Stella raytest (CL very sensitive mode, 10 s, 1 min, 5 min or longer).

2.7 Sequencing and analysis of DNA and RNA

2.7.1 Genome sequencing

Genome sequencing was conducted by the Göttingen Genomics Laboratory (G2L), Germany. Shotgun libraries were generated using the Nextera DNA Sample Preparation Kit, following the manufacturer's instructions. The whole genomes of *S. ply.* HRO-C48 and 3Re-4-18 were sequenced with the Genome Analyzer IIx (Illumina, San Diego, USA). The libraries were sequenced in a 112-bp paired-end single-indexed run, resulting in 9.5 and 7.6 mio paired-end reads, respectively. All shotgun reads were assembled *de novo* using Ray (v1.1.0; Boisvert et al., 2012). The draft genome of *S. ply.* HRO-C48 consisted of 62 contigs (> 500 bp) with a genome size of 5.4 Mb, while

that of *S. ply.* 3Re-4-18 consisted of 68 contigs (> 500 bp) with a similar size of 5.4 Mb. The open reading frames (ORFs) were predicted with Glimmer (Delcher et al., 1999). Gene annotation was performed automatically by transferring the existing annotations of the reference genome of *S. ply.* 4Rx13. To identify putative orthologous genes, bidirectional BLAST analysis was used to compare the encoded protein sequences of seven whole genome protein datasets for the following *Serratia* species: CP007439.1 V4, CP002773.1 AS9, LFJS01000012.1 DB11, NC_009832.1 568, NZ_GG753567.1 4582, NZ_AJB000088.1 PRI-2C, and NC_021591.1 4Rx13. The genomes of isolates HRO-C48 and 3Re-4-18 are currently only draft. The contigs were arranged according to the existing reference genome of *S. ply.* 4Rx13 using Mauve Multiple Genome Alignment Software (Darling et al., 2010).

2.7.2 Genome comparison

To compare whole genomes, the BigBag software tool (bidirectional BLAST to distinguish between the pan and core genome; G2L, Germany) was applied. For visualization and comparison of the genomes of the *Serratia* spp., the program Artemis (Rutherford et al., 2000) and the DNAplotter (Carver et al., 2009) of the Sanger Institute (http://www.sanger.ac.uk/) was used.

2.7.3 Transcriptome sequencing

A transcriptome-sequencing approach was applied to compare differentially expressed genes of a sodorifen-producer (*S. ply.* 4Rx13) and a non-producer (*S. ply.* AS9). Sequencing and library construction were carried out by G2L, Germany. Strand-specific cDNA libraries were generated with the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, USA), with the upstream removal of rRNA by the Ribo-Zero rRNA Removal Kit (Bacteria, Illumina, San Diego, USA). Libraries were sequenced on a Genome Analyzer IIx (Illumina, San Diego, USA) and Shotgun transcriptomic reads were mapped to the reference genome sequence using Bowtie 2 (v1.0.0; Langmead, 2009). Mapping results were used to compare differentially expressed genes, the analysis of which was performed using Transcriptome Viewer (TraV) (Dietrich et al., 2014).

2.7.4 Analysis of transcriptome data

The transcriptome data were visualized with the software tool TraV. This software allows the data to be visualized as a table or a plot. The plot shows the nucleotide activities per kilobase of exon model per million mapped reads (NPKM), which represents the transcriptional activity of all identified regions. To compare the RNA-Seq experiments, the mapped data were normalized and presented as NPKM.

2.7.5 Generation of a phylogenetic tree

Prior to phylogenetic analysis, the protein sequence of the terpene cyclase of *S. ply.* 4Rx13 was run as a query sequence for a BLASTp analysis to identify neighbor sequences in the NCBI database. Multiple alignment of sequences was carried out with ClustalW (Thompson et al., 2002). The phylogenetic tree was constructed with the help of the “One Click” mode of phylogeny.fr (Dereeper et al., 2008), which is available at http://phylogeny.fr/. MUSCLE was used for the multiple alignment, PhylMl for tree building, and TreeDyn for tree output. In addition, the sequences of six other *S. ply.* isolates (AS9, AS12, AS13, A30, V4, and S13) were used to construct the tree, and *P. chloraphis*, *S. tskukubaensis*, and *Burkholderia* were used as outgroups.

3. Results

3.1 Candidate genes for the sodorifen biosynthesis

The aim of this thesis was to shed light on the biosynthesis of the unique and structurally unusual mVOC sodorifen emitted by *Serratia plymuthica* 4Rx13 (formerly *S. odorifera*). The obtained results are presented in three chapters. Firstly, a search for a gene(s) involved in the biosynthesis of sodorifen via genome and transcriptome analysis. Functional evidence of the candidate genes was obtained by knockout mutation. This strategy highlighted a terpene cyclase, which was found to be involved in the sodorifen biosynthesis. Secondly, the location of the terpene cyclase gene in the genome was analyzed. Thirdly, a biosynthetic pathway of sodorifen was postulated on the basis of feeding experiments.

3.1.1 Analysis of the VOC profile and sodorifen emission in *Serratia* species

The genus *Serratia* is known to produce a rich spectrum of volatile organic compounds. Previous investigations of *S. plymuthica* 4Rx13 have illustrated that the VOC profiles of this species are quantitatively and qualitatively different from other *Serratia* spp. under similar growth conditions (Weise et al., 2013). The main compound that is released by *S. ply.* 4Rx13 is sodorifen. Here, additional isolates of *S. ply* were screened in order to find other sodorifen-producers. The VOC profiles were monitored in three different intervals: 0-24 h, 24- 48 h and 48- 72 h, while the bacteria were cultivated in a complex medium (NB). The *S. ply.* isolates HRO-C48, 3Re-4-18 and V4 emit sodorifen (**Figure 3.1**, peak S) but in different quantities. 4Rx13 > 3Re-4-18 > HRO-C48 > V4; **Table 3.1**), with isolates 3Re-4-18, HRO-C48, and V4 only emitting 0.77– 5.33 % of the amount emitted by isolate 4Rx13. Furthermore, in general, the VOC profile of isolate 4Rx13 was more complex than that of the other isolates (**Figure 3.1**). Of particular interest is the finding that isolate 4Rx13 released several sodorifen isomers (#1), which were present in significantly lower amounts or absent from the spectra of the other isolates. Isolate AS9 showed no sodorifen emission, despite there being a high similarity between its genome and that of isolate 4Rx13.
It has previously been shown that the compounds dimethyl trisulfide (#2) and pyrazine (#3) are produced by all isolates, while isolates V4 and 4Rx13 also produce dimethyl disulfide (#5) (Weise, dissertation, 2013). In the present study, it was shown that hexadecen-1-ol is emitted by isolates HRO-C48, 3Re-4-18, and V4, while isolate AS9

emitted various other compounds (2-phenylethanol (#9), undecanone (#10), 2-butanone (#11), and n-heptadecyl ester (#12)), which were not present (**Figure 3.1E**). All sodorifen-producers showed similar growth curves (**Figure 3.1F**). Each grew exponentially until achieving a density of 10^9 cells/ml and reached the stationary phase within 24 h, following which they declined and died after approximately 54 h. A difference can be seen in the first phase of exponential growth for isolate AS9, however, which took longer to reach a comparable density.

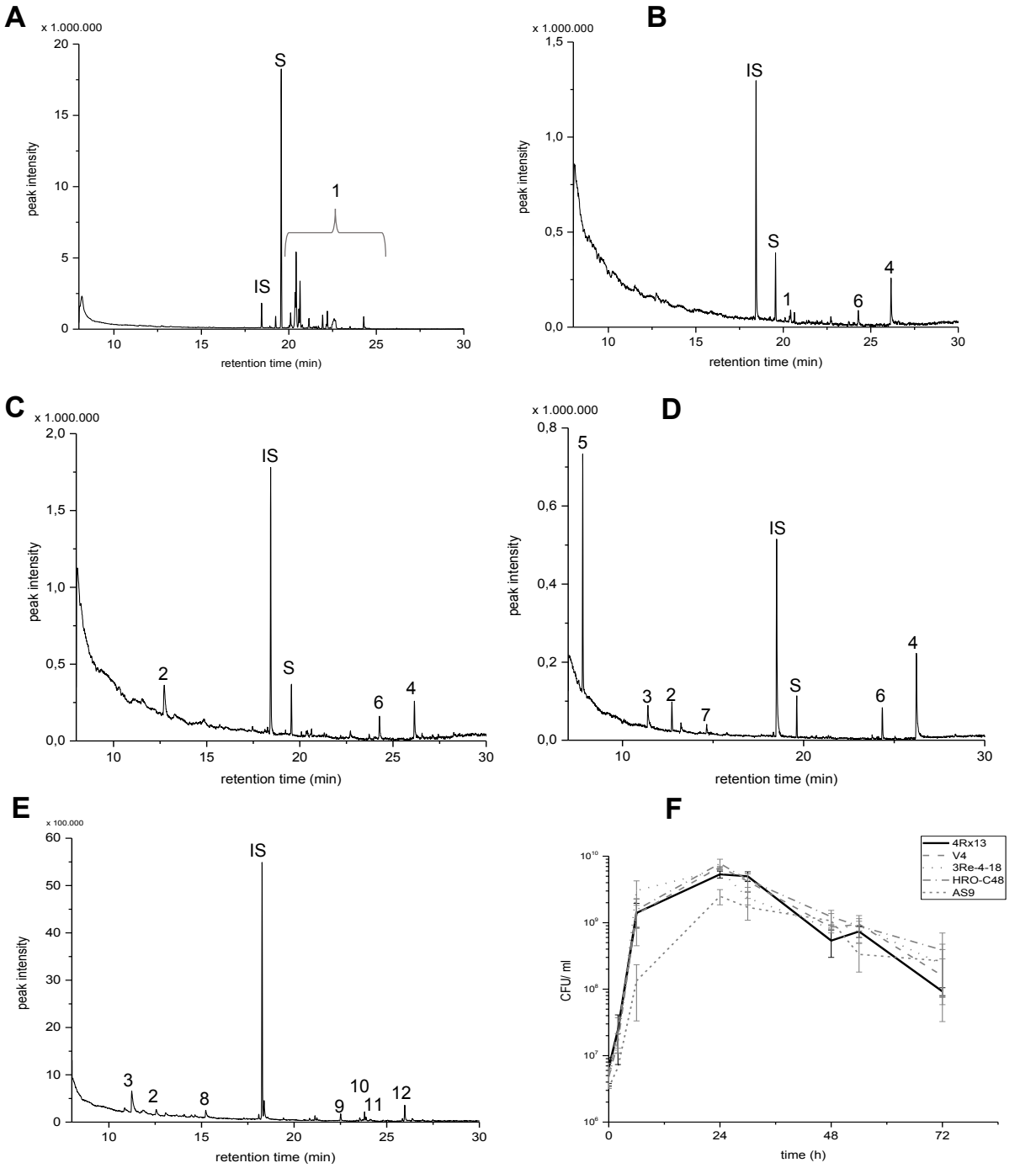


Figure 3.1: VOC profiles and growth curves of *Serratia plymuthica* isolates. The headspace of the VOC profiles of **(A)** *S. ply.* 4Rx13, **(B)** *S. ply.* HRO-C48, **(C)** *S. ply.* 3Re-4-18, **(D)** *S. ply.* V4, and **(E)** *S. ply.* AS9 growing in complex medium (NB II) was analyzed by GC/MS at 24–48 h. Compounds were identified by comparing their mass spectra with those contained within the database Nist107. Peaks are as follows: (IS) nonyl acetate internal standard, (S) sodorifen, (#1) sodorifen isomers, (#2) dimethyl trisulfide, (#3) pyrazine, (#4) hexadecen-1-ol, (#5) dimethyl disulfide, (#6) 2-nonanone, (#7) diethyl ester, (#8) 2-hexanone, (#9) 2-phenylethanol, (#10) undecanone, (#11) 2-butanone, and (#12) n-heptadecyl ester. **(F)** The growth curves of the *S. ply.* isolates V4, 3Re-4-18, HRO-C48, 4Rx13, and AS9 were obtained by determining CFU/ml and calculating standard deviations (*n* = 3) at 0–72 h.

Table 3.1: Relative sodorifen emissions in *Serratia plymuthica* isolates.

Time interval (h)	*Serratia plymuthica* isolates				
	4Rx13	HRO-C48	3Re-4-18	V4	AS9
0-24	100%	3.70 %	5.33 %	0.98 %	0 %
24-48	100%	2.00 %	1.28 %	0.77 %	0 %
48-72	100%	3.07 %	3.72 %	0.92 %	0 %

Sodorifen emissions of the isolates HRO-C48, 3Re-4-18, V4, AS9, and 4Rx13, which were cultivated in complex medium. The adsorbed volatiles were eluted and analyzed in the GC/MS at three time intervals: 0–24 h, 24–48 h, and 48–72 h. The relative sodorifen levels were calculated by setting the peak emission of sodorifen by *S. ply.* 4Rx13 to 100%.

3.1.2 Genome comparison of *Serratia* sodorifen-producers and non-producer strains

A comparative genome analysis of *S. ply.* 4Rx13 with other *Serratia* species was carried out by Weise et al. (2013), in which the non-producer isolates *S. ply.* AS9 (CP002773.1), *S. marcescens* Db11 *(LFJS01000012.1)*, *S. proteamaculans* 568 *(NC_009832.1)*, *S. odorifera* DSM 4582 (NZ_GG753567.1), and *S. ply.* PRI-2C (NZ_AJB000088.1) were compared with the sodorifen-producer *S. ply.* 4Rx13.

The comparison between the sodorifen-producer with non-producers resulted in 246 unique ORFs being identified for isolate 4Rx13. Among these unique ORFs, 138 genes were annotated as hypothetical proteins, which were of great interest as they could potentially be involved in the biosynthesis of sodorifen (Weise, dissertation, 2013). Therefore, further comparison with other sodorifen-producers should lead to common genes being detected that are involved in sodorifen metabolism.

In the present study, two additional sodorifen-producer isolates (HRO-C48 and 3Re- 4- 18) were sequenced to find common genes that may be involved in the production of sodorifen. Their genomes currently exist as draft genomes and have not yet been submitted to NCBI, but can be accessed online via Integrated Microbial Genomes (IMG) (Markowitz et al., 2006). Another recently sequenced isolate, *S. ply.* V4 (CP007439.1), was also used for comparison (Cleto et al., 2014).

The draft genomes of *S. ply.* HRO-C48 and *S. ply.* 3Re-4-18 had genome sizes of 5.48 Mb and 5.40 Mb, respectively, with 5210 and 5169 ORFs detected. Thus, there

was a high ratio of coding sequences to non-coding sequences (96%) within the genomes of both isolates. According to NCBI, *S. ply.* V4 had 4958 ORFs, while isolate AS9 had 5032 ORFs. The genome size of the sodorifen-producers ranged from 5.36 to 5.51 Mb (**Table 3.2**). The GC content of the *S. ply*. isolates ranged from 55.32% to 56.20%. The non-producer isolate AS9 had a similar genome size of 5.47 Mb, which placed it between isolates 4Rx13 and V4, and had a GC content of 56.20%, which is also similar to the values for the other isolates (**Table 3.2**).

Table 3.2: Characterization of the genome data for different *Serratia plymuthica* isolates.

Isolate	Genome size (Mb)	GC content (%)	Presence of plasmid DNA	Reference
4Rx13	5.36	56.20	Yes	Weise et al., 2014
HRO-C48	5.48	56.22	Yes	Unpublished data
3Re-4-18	5.40	55.31	Yes	Unpublished data
V4	5.51	56.20	Yes	Cleto et al., 2014
AS9	5.47	56.20	Yes	Tatusova et al. 2014

Comparison of the sodorifen-producer and non-producer including the isolates HRO- C48, 3Re-4-18 and V4 at the genome level provided an overview of the genes and proteins that are shared and are unique. This detailed comparison was performed with help of a bidirectional Biblast. To visualize the similarities and differences between the genomes, the DNAplotter (Carver et al., 2009) of the Sanger Institute (http://www.sanger.ac.uk/) was applied, which plots the genes against each other in a circle (**Figure 3.2**).

It has previously been shown that, in general *S. ply.* 4Rx13 has a similar genome size to other *Serratia* species, with a whole genome sequence alignment showing an identity of 94–96% (Weise et al., 2014). In the present study, the genomes of the sodorifen- producer isolates HRO-C48, 3Re-4-18 and V4 were also included. In **Figure 3.2**, the core genome in red depicts the similarities (90–100% identity) at the nucleotide level, while white depicts the differences (0–20% identity). On the basis of a whole genome sequence alignment (**Table 3.3**), all sodorifen-producer isolates of

S. ply. (red circles: 3-6, from outside to inside) are similar with the exception of isolate AS9, which belongs to the non-producer. The non-producer (circle 6-10) are less similar compared to the sodorifen-producer.

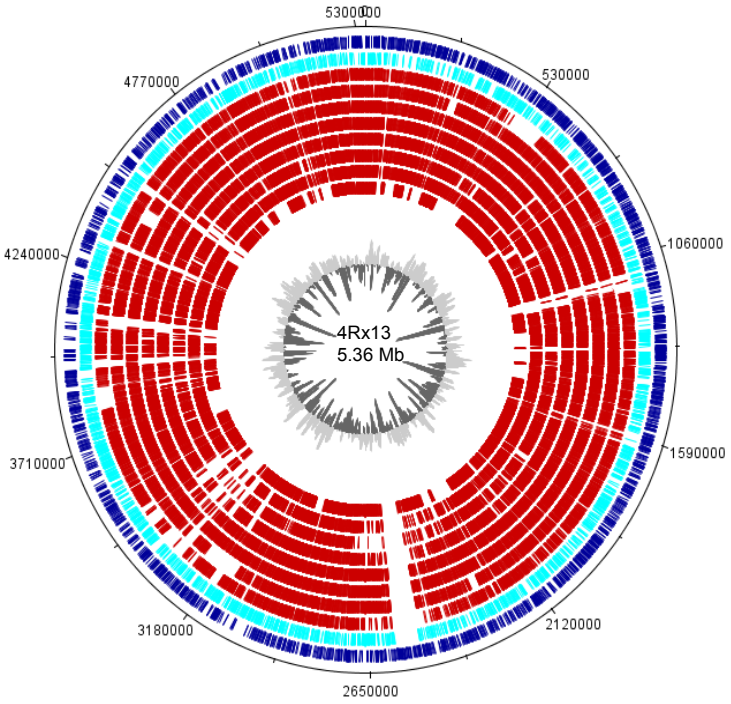


Figure 3.2: The genome of *Serratia plymuthica* 4Rx13 plotted against the genomes of other *Serratia* spp. Bidirectional BiBlasts of the genomes were plotted against each other using DNAplotter (Carver et al., 2009). The circles from outside to inside are as follows: 1. open reading frames (ORFs) in the sense strand of *S. ply.* 4Rx13 (dark blue), 2. antisense strand of *S. ply.* 4Rx13 (light blue), 3. *S. ply.* HRO-C48, 4. *S. ply.* 3Re-4-18, 5. *S. ply.* V4, 6. *S. ply.* AS9, 7. *S. proteamaculans* 568, 8. *S. odorifera* DSM 4582, 9. *S. marcescens* Db11, 10. *S. ply.* PRI-2C, and 11. GC content (light gray: G, dark gray: C). Red: core genome; white: pan genome.

Table 3.3 summarizes additional features of the sodorifen producers. This shows, for example, that *S. ply.* 4Rx13 and V4 shared the most ORFs (3023), with only 313 different ORFs being found. In addition, isolate HRO-C48 had 2861 ORFs and 3Re- 4- 18 had 2400 ORFs in common with isolate 4Rx13. Isolate 3Re-4-18 had the most genes that were different from isolate 4Rx13 (919 ORFs), followed by isolate HRO-C48 (556 ORFs).

By comparing sodorifen non-producers with *S. ply.* 4Rx13, it was possible to clarify the relationship between other *Serratia* species. For example, *S. ply.* AS9 had the most ORFs (2901) in common with isolate 4Rx13, followed by *S. proteamaculans* 568 (2512

ORFs), *S. marcescens* (2258 ORFs), *S. ply.* PRI-2C (2059 ORFs), and *S. odorifera* DSM 4582 (2015 ORFs). Furthermore, similar results were also found when analyzing the ORFs that differed between the *Serratia* species, with *S. ply.* AS9 showing 436 different ORFs from isolate 4Rx13, followed by *S. proteamaculans* 568 (784 ORFs), *S. marcescens* Db11 (956 ORFs), and *S. odorifera* DSM 4582 (1221 ORFs). Isolate *S. ply.* PRI-2C differed most from *S. ply.* 4Rx13 (1579 different ORFs), despite this bacterium belonging to the same species. In general, these findings support the previous 16S-rRDNA analysis that was used to construct a phylogenetic tree for *Serratia* species (Weise et al., 2014).

The genome comparison showed that isolate V4 had the most genes in common with isolate 4Rx13. Intriguingly, however, isolate AS9, which is a sodorifen non-producer, had even more conformity with isolate 4Rx13 than the sodorifen-producer isolates 3Re-4-18 and HRO-C48.

Table 3.3: Comparison of the genomes of sodorifen-producing and non-producing *Serratia* spp.

Serratia spp. isolate	Sodorifen producer (+) of non-producer (−)	Number of common genes with *S. ply*. 4Rx13	Number of different genes from *S. ply*. 4Rx13 genes	Sequence identity (%)
S. ply. 3Re-4-18*	+	2400	919	83.4
S. ply. HRO-C48*	+	2861	556	96.5
S. ply. V4	+	3023	313	95.5
S. ply. AS9	-	2901	436	94.5
S. ply. PRI-2C	-	2059	1579	71
S. proteamaculans 568	-	2512	784	87.5
S. odorifera DSM 4582	-	2015	1221	74.1
S. marcescens Db11	-	2258	956	71.6

Open reading frame (ORF) sequences of *Serratia* spp. were compared using the BigBag software tool. ORFs with an e-value > −100 and a Needleman-Wunsch percent identity (NW-identity) > 70% were assigned as common ORFs, while ORFs with an e-value of e^{-20} −1 and NW identity of 0–20% were assigned as different genes. * draft genomes.

3.1.3 Analysis of the plasmid-DNA of *S. plymuthica*

In addition to investigating chromosomal DNA, the isolates were also checked for the presence of plasmid DNA because it was hypothesized that the genes involved in the biosynthesis of sodorifen could be localized extrachromosomally. It has previously been shown that isolates 4Rx13, HRO-C48, and 3Re-4-18 contain at least one plasmid

(Weise, dissertation, 2013). Therefore, the sodorifen-producer isolate V4 was investigated for the presence of plasmid DNA (**Figure 3.3**).

Plasmid DNA was detected in isolate V4, but the plasmid was smaller (< 50 Kb) than that of isolate 4Rx13 (75 Kb). The isolation procedure included a size-selective step, which indicated that the plasmid in isolate V4 was smaller than 50 Kb. However, it should be noted that this procedure only allows the presence of plasmid DNA to be detected, with no size determination possible. Since all sodorifen producers have at least one plasmid in common (Weise, dissertation, 2013), they could hypothetically be involved in the production of secondary metabolites. The possibility of the genes in the plasmid being involved in the biosynthesis of sodorifen or the production of other secondary metabolites has not been entirely excluded. However, the plasmid genes were not further considered in the present study.

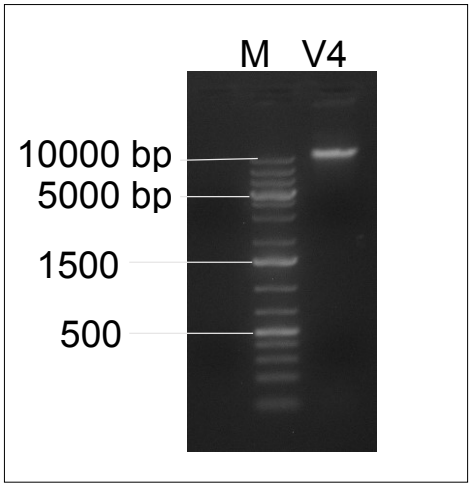


Figure 3.3: Isolation of plasmid DNA in *Serratia plymuthica* V4. The plasmid DNA was obtained using the NucleoBond Xtra Midi/Maxi Kit and analyzed by 1% agarose gel-electrophoresis. M: the 1 Kb Plus DNA ladder.

3.1.4 Candidate genes for sodorifen biosynthesis

The genome comparison showed that i) the sodorifen producers are more similar to each other than the non-producer strains are to isolate 4Rx13, ii) isolate V4 is the closest relative to isolate 4Rx13, and iii) isolate AS9 shares the most genes with isolate 4Rx13, despite it not being able to produce sodorifen.

Therefore, when searching for and selecting candidate genes for the biosynthesis of sodorifen, genes were separated into three groups: Group A includes two genes that all sodrorifen-producers have in common but are absent from the non-producers, of

the isolate AS9, Group B includes a selection of 17 genes that are present in the majority of sodorifen-producers and the closely related non-producers AS9 and PRI- 2C, and Group C is an extension to Group B that includes the sodorifen non-producer DSM 4582 and highlights 24 potential genes (**Table 3.4**).

It was a disadvantage that the sodorifen-producer isolates *S. ply.* 3Re-4-18 and HRO- C48 only existed as draft genomes due the uncomplete sequence and arrangement in contigs, it is possible that not all genes are present. To circumvent this disadvantage the presence or absence of the candidate genes was additionally verified by PCR and then added to the relevant group. Especially, the isolate 3Re-4-18 had to be additionally checked due to the absence of genes in the genome data but the PCR revealed the presence of the genes.

Table 3.4: Search for candidate genes involved in sodorifen production

Gene ID	Annotation	Presence of genes in *Serratia* spp.							
		3Re-4-18	HRO-C-48	V4	AS9	Db11	568	DSM 4582	PRI-2C
Group A									
SOD_c13130	Hypothetical protein	x	x	x	x	-	-	-	-
SOD_c44800	Hypothetical protein	x	x	x	x	-	-	-	-
Group B									
SOD_c03810	Hypothetical protein	-	x	x	x	-	-	-	x
SOD_c10340	Hypothetical protein	-	x	x	x	-	-	-	x
SOD_c17850	Hypothetical protein	-	x	x	x	-	-	-	x
SOD_c17950	CatD1:3-oxoadipate enol-lactonase 2 (3.1.1.24)	-	x	x	x	-	-	-	x
SOD_c20750	Putative terpene cyclase	-	x	x	x	-	-	-	x
SOD_c20760	Hypothetical protein	-	x	x	x	-	-	-	x
SOD_c20770	Dxs:1-deoxy-D-xylulose-5-phosphate synthase Dxs	-	x	x	x	-	-	-	x

SOD_c21320	AglA:pTS system alpha-glucoside-specific EIICB component	-	x	x	x	-	-	-	x
SOD_c22790	QorB:quinone oxidoreductase 2	-	x	x	x	-	-	-	x
SOD_c24590	Hypothetical protein	-	x	x	x	-	-	-	x
SOD_c29310	Hypothetical protein	-	x	x	x	-	-	-	x
SOD_c30020	Hypothetical protein	-	x	x	x	-	-	-	x
SOD_c35130	Hypothetical protein	-	x	x	x	-	-	-	x
SOD_c39970	Hypothetical protein	-	x	x	x	-	-	-	x
SOD_c45440	Hypothetical protein	-	x	x	x	-	-	-	x
SOD_c29200	VdlC:putative short-chain type dehydrogenase/reductase	-	x	x	x	-	-	-	x
SOD_c29250	Hypothetical protein	-	x	x	x	-	-	-	x

Group C									
SOD_c00690	Hypothetical protein	-	x	x	-	-	-	x	x
SOD_c14150	Hypothetical protein	-	x	x	-	-	-	x	x
SOD_c22570	Hypothetical protein	-	x	x	-	-	-	x	x
SOD_c22780	Hypothetical protein	-	x	x	-	-	-	x	x
SOD_c23170	Hypothetical protein	-	x	x	-	-	-	x	x
SOD_c23180	Hypothetical protein	-	x	x	-	-	-	x	x
SOD_c30110	Hypothetical protein	-	x	x	-	-	-	x	x
SOD_c30140	Hypothetical protein	-	x	x	-	-	-	x	x
SOD_c30150	AspC2:aspartate aminotransferase	-	x	x	-	-	-	x	x
SOD_c30160	Hypothetical protein	-	x	x	-	-	-	x	x
SOD_c30170	Hypothetical protein	-	x	x	-	-	-	x	x
SOD_c30180	AspC:aspartate aminotransferase	-	x	x	-	-	-	x	x
SOD_c30200	Hypothetical protein	-	x	x	-	-	-	x	x
SOD_c30710	Hypothetical protein	-	x	x	-	-	-	x	x

SOD_c13290	Putative isomerase	-	x	x	-	-	-	x	x
SOD_c29090	Putative oxidoreductase	-	x	x	-	-	-	x	x
SOD_c29100	YulF:putative oxidoreductase YulF	-	x	x	-	-	-	x	x
SOD_c38740	KamA:L-lysine 2,3-aminomutase	-	x	x	-	-	-	x	x
SOD_c38750	Hypothetical protein	-	x	x	-	-	-	x	x
SOD_c38760	Hypothetical protein	-	x	x	-	-	-	x	x
SOD_c29110	YrbE:putative oxidoreductase YrbE	-	x	x	-	-	-	x	x
SOD_c22540	Putative oxidoreductase	-	x	x	-	-	-	x	x
SOD_c30130	Putative oxidoreductase	-	x	x	-	-	-	x	x
SOD_c36720	YdbC:oxidoreductase YdbC	-	x	x	-	-	-	x	x

The sodorifen-producer isolates are highlighted in gray, while non-producers have a white background. Genes were grouped as follows: A) genes that are present in the sodorifen producers and absent from non-producers; B) genes that are present in most sodorifen-producer strains, and *S. ply.* AS9 and PRI- 2C; and C) genes that are present in most sodorifen producers, and in *S. ply.* PRI-2C and *S. odorifera* DSM 4582. X: gene is present; -: gene is absent.

3.1.5 Mutagenesis of two candidate genes based on genome comparisons

The grouping of genes as outlined above resulted in 45 candidate genes for sodorifen biosynthesis being identified. Following this, application of the most stringent criterion for selection, i.e., that all of the sodorifen-producing isolates had the genes for synthesizing sodorifen in common, and that these were absent from the sodorifen non- producers, with the exception of isolate AS9, led to the identification of two candidate genes (*SOD_c13130* and *SOD_c44800*; Group A).

The mutagenesis of these candidate genes was successfully performed according to the established protocol by Weise (dissertation, 2013), following which the VOC profile was analyzed in a modified VOC-collection system (Kai et al., 2010). This showed that the mutants presented an unchanged phenotype (**Figure 3.4**). Gene *SOD_c30110* of Group C was also knocked out, was still able to produce sodorifen. The VOC profile can be found in **supplementary Figure S9B**. Although mutagenesis led to a dysfunctional protein, the gene was still present, with an integrated resistance cassette

and a deletion of 10 bp within the gene. Thus, it led to a functional knockout, and so from here on these mutants are referred to as “knockout mutants.”

A) **B)**

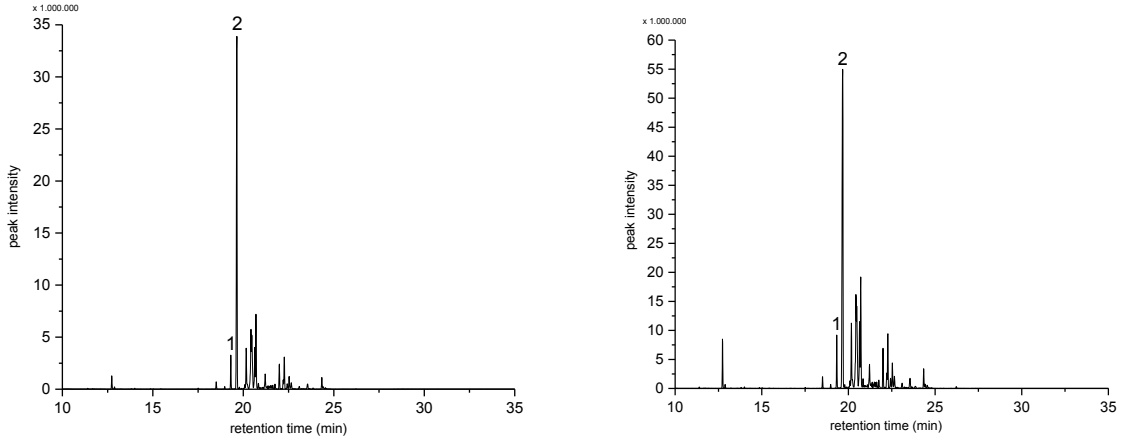


Figure 3.4: Chromatogram of knockout mutants of candidate genes for sodorifen biosynthesis. Knockout of gene **A)** *SOD_c13130* and **B)** *SOD_c44800*. The emitted volatiles of the mutants were examined in a VOC-collection system. Peaks are as follows: 1. nonyl acetate internal standard (concentration = 5 ng / 10 µl), 2. sodorifen.

3.1.6 Analysis of the transcriptomes of *S. plymuthica* 4Rx13 and AS9

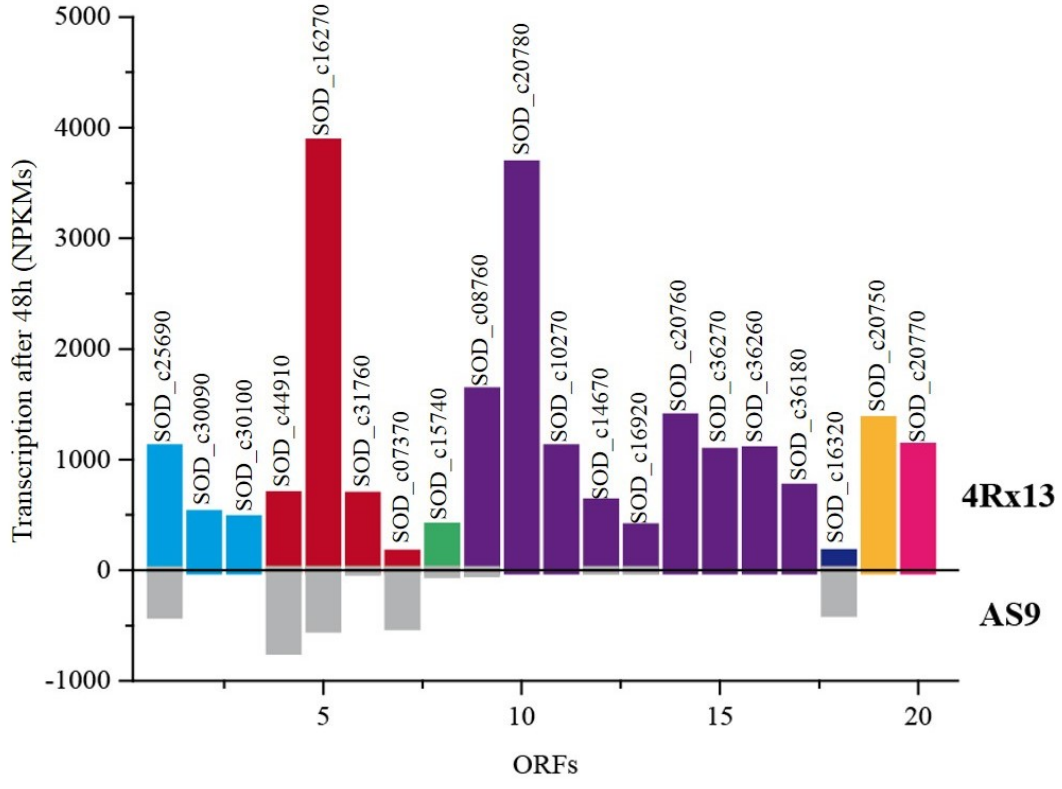
As an alternative approach to finding candidate genes for sodorifen biosynthesis, the transcription levels of genes in the sodorifen producer *S. ply.* 4Rx13 and the closely related non-producer *S. ply.* AS9 were compared based on RNA-sequencing data. This allowed pseudogenes or truncated genes that were not transcribed to be eliminated. Growth conditions were selected that yielded high sodorifen emissions, as these should lead to higher expression levels of the genes involved in the biosynthesis of sodorifen, allowing them to be distinguished from housekeeping genes.

Thus, isolates 4Rx13 and AS9 were cultivated in a minimal medium supplemented with succinate, which has previously been shown to yield the highest sodorifen emissions (Weise, dissertation, 2013). Total RNA was isolated after 24 h (low sodorifen emissions) and 48 h (high sodorifen emissions) of growth (Domik, diploma thesis, 2012), and was then sequenced, allowing the transcription levels of genes to be compared. To analyze the transcriptome data, the sequenced mRNA was visualized using the TraV software tool (Dietrich et al., 2014).

Following the standard protocol for genome investigations, all enzymes, proteins, and genes that are involved in the general primary metabolism of the organism, such as transcription factors, proteins involved in translation, and all housekeeping genes, were excluded from further analysis. In addition, for a gene or enzyme to be considered as a candidate for sodorifen biosynthesis, it needed to have a higher transcription level (NPKM) in *S. ply.* 4Rx13 than in the non-producer *S. ply.* AS9.

In total, 21 genes were identified that met these criteria, the transcription profiles for which are illustrated in **Figure 3.5A**. These genes were involved in various different metabolic pathways, such as carbohydrate metabolism (three genes), stress response (four genes), and nucleotide metabolism (one gene) (**Figure 3.5B**), and included nine hypothetical proteins (violet bars in **Figure 3.5**), which should be further investigated due to the likelihood that novel enzymes are involved in the biosynthesis of sodorifen. Two of these genes (*SOD_c20750* and *SOD_c20770*) were also found to be involved in isoprenoid metabolism, which is interesting given that it is believed that sodorifen could be a terpenoid.

A)



B)

Genes	Occurence	Annotation
SOD_c25690	Carbohydrate metabolism	Glyceraldehyde-3-phosphate dehydrogenase
SOD_c30090	Carbohydrate metabolism	Phosphoenolpyruvate phosphomutase
SOD_c30100	Carbohydrate metabolism	Phosphonopyruvate decarboxylase
SOD_c44910	Stress response	Universal stress protein A
SOD_c16270	Stress response	Chaperone protein ClpB
SOD_c31760	Stress response	Heat shock protein
SOD_c07370	Stress response	Osmotically-inducible lipoprotein E
SOD_c15740	Nucleotide biosynthesis	Cytidylate kinase Cmk
SOD_c08760	Hypothetical protein	Hypothetical protein
SOD_c20780	Hypothetical protein	Hypothetical protein (IPP Isomerase)
SOD_c10270	Hypothetical protein	Hypothetical protein
SOD_c14670	Hypothetical protein	Hypothetical protein
SOD_c16920	Hypothetical protein	Hypothetical protein
SOD_c20760	Hypothetical protein	Hypothetical protein (Methyltransferase)
SOD_c36270	Hypothetical protein	Hypothetical protein
SOD_c36260	Hypothetical protein	Hypothetical protein
SOD_c36180	Hypothetical protein	Hypothetical protein
SOD_c16320	Membrane	Modulator of FtsH protease YccA
SOD_c20750	Isoprenoid metabolism	Putative terpene cyclase
SOD_c20770	Isoprenoid metabolism	1-deoxy-D-xylulose-5-phosphate synthase

Figure 3.5: Analysis of the transcriptomes of *Serratia plymuthica* 4Rx13 and AS9. Both isolates were grown in minimal medium supplemented with succinate over 48 h. Samples for RNA isolation were taken after 24 h and 48 h, and mRNA was sequenced to determine the expression levels of genes. **A)** Genes expressed in high levels in *S. ply.* 4Rx13 and expressed at a low level or not at all in *S. ply.* AS9. **B)** Differentially expressed genes in *S. ply.* 4Rx13 and AS9 with their annotations, which assigned them to various metabolic pathways.

3.1.7 Analysis of terpene cyclase mutant and complementation

Not all 45 potential candidate genes that were identified at the genome level were investigated by mutagenesis. It was found that mutants of the genes *SOD_c13130* and *SOD_c44800* were still able to produce sodorifen. Therefore, overlaps between the candidate genes that were detected using the genome data and the transcriptome data

were identified to help determine which genes were involved in isoprenoid metabolism. The gene *SOD_c20750*, which is annotated as terpene cyclase, showed pronounced expression in *S. ply.* 4Rx13 (NPKM after 48 h = 1357) but no transcription in *S. ply.* AS9, and so was chosen as a candidate gene for mutagenesis. Verification of the mutants was performed phenotypically and genotypically, as illustrated in **Figure 3.6 A–D**. The gene knockout exhibited a sodorifen-negative phenotype that also included a new compound in its VOC profile (**Figure 3.6A**). The stability of the mutation was investigated by PCR with gene specific primer. Thus, the presence of the resistance cassette was checked over 72 h (**Figure 3.6 B).** The mutated gene is 1.7 kb bigger (lane 2, 4, 6 in **Figure 3.6 B**) than the wild type (lane 1, 3, 5) due to the integrated resistance cassette into the mutant but absent in the wild type gene. The correct integration of the resistance cassette was verified by a combination of gene specific and resistance cassette primer (**Figure3.7C**). **Figure 3.6C**. The wild type and mutant had similar growth behaviors (**Figure 6D**), indicating that the mutation had no effect on cell differentiation or metabolism.

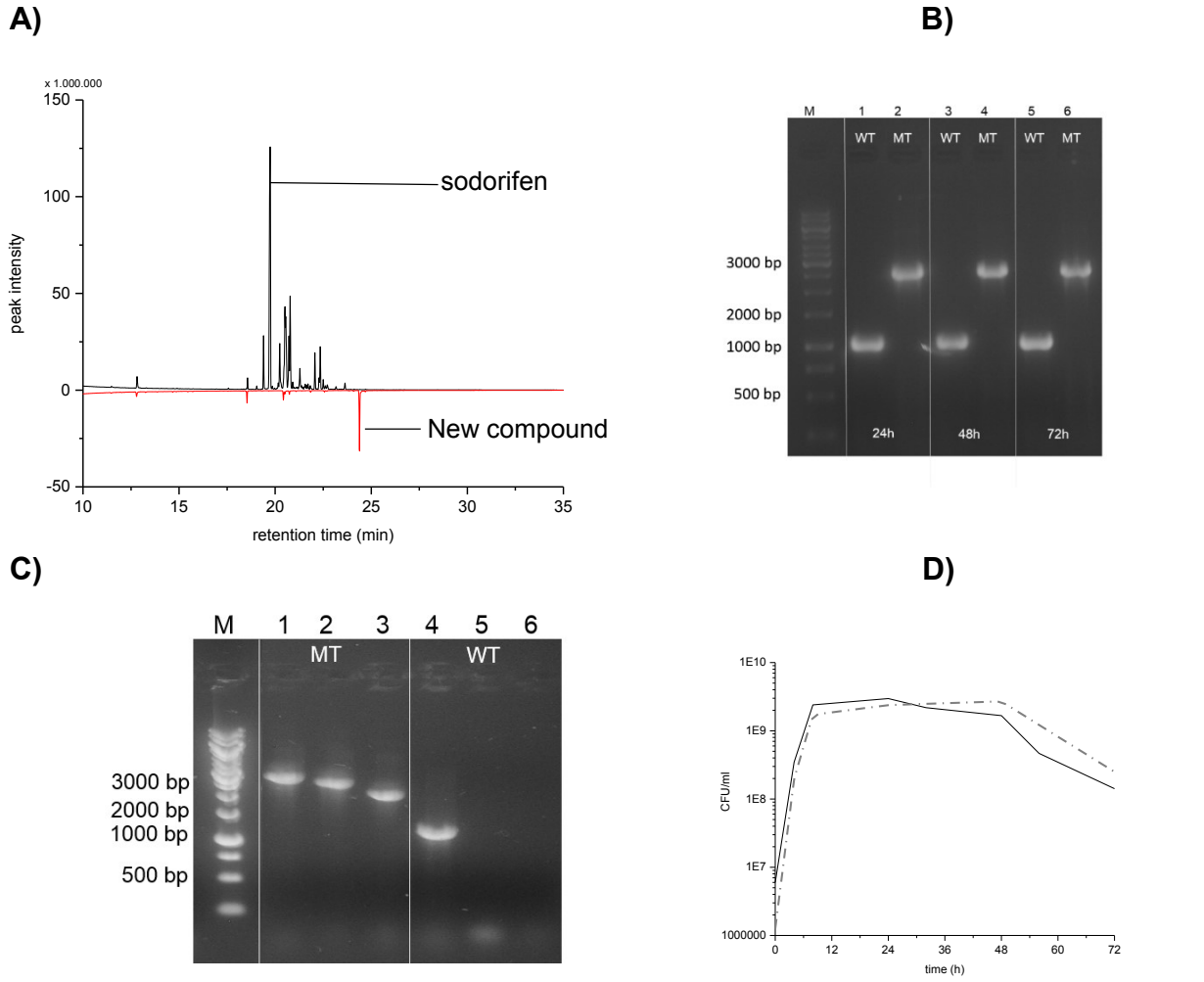


Figure 3.6: Analysis of the terpene cyclase knockout mutant of *Serratia plymuthica* 4Rx13. A) A typical volatile organic compound (VOC) profile of the mutant (red line) and the wild type (black line) grown in complex medium after 48 h (*n* = 2). **B)** Polymerase chain reaction (PCR) products of the wild type gene (WT; 1.1 kb) and mutant (MT; 2.8 kb) cultivated in complex medium over 72 h. **C)** Integration of the resistance cassette (1.7 kb) was verified by PCR with specific primer combinations: lanes 1–3, the mutant with the integrated resistance cassette; lanes 4–6, the wild type without the inserted resistance cassette (FRT-PGK-gb2-neo-FRT-cassette); lanes 1+4, the amplicon with gene-specific sense and antisense primers for the mutated gene; lanes 2+5, the amplicon with a specific sense primer for the inserted resistance cassette and a gene-specific antisense primer; lanes 3+6, the amplicon with a gene-specific sense primer and an antisense primer specific for the resistance cassette; M: GeneRuler 1 kb ladder. **D)** Growth curve of the mutant (dotted line) and wild type (black line), measured in colony-forming units (CFU)/ml (*n* = 2).

The mutation of the gene *SOD_c20750* was reversed by plasmid-assisted complementation, whereby a recombinant plasmid harboring the wild type gene was reintroduced into *S. ply.* 4Rx13 under the control of a strong promoter, leading to the

expression of the gene and restoring its function. Thus, the complemented mutant produced sodorifen. However, the quantity of sodorifen produced was significantly reduced compared with the wild type (< 1% after 72 h growth; **Figure 3.7**). The chromatograms for the regained sodorifen emissions are provided in **supplementary Figure S2**.

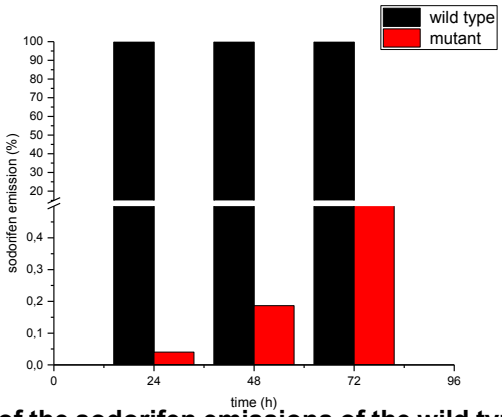


Figure 3.7: Quantification of the sodorifen emissions of the wild type and complemented terpene cyclase mutant of *Serratia plymuthica* 4Rx13. The emission of sodorifen was restored by introducing a recombinant plasmid consisting of the plasmid pUC19 and the wild type terpene cyclase gene into the mutant. The emission of the complemented terpene cyclase mutant (red) is plotted against the wild type (black). Wild type equals 100%.

3.1.8 Presence of the terpene cyclase of *S: plymuthica* 4Rx13 in other bacteria

Alignment of the gene sequence of *SOD_c20750* with sequences stored in the NCBI and Swiss-prot databases showed that this gene had high conformity (99%) with other sequenced *S. ply.* isolates: AS9, AS12, AS13, S13, and A30 (**Table 3.5**). Lower identities were also observed with more distantly related bacteria, such as *Pseudomonas chlororaphis* O6 (43.4%), *Streptomyces tskubaensis* NRRL 18488 (41.8%), and *Burkholderia pyrrocinia* (41%). For the latter, the protein found in the database was annotated as a hypothetical protein. The related genes in *Pseudomonas agarici* (31%) and *Streptomyces partensis* (27.3%) were annotated as terpene synthases, which are similar to the terpene cyclase of isolate 4Rx13 (**Table 3.5**).

Table 3.5: Homologous protein sequences of the terpene cyclase of *Serratia plymuthica* 4Rx13 in other bacteria.

Description	Organism	E value	Identity (%) at amino acid level
Putative terpene cyclase	*S. ply.* 4Rx13	0.0	100
Terpene cyclase	*S. ply.* AS9; AS12; AS13;S13 and A30	0.0	99
Uncharacterized protein	*Pseudomonas chlororaphis* O6	31e-99	43.4
Uncharacterized protein	*Streptomyces tskubaensis* NRRL 18488	190e-93	41.8
Hypothetical protein	*Burkholderia pyrrocinia*	5e-85	41
Terpene synthase	*Pseudomonas agarici*	2e-45	31
Terpene synthase	*Streptomyces pratensis*	45e-33	27.3

The BLASTp search with the terpene cyclase of *S. ply.* 4Rx13 as the query sequence was carried out in the National Center for Biotechnology Information (NBCI) Protein Database and the Swiss-prot database.

3.1.9 Protein motifs within the terpene cyclase

Bacterial terpene cyclases contain one of the two universally conserved motifs that are typically found in the terpene synthases of plants and fungi. A slight variation of the aspartate-rich **DD**XX(**D**/**E**) motif is present in the terpene cyclase of *S. ply.* 4Rx13, which contains an additional variable amino acid (aa) to make **DD**XXX**DE**. As is the case for other terpene cyclases, this motif is located between aa 101 and 109 (Yamada et al., 2014). The homologous terpene cyclase in isolate AS9 was extremely similar (99%) to that of isolate 4Rx13 and contained an identical motif.

The NSE/DTE motif (N/D)DXX(S/T)XX(K/R)(D/E), which is involved in binding cations (particularly Mg^{2+}), which is required for cyclization of the acyclic terpene precursor (Cane et al., 2012), is absent from *S. ply.* 4Rx13.

Thus, terpene cyclases are widespread among bacteria and the characteristic motifs can be found in distantly related species. To the best of our knowledge, the products of the terpene cyclases of other bacterial species or other *S. ply.* isolates have not yet been determined. Here it was shown for the first time that the gene *SOD_c20750* is indispensable for the synthesis of sodorifen.

3.2 Analysis of the sodorifen cluster

3.2.1 Localization of the terpene cyclase within the genome

The knockout of the gene *SOD_c20750* exhibited a sodorifen-negative phenotype and therefore this gene was considered important due to its involvement in sodorifen biosynthesis. Therefore, it was believed that further investigation into the location of this gene within the genome may lead to the identification of additional biosynthetic genes. To localize the gene in a potential cluster, the software antiSMASH (Medema et al., 2011) was applied. This resulted in a small cluster of four genes (*SOD_c20750*, *SOD_c20760*, *SOD_c20770*, and *SOD_c20780*) being identified, which are positioned in the antisense orientation within the genome (**Figure 3.8**). The four genes in this cluster have individual lengths of I: 540 bp, II: 1840 bp, III: 978 bp and IV: 1100 bp, giving an expected total transcript length of 4.4 kb. Upstream the cluster is embedded by the gene *SOD_c20740*, which was annotated as a regulator for araBAD and araFGH operons, and other genes involved in the transport and catabolism of L-arabinose. Downstream the gene *SOD_c20790* is located, which was annotated as a putative oxidoreductase. Both genes (*SOD_c20740 and SOD_c20790*) are orientated in the sense direction.

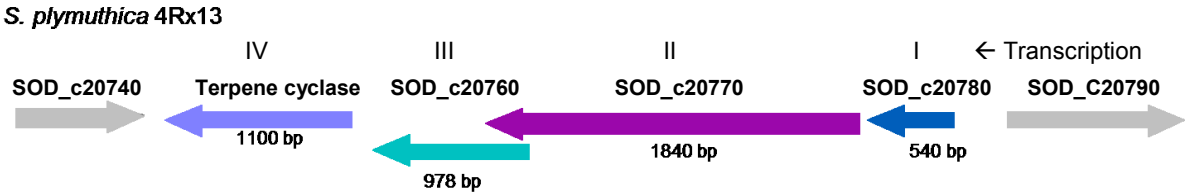


Figure 3.8: Analysis of the localization of the terpene cyclase within the genome of *Serratia plymuthica* 4Rx13. The terpene cyclase forms a cluster together with three additional genes, which are positioned in the antisense direction within the genome. This cluster is bordered by two genes in the sense orientation.

3.2.2 Analysis of the transcription levels of the sodorifen cluster genes

The transcriptome data for the genes within the sodorifen cluster were evaluated according to the following criteria: i) the transcription level should be above the level of the housekeeping genes, ii) gene expression should be high in isolate 4Rx13 and very low or absent in isolate AS9, and iii) genes belonging to the general cell cycle, such as

transcription and translation factors, housekeeping genes, or stress response genes, should be excluded.

All of these criteria were fulfilled by the terpene cyclase, which was highly expressed (NPKM = 1357) in isolate 4Rx13 but had little to no expression in isolate AS9 after 48 h (**Figure 3.9**). In addition, the other three genes in the cluster were also highly expressed in isolate 4Rx13 (*SOD_c20760*: NPKM = 1382; *SOD_C20770*: NPKM = 1118; *SOD_c20780*: NPKM = 3669) and not expressed in isolate AS9.

The genes within this cluster have much lower transcription levels in *S. ply.* AS9 than in *S. ply.* 4Rx13 (**Figure 3.9**). After 24 h and 48 h of growth the homologous gene of *SOD_c20780* in isolate AS9 was not transcribed and almost no activity was seen for the homologous gene *SOD_c20770*. Furthermore, the activity of the homologous gene of *SOD_c20760* had an NPKM value of 4 in isolate AS9, compared with 907 in isolate 4Rx13; and the homologous gene of *SOD_c20750* had an NPKM value of 1 in isolate AS9, compared with 939 in isolate 4Rx13. Thus, the homologous genes of this cluster show no or very little activity in isolate AS9. These findings support the hypothesis that a lack of expression of genes in this cluster resulted in the lack of sodorifen emission. The transcribed mRNA was visualized using the software TraV (Dietrich et al., 2014), which depicted all four genes of the cluster as a single mRNA unit (**Figure 3.9**), which could indicate cotranscription of these genes.

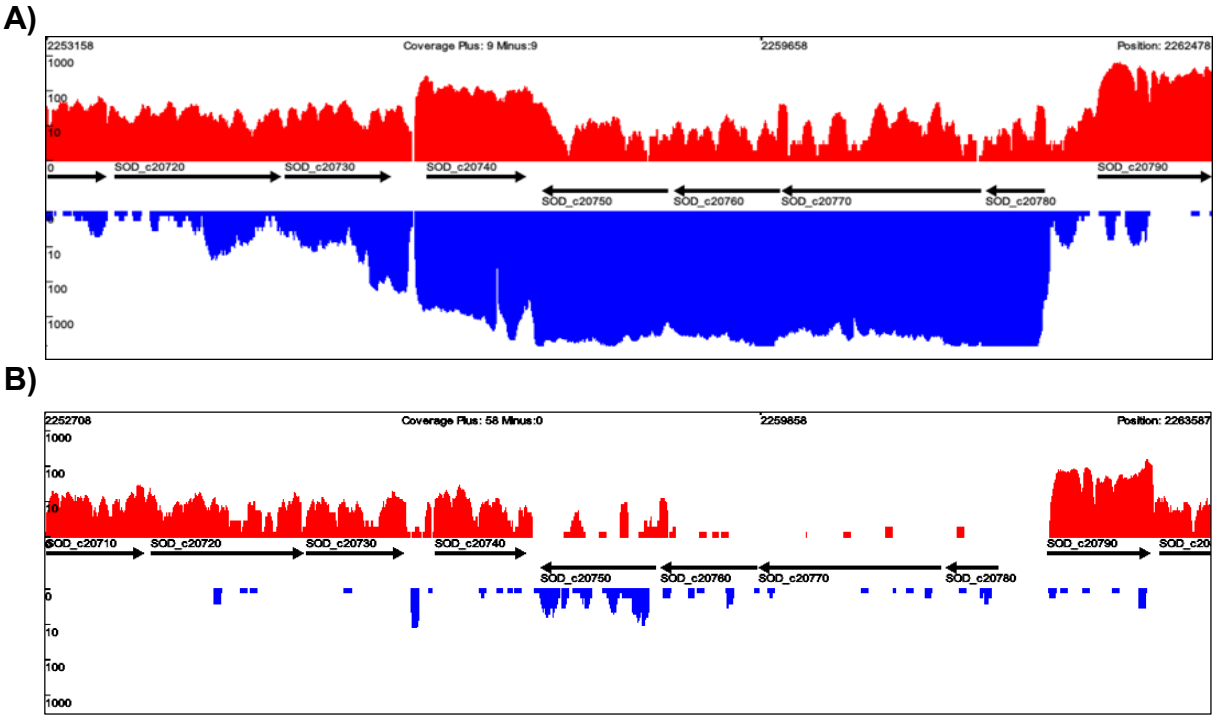
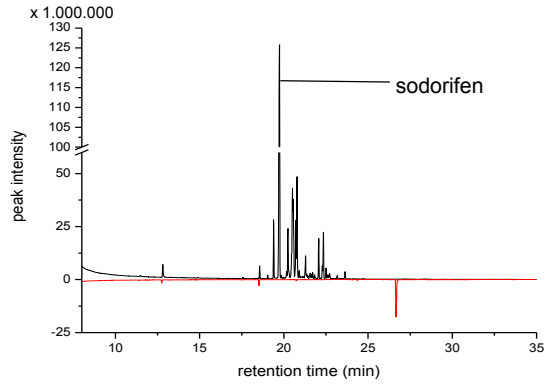


Figure 3.9: Visualization of the expression of the sodorifen cluster of genes in *Serratia plymuthica* 4Rx13 after 48 h. The transcriptional activity of the genes of the sodorifen cluster (*SOD_c20750–SOD_c20780*) on the negative strand is shown in **blue**, while the transcriptional activity on the positive strand is shown in **red**. Each black arrow indicates an open reading frame (ORF). This graph was obtained using the software TraV (Dietrich et al., 2014). The transcriptional activity was measured in nucleotide activities per kilobase of exon model per million mapped reads (NPKM) and the plotted data are presented on a logarithmic scale. **A)** *S. plymuthica* 4Rx13 and **B)** *S. plymuthica* AS9.

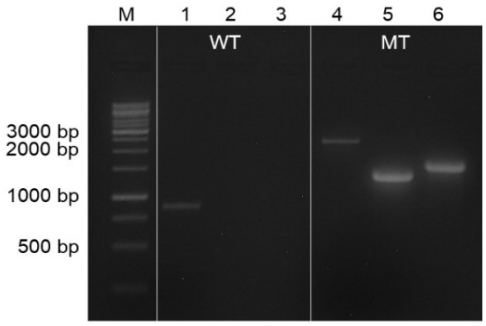
3.2.3. Analysis of the sodorifen cluster mutants

Since the terpene cyclase mutant showed a sodorifen-negative phenotype (see section 3.1.7), the function of the other three genes of the cluster was also considered to be of interest. Therefore, these genes were mutated and analyzed with regard to sodorifen emission.

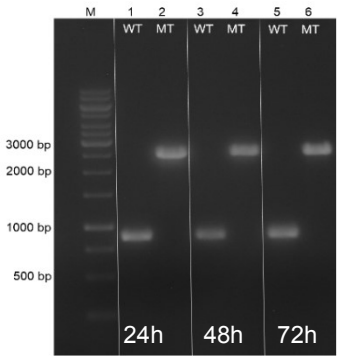
A)


x 1.000.000
peak intensity
sodorifen
retention time (min)

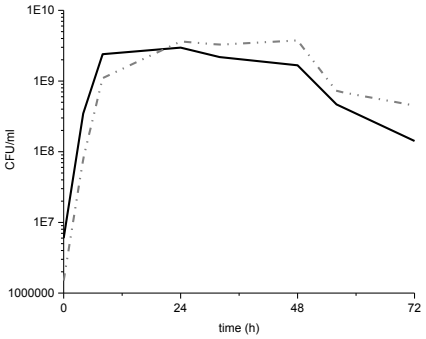
B)


M 1 2 3 4 5 6
WT
MT
3000 bp
2000 bp
1000 bp
500 bp

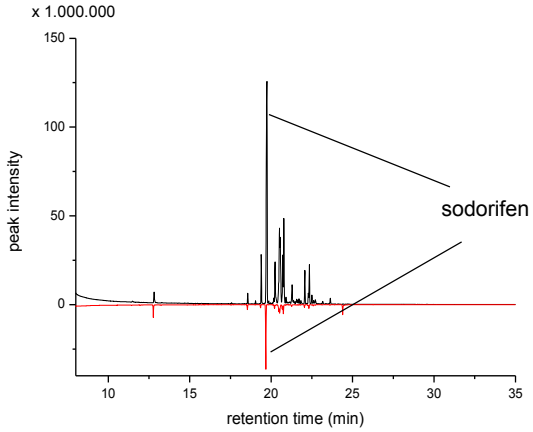
C)


M 1 2 3 4 5 6
WT MT WT MT WT MT
3000 bp
2000 bp
1000 bp
500 bp
24h
48h
72h

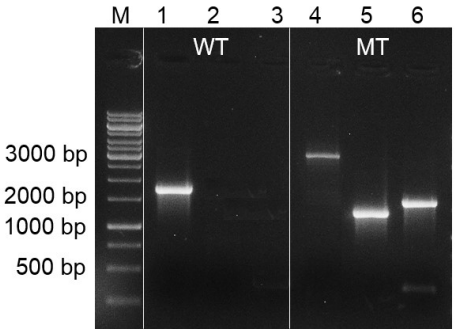
D)


CFU/ml
time (h)

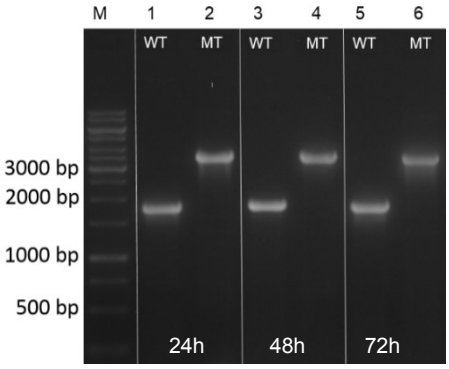
E)


x 1.000.000
peak intensity
sodorifen
retention time (min)

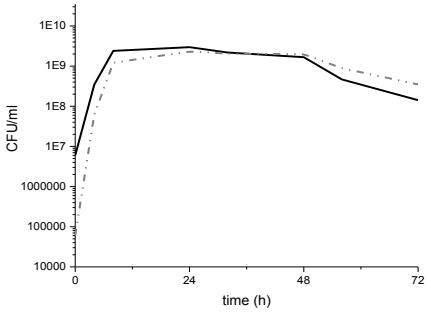
F)


M 1 2 3 4 5 6
WT
MT
3000 bp
2000 bp
1000 bp
500 bp

G)


M 1 2 3 4 5 6
WT MT WT MT WT MT
3000 bp
2000 bp
1000 bp
500 bp
24h
48h
72h

H)


CFU/ml
time (h)

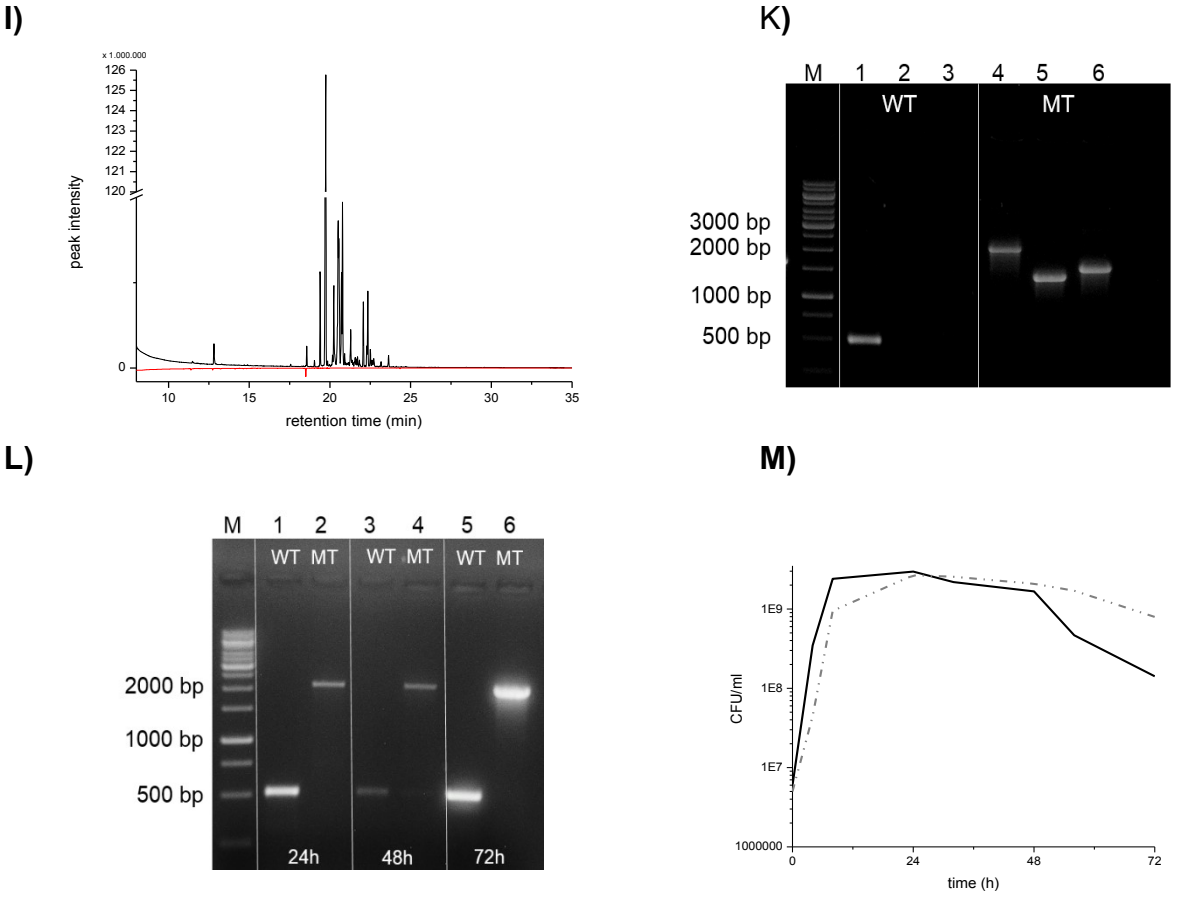


Figure 3.10: Analysis of the knockout mutants of the sodorifen cluster of *Serratia plymuthica* 4Rx13. A–D): Mutant of *SOD_c20760*; **E–H)**: mutant of *SOD_c20770*; **I–L)**: mutant of *SOD_c20780*. **A**, **E, I)** Typical volatile organic compound (VOC) profiles of the mutants (red lines) and the wild type (black lines) grown in complex medium after 48 h (*n* = 2). **B, F, J)** Integration of the resistance cassette was verified by polymerase chain reaction (PCR) with specific primer combinations: lanes 1–3, the mutant with the integrated resistance cassette; lanes 4–6, the wild type without the inserted resistance cassette (FRT-PGK-gb2-neo-FRT-cassette); lanes 1+4, the amplicon with gene- specific sense and antisense primers for the mutated gene; lanes 2+5, the amplicon with a gene- specific sense primer and an antisense primer specific for the resistance cassette; lanes 3+6, the amplicon with a specific sense primer for the inserted resistance cassette and a gene-specific antisense primer; M: GeneRuler 1 kb ladder. **C, G, K)** PCR products of the gene in the wild type and mutants cultivated in complex medium over 72 h. **D, H, L)** Growth curves of the mutants (dotted lines) and wild type (black lines), measured as colony forming units (CFU)/ml (*n* = 2).

The mutants of the cluster were analyzed to determine their VOC profiles, verification of the mutation, the stability of the mutation, and their growth compared with the wild type (**Figure 3.10A–L**). The VOC profiles of the mutants of the genes *SOD_c20760* and *SOD_c20780* did not contain sodorifen (**Figure 3.10A, I**). However, the knockout mutant of *SOD_c20770* did still emit sodorifen (**Figure 3.10E**). For each mutant, it was

ensured that the resistance cassette was not only integrated into the correct gene, but also placed correctly within the gene (**Figure 3.10B, F, J**). To check this different primer combinations were applied. The first lane shows the amplicon generated with gene-specific primer. The wild type showed always a smaller amplicon than the mutant due to the integration of the 1.7 kb resistance cassette. Lane two and three proved the correct location of the cassette by applying a combination of a gene-specific primer and a primer for the resistance cassette. The wild type indicated no bands in lane two and three due to the absence of the resistance cassette. The stability of the mutation was observed over 72 h and proved positive in all mutants (**Figure 3.10C, G,** Therefore, samples at certain time points were taken and the presence of the resistance within the gene was checked with gene-specific primers. It was expected that the gene size of the mutant was 1.7 kb bigger than the gene size of the wild type. In all mutants the integration of the resistance cassette proofed positive **K**). In addition, no significant variation in growth was observed between the wild type and the mutants in complex medium (**Figure 3.10D, H, L**).

The sodorifen negative phenotype of the knockout of the genes *SOD_c20760* and *SOD_c20780* together with the knockout of *SOD_c20750*, supported the initial hypothesis of the presence of a biosynthetic sodorifen cluster **(Table 3.6)**. An exception was displayed by the knockout of the gene *SOD_c20770*, which still showed sodorifen emission (**Figure 3.10E**). However, the quantity of sodorifen emission was significantly reduced. As expected, the knockout of the adjacent gene *SOD_c20790* did not alter the phenotype.

Furthermore, the involvement of the genes *SOD_c20760* and *SOD_c20780* in sodorifen biosynthesis was verified by plasmid-assisted complementation of the mutants. The wild-type phenotype of the mutants of *SOD_c20760* and *SOD_c20780* was restored by introducing a recombinant plasmid into each mutant. This plasmid (pUC19) consisted of the integrated wild type gene together with a strong promoter. The chromatograms are shown in **supplementary Figures S2–S4**. However, the emission was significantly lower compared to the wild type.

Table 3.6: Functional analysis of the sodorifen cluster genes and an upstream gene.

Gene	Annotation of encoded protein	Selection	Sodorifen emission of mutant
SOD_c20750	terpene cyclase	Identified by transcriptome analysis	No
SOD_c20760	methyl transferase	Cluster search with antiSMASH	No
SOD_c20770	1-Deoxy-D-xylulose 5-phosphate (DOXP)-synthase		Yes
SOD_c20780	Isopentenyl diphosphate (IPP) isomerase		No
SOD_c20790	putative oxidoreductase YdgJ		Yes

To verify that the resistance cassette was integrated only once into the genome of the mutants, a Southern blot was performed (**Figure 3.11**). All mutants of the sodorifen cluster (*SOD_c20750–SOD_c20780*) and mutants of genes with a potentially complementary function (*SOD_c09170–SOD_c09190*) were included in the analysis. The results demonstrated a single integration of the resistance cassette in each mutant (**Figure 3.11**), indicating the correct setup for the mutagenesis procedure.

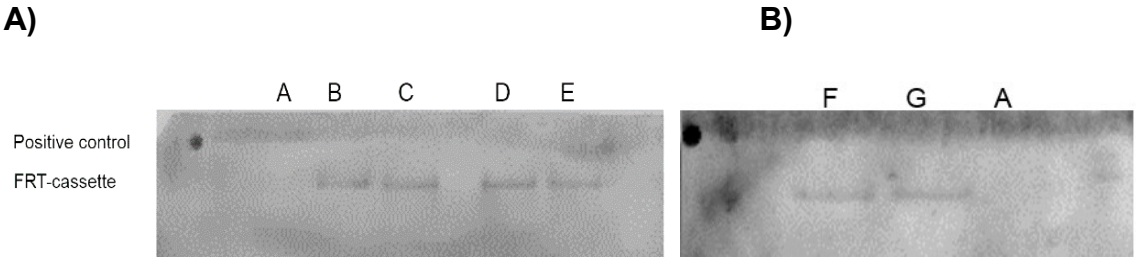


Figure 3.11: Confirmation of the single integration of the FRT-neo cassette into the generated mutants by Southern blot analysis. A) Wild type, no functional cassette detectable; **B)** mutant *SOD_c20750*::FRT-PGK-gb2-neo-FRT cassette; **C)** mutant *SOD_c20760*::FRT-PGK-gb2-neo-FRT cassette; **D)** mutant *SOD_c20770*::FRT-PGK-gb2-neo-FRT cassette; **E)** mutant *SOD_c20780*:: FRT-PGK-gb2-neo-FRT cassette; **F)** mutant *SOD_c09180*::FRT-PGK-gb2-neo-FRT cassette; **G)** mutant *SOD_c20770*::FRT-PGK-gb2-neo-FRT cassette; and Δ mutant *SOD_c09180*. Positive control: dark spot in upper left corner.

3.2.4 Role of the *dxs* gene in the sodorifen biosynthesis

Based on the gene annotation and the protein motifs that are present, the gene *SOD_c20750* probably encodes a putative terpene cyclase, while the gene *SOD_c20760* was annotated as a methyl transferase, the gene *SOD_c20770* as a 1- deoxy-D-xylulose 5-phosphate (DOXP)-synthase, and the gene *SOD_c20780* as an isopentenyl diphosphate (IPP) delta-isomerase (**Table 3.6**). Thus, three genes of this cluster are involved in the terpenoid biosynthetic pathway. This, combined with the findings from the mutagenesis experiment, which showed that the knockout mutants of these genes exhibited sodorifen-negative phenotypes, suggests that sodorifen is at least partially derived from isoprenoid metabolism and may be classified as a terpene. Since the knockout mutant of DOXP-synthase (*SOD_c20770*) still emitted sodorifen, it was speculated that a second DOXP-synthase gene is able to complement the function of this gene, and confirming this, another search produced a second *dxs* gene (*SOD_c09180*).

These two *dxs* genes (*SOD_c20770* and *SOD_c09180*) had only a 52.78% identity at the amino acid level, but high accordance was found within the protein domains (**Figure 3.12**). The alignment of both sequences using the online tool ClustalW2 (http://www.ebi.ac.uk/Tools/) highlighted gaps and several amino acid exchanges in the C-terminal as well as the N-terminal regions, which probably includes the active pocket.

Additional differences appeared in the transcription level, with *SOD_c20770* of the sodorifen cluster being highly expressed (NPKM = 1118 after 48 h) but *SOD_c09180* (outside the cluster) only reaching a much lower value (NPKM = 92). This value is still considerably higher than housekeeping genes such as lactate dehydrogenase, however, which has an average NPKM value of 14.5 over 48 h.

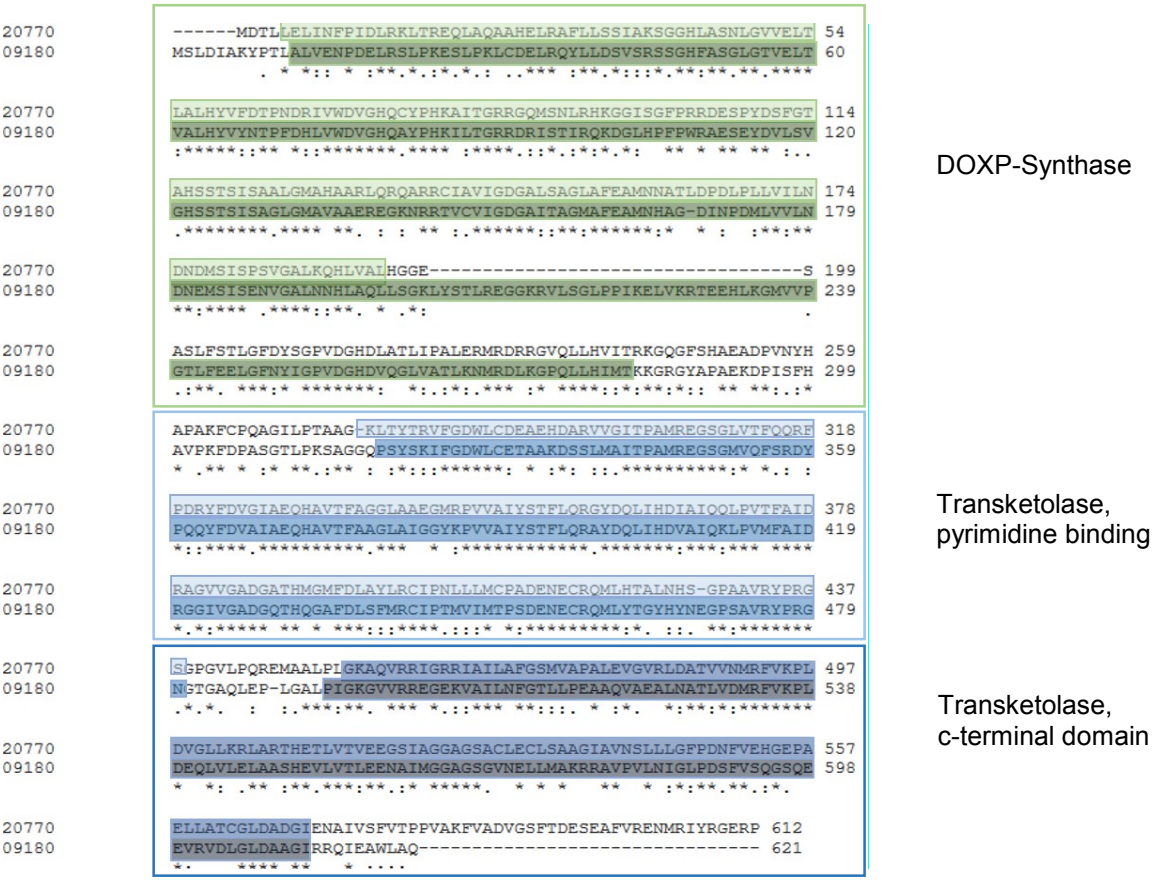


Figure 3.12: Motif analysis in the *dxs* genes *SOD_c20770* and *SOD_c09180* in the genome of *Serratia plymuthica* 4Rx13. The protein sequences were aligned using ClustalW2 (Analysis Tool Web Services from the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI), 2013*) and* the protein domains are highlighted in different colors within the sequence. The identity of the sequences was calculated by their degree of similarity. Star: amino acids are identical; double dot: conserved substitution; single dot: semi-conserved substitution; blank: mismatch.

To investigate the role of the second *dxs* gene, a knockout mutant of the gene *SOD_c09180* was generated. This mutant was still able to produce sodorifen but, as for the mutant of *SOD_c20770*, the amount was significantly reduced–the emission was decreased to 29% of the wild type, compared with 30% for the mutant of *SOD_c20770*. No other distinct differences were detected in the other compounds within the VOC profile (**Figure 3.13**).

Since the single-knockout mutants did not result in the expected sodorifen-negative phenotype, a double-knockout mutant was generated. This double-knockout mutant (*SOD_c20770*::FRT-neo-cassette, Δ*SOD_c09180*) was still able to synthesize sodorifen, but the sodorifen emission was reduced to 12.5%.

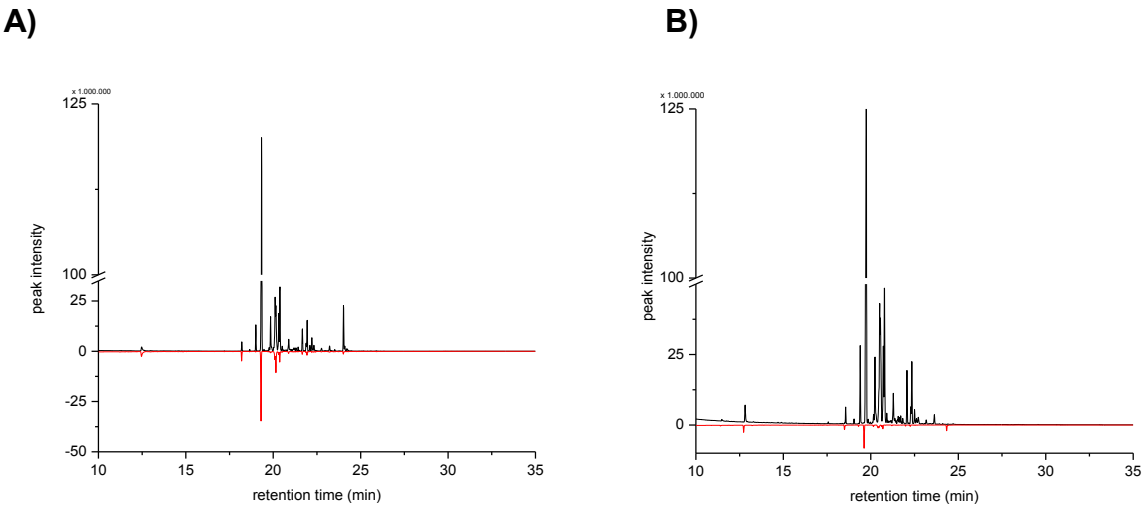


Figure 3.13: Volatile spectra of *dxs* gene mutants in comparison to the wild type for *Serratica plymuthica* 4Rx13. The sodorifen emissions of mutants with the knockout of heterologous *dxs* genes (black) were compared with those of the wild type (red) after 48 h inoculation in complex medium (n = 2). **A)** Double mutant *SOD_c20770*::FRT-neo-cassette, Δ*SOD_c09180* and **B)** mutant of *SOD_c09180*.

Beside the phenotypical analysis of the volatile spectrum (**Figure 3.13A**), the genotypic verification of the double mutant was done with gene-specific and resistance cassette primer in comparison to the wild type (**Figure 3.14**). To knockout a second gene in the genome of *S. ply.* 4Rx13, the resistance cassette within gene *SOD_c09180* of the mutant had to be removed and integrated into *SOD_c20770* of the same mutant. The iintegration of the resistance cassette was verified by polymerase chain reaction (PCR) with specific primer combinations: The resistance cassette in *SOD_c09180* (lane 5 and 10) and the wild type (lane 1, 7 and 8) is absent but present in *SOD_c20070* (lane 2, 4 and 9) (**Figure 3.14A**). The double mutant contains the two altered genes *SOD_c09180* and *SOD_c20770*, while into the latter gene the 1.7 kb resistance cassette was introduced with a gene size about 3.6 kb in comparison to the wild type genes (*SOD_c20770* with 1.9 kb and *SOD_c09180* with 1.9 kb). The correct integration of the resistance cassette was checked with a combination gene-specific and resistance cassette primer. Additionally, the stability of the mutation was observed over 72 h and proved positive in the double mutant (**Figure 3.14B**).

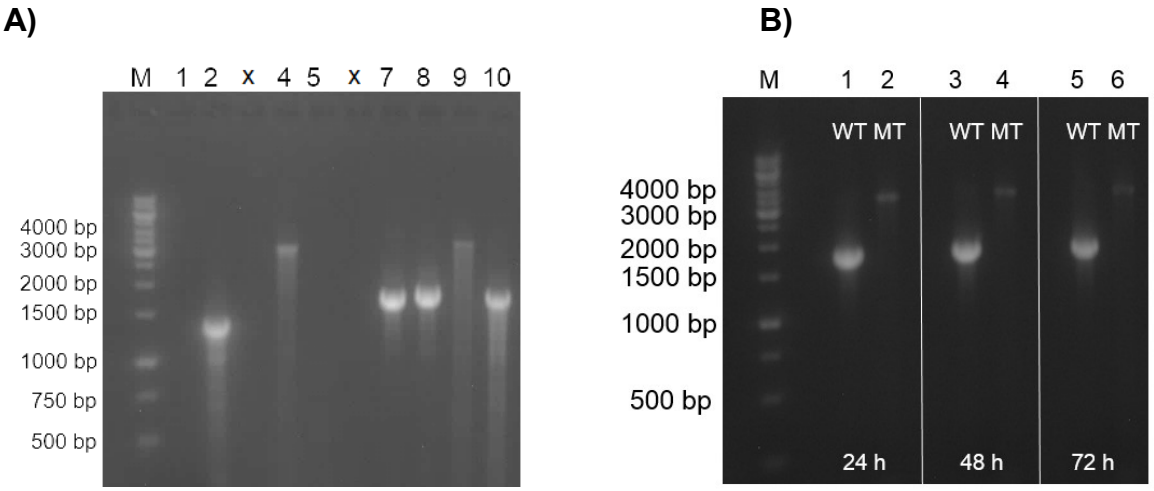


Figure 3.14: Analysis of the *dxs* double knockout mutant of *S. plymuthica* 4Rx13. A) Integration of the resistance cassette was verified by polymerase chain reaction (PCR) with specific primer combinations: lanes 2, 4, and 9, the double mutant of gene *SOD_c20770* with the integrated resistance cassette; lanes: 5, and 10, the double mutant of gene *SOD_c09180* without the integrated resistance cassette; lanes 1, 7 the wild type gene *SOD_c20770* and lane 8 , the wild type gene *SOD_c09180* without the inserted resistance cassette (FRT-PGK-gb2-neo-FRT-cassette); lanes 4, 7,8, 9 and 10 the amplicon with gene-specific sense and antisense primers; lanes 1, 2 and 5, the amplicon with a specific sense primer for the inserted resistance cassette and a gene-specific antisense primer; M: GeneRuler 1 kb ladder. **B)** PCR products of the gene *SOD_c20770* in the wild type and mutant cultivated in complex medium over 72 h.

There are two possible explanations for the finding that the double-knockout mutant of both *dxs* genes was still able to emit sodorifen: i) a third gene exists that is able to substitute the function of both the *SOD_c20770* and *SOD_c09180* genes, or ii) the *dxs* gene is not necessary for the biosynthesis of sodorifen. Therefore, the genome was screened again to search for a third DOXP-synthase or homologous genes. A cluster of three genes was found that included *SOD_c09180*, a farnesyl diphosphate synthase (*SOD_c09190*), and a putative oxidoreductase YajO (*SOD_c09170*) (**Table 3.7**). Sponsel (2002) previously demonstrated that a cluster of three genes is involved in isoprenoid metabolism in *E. coli*, and so these genes were further analyzed.

The generation of a mutant of the gene *SOD_c9190* was unsuccessful, indicating that this gene is required for the survival of the cell, making a knockout lethal (**Table 3.7**). In contrast, the mutant of the gene *SOD_c09170* had an unchanged phenotype. The chromatogram illustrating the VOC profile of this mutant is presented in **supplementary Figure S8**.

Since homologous genes of the *dxs* were found in *S. ply.* 4Rx13, it was also analyzed whether homologous genes of the remaining genes of the sodorifen cluster exist in the genome of the isolate 4Rx13. The terpene cyclase is unique in *S. ply.* 4Rx13. Within the genome another annotated methyltransferases (*SOD_c00380*) existed but had no significant similarity to the enzyme in the sodorifen cluster. A homologous gene of the IPP-isomerase (*SOD c20780*) was found: *SOD_c20170*, which appeared to have a similar length and similar protein domains resulting in an overall identity of 37.78 % at the amino acid level. A mutant of the latter was generated but was still able to produce sodorifen **(Figure S7)**.

Table 3.7: Functional phenotype analysis following the single and double knockout of the heterologous *dxs* genes, and the single knockout of the adjacent genes on the chromosome.

Gene	Annotation	Sodorifen emission of mutant
SOD_c09170	Putative oxidoreductase YajO	Yes
SOD_c09180	1-deoxy-D-xylulose 5-phosphate (DOXP)	Yes
SOD_c09190	Farnesyl diphosphate (FPP) synthase	Yes
SOD_c20770 + SOD_c09180	double mutant	Yes

3.2.5 Protein motifs of the genes of the sodorifen cluster

In addition to investigating the annotation of genes, the functional and structural domains at the amino acid level were also searched for in the Pfam database (Bateman et al., 2004) (http://www.sanger.ac.uk/Software/Pfam/). This search yielded a terpene synthase C domain for the terpene cyclase, a methyl transferase domain for the methyl transferase, and three domains for the DOXP-synthase (1-deoxy-D-xylulose-5-phosphate synthase, transketolase, pyrimidine binding domain, and a transketolase, C-terminal domain). In addition, the IPP-isomerase carries an isopentenyl-diphosphate delta-isomerase domain (**Figure 3.15A**).

As outlined in section 3.1.9, the terpene cyclase in *S. ply.* 4Rx13 contains one of the two universally conserved motifs that are typically found in terpene synthases of plants and fungi. The aspartate-rich DDxx(D/E) motif has an additional aa variation (DDxxxD/E) and is located between amino acids 101 and 109. Typically, this motif is

found within 80–120 aa of the N-terminus in bacterial and fungi terpene synthases. By contrast, the NSE/DTE motif is usually found 140 aa downstream of this, closer to the C-terminus (Yamada et al., 2015), but is absent in isolate 4Rx13. Both of these motifs are involved in binding cations (particularly Mg^{2+}), which is required for the acyclic terpene precursor (Cane et al., 2012). Both of the DOXP-synthases in isolate 4Rx13 have the same arrangement of the three protein domains (**Figure 3.15B**).

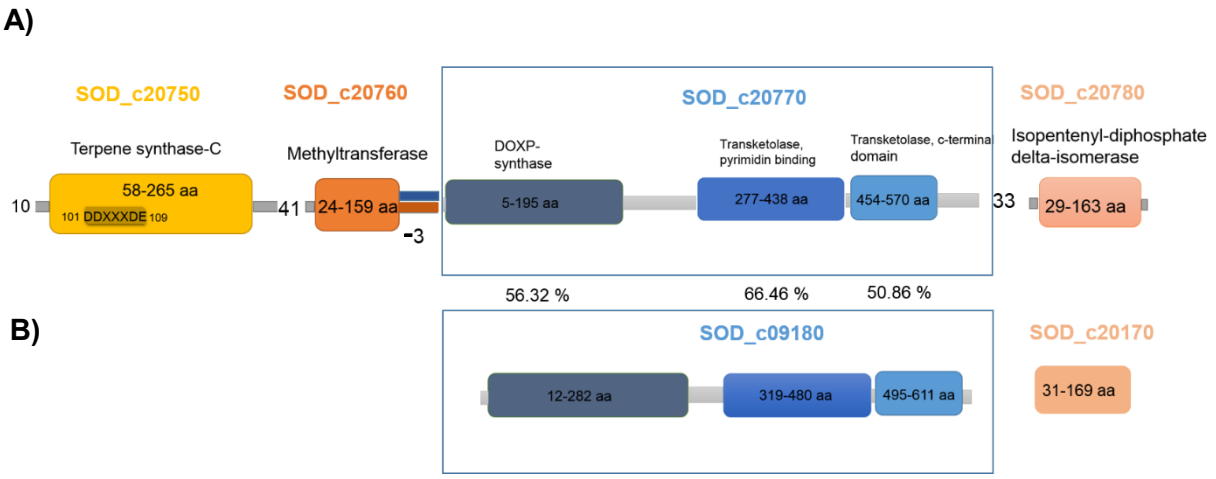


Figure 3.15: Search for protein domain motifs in genes of the sodorifen cluster and homologous *dxs* genes. The protein domain search was performed using the Pfam protein family database. **A)** Illustration of the position and length of the protein domains present in the genes of the sodorifen cluster (*SOD_c20750–SOD_c20780*). **B)** Motif of the homologous *dxs* gene *SOD_c09180*. Grey: the distance between the genes and domains; and purple: the overlap of amino acids. The identities between the domains are provided as percentages. The numbers between the genes indicate the intergenic distances between contiguous genes, with negative numbers representing overlapping genes.

The presence of the terpene cyclase in other bacterial species was investigated and a phylogenetic tree containing species that are closely related to *S. ply.* was generated by BLASTp analysis (NCBI, http://blast.ncbi/), using *Burkholderia pyrrocinia* as an outgroup (**Figure 3.16**). The terpene cyclase occurred only in *S. ply.* and three other species: *Streptomyces tskubaensis, Pseudomonas chlororaphis*, and *Burkholderia pyrrocinia*. The phylogenetic tree was generated online at phylogeny.fr (Dereeper et al., 2008) and showed high similarities (97–99%) between the closely related *S. ply.* isolates AS9, AS12, AS13, A30, V4, and S13. Interestingly, the terpene cyclase is absent in other *Serratia* species, including *S. quinivorans*,

S. proteamaculans, and *S. odorifera* (data not shown). The identity with other more distantly related species was low (43% for *Pseudomonas chlororaphis*, 41% for *Streptomyces tskubaensis*, and *Burkholderia pyrocinia* had only parts of the protein sequence; **Table 3.8**).

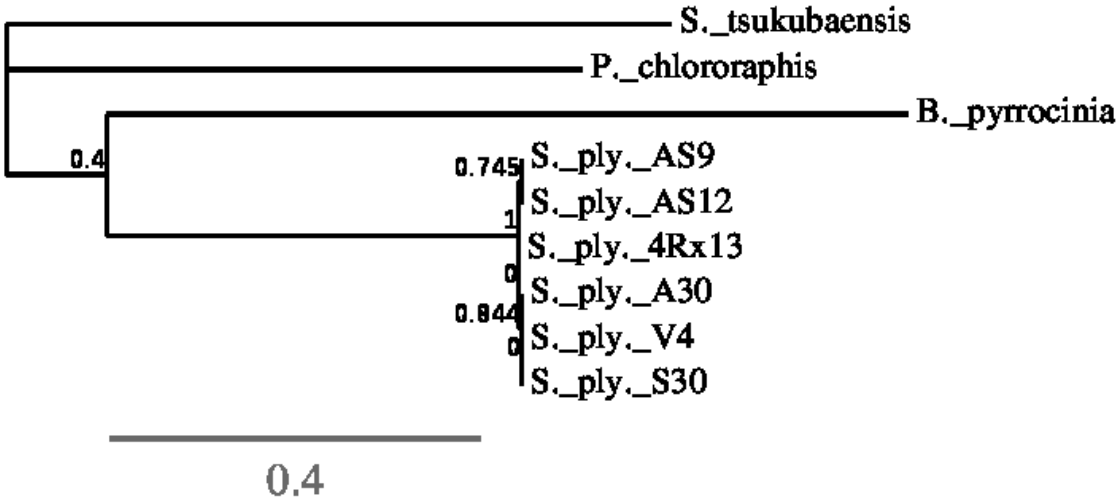


Figure 3.16: The phylogenetic tree of the terpene cyclase in different bacterial species and isolates. The tree was calculated using phylogeny.fr (Dereeper et al., 2008) based on the protein sequences of the terpene cyclase of *Serratia plymuthica* 4Rx13. The protein sequences of the *S. ply.* isolates AS9, AS12, AS13, A30, V4, and S13 were aligned to 4Rx13. *Streptomyces tsukubaensis*, *Pseudomonas chlororaphis*, and *Burkholderia pyrrocinia* were selected as outgroups. The tree was built on the basis of the approximation of the standard Likelihood Ratio Test (aLRT). Numbers indicate branch support values.

3.2.7 Presence of the sodorifen cluster in other bacterial species

The sodorifen cluster genes were searched for in other bacterial species using the online software antiSMASH (Medema et al., 2011). The sodorifen cluster of *S. ply.* 4Rx13 is present in closely related isolates belonging to the same species, including the sodorifen-producer isolate V4 and the non-producer isolate AS9. The sequences of isolates HRO-C48 and 3Re-4-18 only exist as draft genomes and are currently not publicly available and therefore not found in any databases. However, sequences for an alignment were available for isolate HRO-C48, which showed the presence of the cluster with an overall identity of 98%.

Comparison of the cluster structure also revealed that three out of the four genes in the cluster are present in *Streptomyces tskubaensis* NRRL 18488 (the IPP-isomerase is missing; **Figure 3.17**). However, the methyl transferase is 60 bp shorter and the *dxs* gene is 580 bp shorter than in isolate 4Rx13. In *Pseudomonas chlororaphis*, all four genes of the cluster are present but they differ in length–the methyl transferase is 12 bp shorter, the *dxs* gene is 140 bp longer, and the IPP-isomerase is 69 bp shorter.

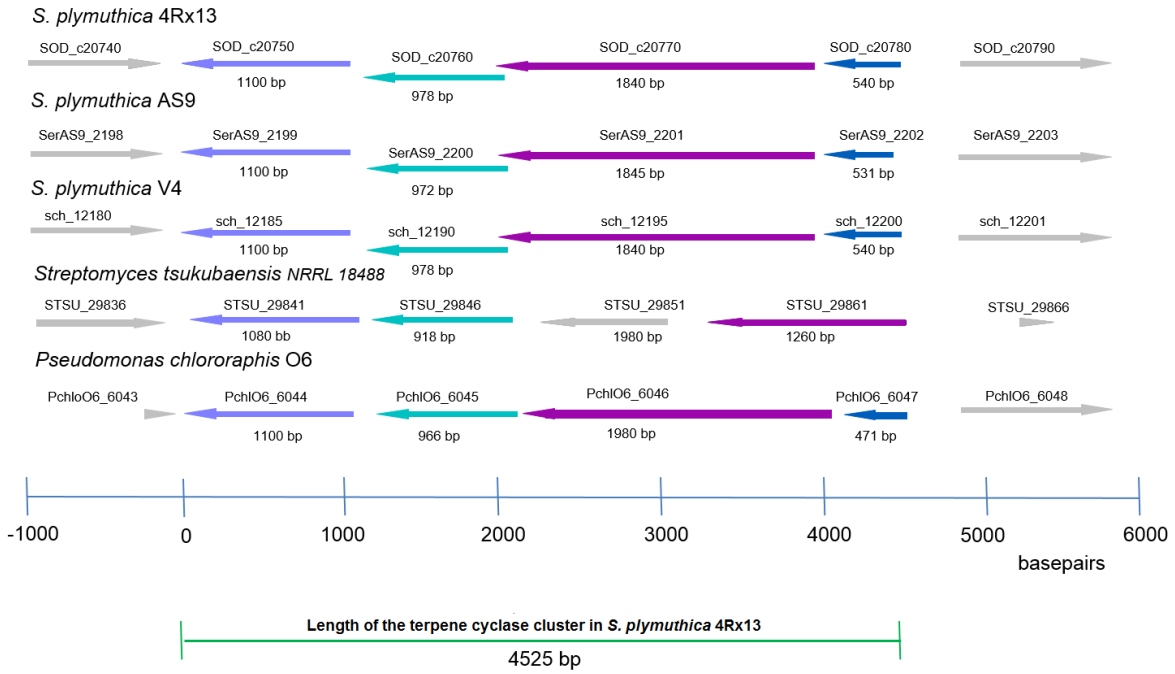


Figure 3.17: The presence of the sodorifen cluster genes in other bacteria. The presence of the sodorifen cluster was searched for using the online tool antiSMASH. Homologous genes are presented in the same color.

Alignment of the sodorifen cluster genes at the amino acid level was performed for all species using the online software ClustalW (Thomopson et al., 1994). The different *S. ply.* isolates had very high sequence identities at the amino acid level within the sodorifen cluster genes (> 95%) and no difference was observed between the sodorifen-producer isolates and the non-producer isolate AS9 (**Table 3.8**). Therefore, it was concluded that the presence of the cluster itself is not an indicator of the ability to produce sodorifen, possibly due to the cluster being silent. For example, isolate AS9 carries the complete cluster but is a sodorifen non-producer, and the transcriptome data demonstrated that these genes are not expressed.

Burkholderia pyrrocinia was also found to contain a hypothetical protein that had an identity of 41% with the terpene cyclase of *S. ply.* 4Rx13. The methyl transferase (64%) and DOXP-synthase (62%) were quite similar, but the IPP-isomerase was found to have no similarity. However, these genes are not organized in one cluster (**Table 3.8**).

Table 3.8: Identities of the sodorifen cluster genes at the amino acid level in *Serratia plymuthica* and other closely related species.

Gene / Organism	Terpene cyclase	Methyl transferase	1-deoxy-D-xylulose 5-phosphate synthase (DOXP)	Isopentenyl diphosphate (IPP)-isomerase	Amino acid alterations compared with the terpene cyclase of *S. ply*. 4Rx13
S.ply. 4Rx13	100 %	100 %	100 %	100 %	x
S.ply. AS9	99 %	97 %	95 %	96 %	M14T; E328Q; N357S; Y381H
S.ply. V4	99 %	98 %	98 %	99 %	A275T; N375S; Y381H; G382S
S.ply. HRO-C48	99 %	98 %	98 %	approximately 99%	A275T; N375S; Y381H; G382S
Pseudomonas chlororaphis	43 %	59 %	65 %	50 %	< 40
Streptomyces tsukubaensis	41 %	52 %	50 %	-	< 40
Burkholderia pyrrocinia	41 %	64 %	62 %	-	< 40

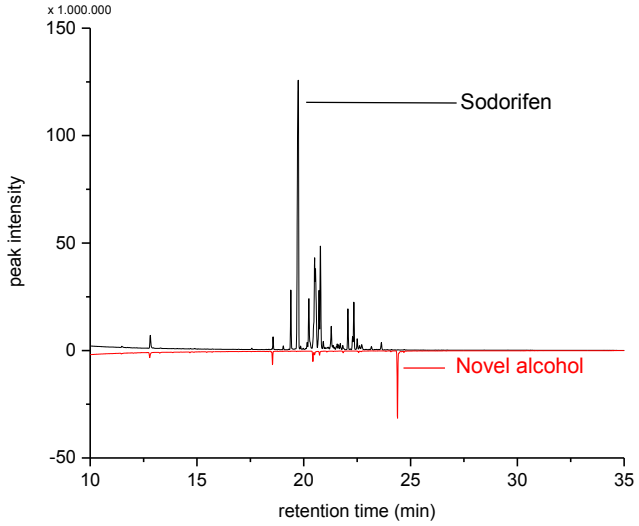
3.3 Proposed biosynthesis of the new compound and sodorifen

Within the genome of *Serratia plymuthica* 4Rx13 a novel terpene cyclase was detected that is located in a cluster of four genes (*SOD_c20750*: terpene cyclase, *SOD_c20760*: methyl transferase, *SOD_C20770*: DOXP-synthase, *SOD_c20780*: IPP-isomerase). To investigate the role of genes in the biosynthesis of sodorifen mutants were generated and the VOC profile was analyzed. While the VOC spectrum of the wild type is dominated by sodorifen, it was absent in the terpene cyclase, methyl transferase and IPP-isomerase. The compound of the methyl transferase mutant was identified as farnesyl acetone. In the spectrum of the terpene cyclase mutant a novel compound was observed. The compound was curious and therefore further investigated because it might represent a biosynthetic intermediate in the biosynthesis of sodorifen. Its structure was identified by NMR spectroscopy and its biosynthesis elucidated using feeding experiments with stable isotope labeled precursors. Thus, conclusions for the biosynthesis of sodorifen were drawn and a hypothetical biosynthetic pathway based on the current results was proposed.

3.3.1 Analysis of the VOC profile and mass spectrum of the terpene cyclase mutant

The chromatogram of the wild type and the terpene cyclase mutant, no longer emits sodorifen but instead a new compound appeared that is absent in the wild type (**Figure 3.18A)**. The mutant emitted about a third of the new compound compared to the emission of sodorifen of the wild type after 48h cultivation in complex medium. The VOC profile demonstrates that the new compound appeared at a later retention time of 24.6 min than sodorifen (19.6 min), which equates to the Kovats retention indices of 1746 for the new compound and 1377 for sodorifen, respectively. The mass spectra of the new compound (**Figure 3.18B**) had a molecular ion mass peak of *m/z*=236, suggesting a molecular formula of $C_{16}H_{28}O$, assuming that it is structurally related to sodorifen. This similarity was indicated by the characteristic fragmentation pattern yielding *m/z* 136, 137 and 138 reminiscent of sodorifen. The new compound will further also referred to as the novel alcohol.

A)



B) **C)**

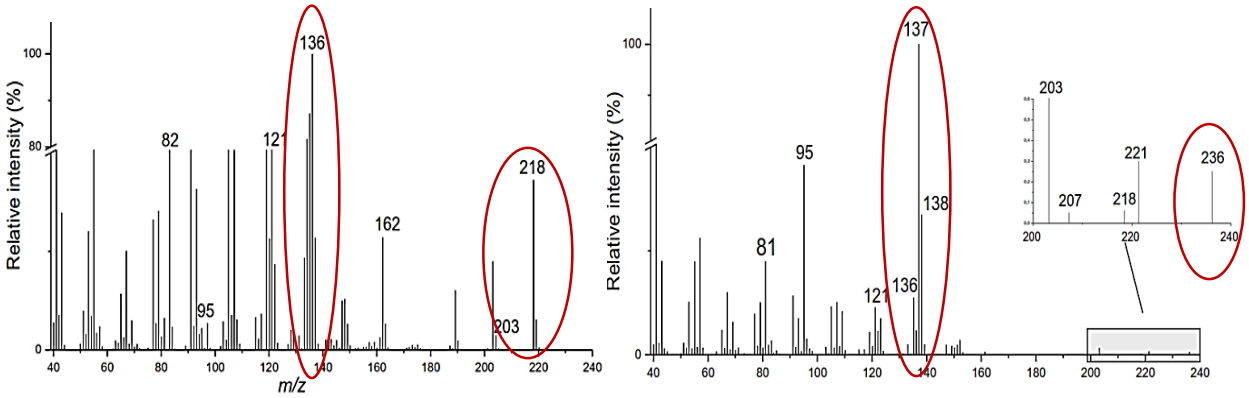


Figure 3.18: VOC profile and mass spectra of the wild type and the terpene cyclase mutant in *Serratia plymuthica* 4Rx13. A) The headspace of the VOC profiles of *S. plymuthica* 4Rx13 (black) and the terpene cyclase (*SOD_c20750*) mutant (red) grown in a complex medium for 48 h. Sodorifen peak and that of the novel alcohol are marked. Mass spectra of the **B)** main compound sodorifen and **C)** the novel alcohol of the terpene cyclase mutant are shown. Red circles: Characteristic signals are discussed in the text.

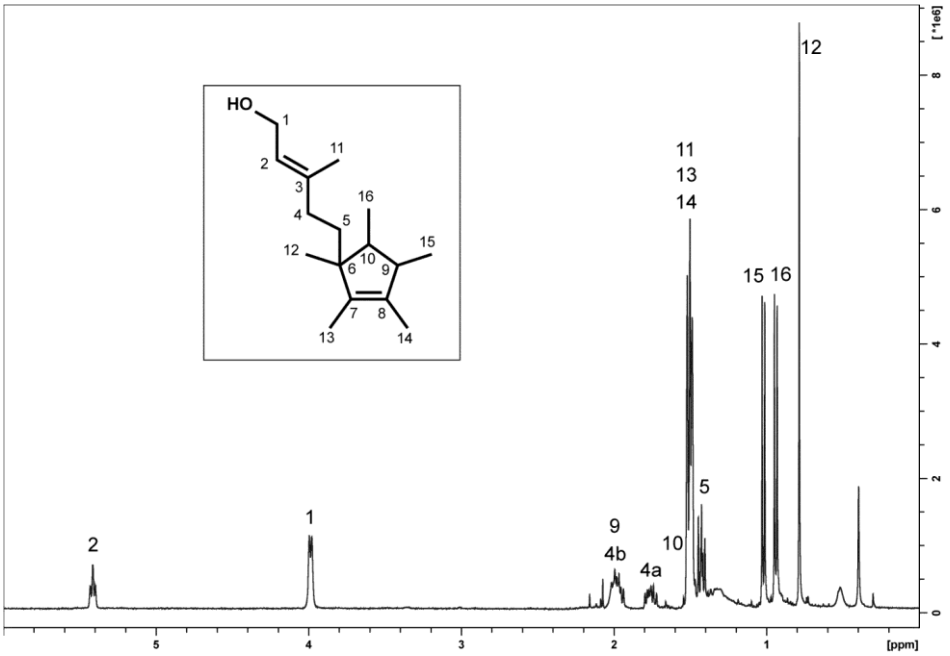
3.3.2 Structure elucidation of the novel alcohol by NMR spectroscopy

The structure of the novel alcohol was elucidated by a combination of one- and two- dimensional NMR spectroscopy. Inspection of the ^{1}H NMR spectrum (**Figure 3.19A**) indicated one olefinic methine group (5.42 ppm), one hydroxymethylene group (3.99 ppm), three olefinic methyl groups (1.49, 1.50, 1.52 ppm), two dublet methyl groups (0.94 and 1.02 ppm), and one singlet methyl group (0.79 ppm), along with overlapping signals for 6 additional protons. Analysis of the broadband decoupled ^{13}C NMR spectrum (**Figure 3.19B**) confirmed the presence of 16 carbon atoms along with two double bonds (124.3, 134.4, 137.1, 139.3 ppm), and one hydroxylated carbon (59.4 ppm). The structure of the novel alcohol (**Figure 3.20)** was finally deduced from inspection of two-dimensional *dqf*-COSY (correlated spectroscopy), HSQC (heteronuclear single quantum correlation), and HMBC (heteronuclear multiple bond correlation) spectra (**Supplement S10-12**), and the relative configuration was deduced from analysis of the H, H-NOESY (nuclear Overhauser enhancement spectroscopy) spectrum (**Supplement S13**).

Structure comparison of both sodorifen and the novel alcohol made some similarities and differences apparent (**Figure 3.20**). Sodorifen consisted of two rings, while the novel alcohol is composed of a similar five ring structure with a long chain and hydroxyl group attached. Additionally, the orientation of methyl groups differed in both compounds.

Beside the elucidation of the compound produced by the terpene cyclase mutant, the compound present in the spectrum of the methyl transferase mutant was identified as farnesyl acetone (data not shown). The compound present in the spectrum of the IPP- isomerase mutant was not yet elucidated by NMR spectroscopy.

A)



B)

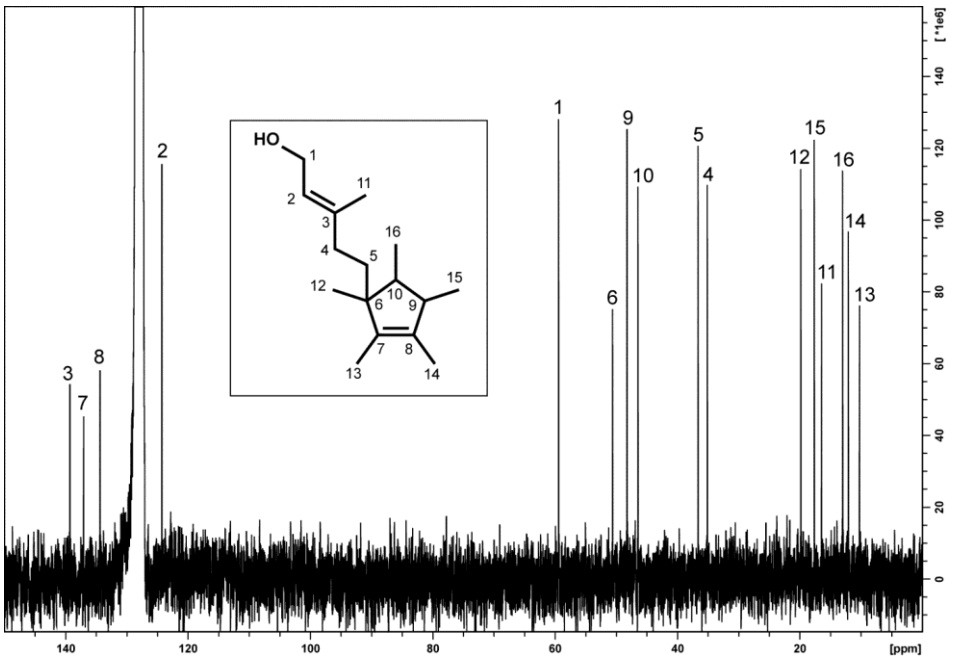


Figure 3.19: NMR spectra of the novel alcohol emitted by the terpene cyclase mutant. *Serratia plymuthica* 4Rx13 was cultivated in a complex medium. Headspace volatiles were eluted in D6-benzene every 24 h over 96 h. Samples were analyzed by NMR spectroscopy. **A)** Inspection of ^{1}H NMR spectrum and **B)** analysis of the broadband decoupled ^{13}C NMR spectrum. Positions of carbon atoms are numbered in both the spectra and the novel alcohol. (constructed by Dr. S. v. Reuss).

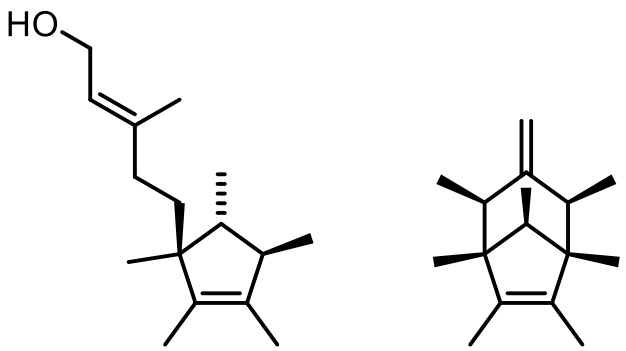


Figure 3.20: Structures of both the novel alcohol of the terpene cyclase mutant and sodorifen of the wild type in *S. plymuthica* 4Rx13. (constructed Dr. S. v. Reuss).

3.3.3 Investigation of the incorporation patterns of isotope feeding in *Serratia plymuthica* 4Rx13

Given the close structural similarity of sodorifen and the new alcohol obtained from the terpene cyclase knockout mutant, it was the aim to decipher the biosynthesis of this putative biosynthetic intermediate, which might provide insights into the biosynthesis of sodorifen. Four stable isotope labeled compounds: [1.4-$^{13}C_2$]-succinate, [2.3- $^{13}C_2$]- succinate L-[*S*-$^{13}CH_3$]-methionine and L-[3-^{13}C]-alanine were chosen as a carbon source for the terpene cyclase mutant, this selection was based on similar feeding experiments performed previously with the wild type of *S. ply.* 4Rx13 (Weise, dissertation, 2013). Emitted volatiles of the terpene cyclase mutant were trapped and collected in D6-benzene and incorporation of isotope labeled precursors was confirmed by GC-MS (**Supplement S15**). The position of isotope labels of the novel compound was determined by NMR spectroscopy performed by Dr. S. v. Reuss at the Max-Planck Institute in Jena.

The feeding experiments demonstrated that seven positions within the molecule of the novel alcohol are labeled by L-[3-^{13}C]-alanine (**Figure 3.21A**) of which those in the sidechain correspond to the labeling pattern expected for a DOXP-derived isoprene unit. C_2-bond labelling using [2.3- $^{13}C_2$]-succinate (**Figure 3.21B**) identified five C_2 units that originate from succinate along with three additional succinate-derived C_1 units. The remaining carbons were successfully labeled by incorporation of [1.4- $^{13}C_2$]- succinate (**Figure 3.21C**). Taken together these results were indicative of

a 1-deoxy-Dxylulose 5-phosphate (DOXP) pathway related terpenoid origin of the novel C_{16}-alcohol. This pathway would account for a 15 carbon atom intermediate. The remaining C_1 unit was hypothesized to originate from methionine via SAM-associated methyl group transfer, which is in agreement with the identification of a methyl transferase within the sodorifen biosynthetic gene cluster. Therefore, another feeding experiment was done with L-[*S*-$^{13}CH_3$]-methionine (**Figure 3.21D**) that would demonstrate which one of the methyl groups is attached to the terpenoid derived C_{15} skeleton. Therefore, the mutant of the terpene cyclase was cultivated in a minimal medium supplemented with 20mM alanine, 20mM threonine and 20mM of 100% labeled methionine. This combination of carbon sources was chosen due to the observation of suitable growth whereas methionine is an unfavorable sole carbon source for the isolate 4Rx13 (Weise, 2013). It is interesting to note that the ^{13}C label of L-[*S*-$^{13}CH_3$]-methionine was only once incorporated in the novel compound (**Figure 3.12D**) and sodorifen (**Figure 3.22**). It is a distinct methyl group attached to the cyclopentenyl ring confirming our hypotheses that a sesquiterpenoid origin with additional SAM-related methylation for the biosynthesis of the alcohol. A summary of the results of the incorporation patterns of all labeling experiments is illustrated in **Figure 3.23**. Thus, the origin of all carbon atoms of the novel alcohol was established.

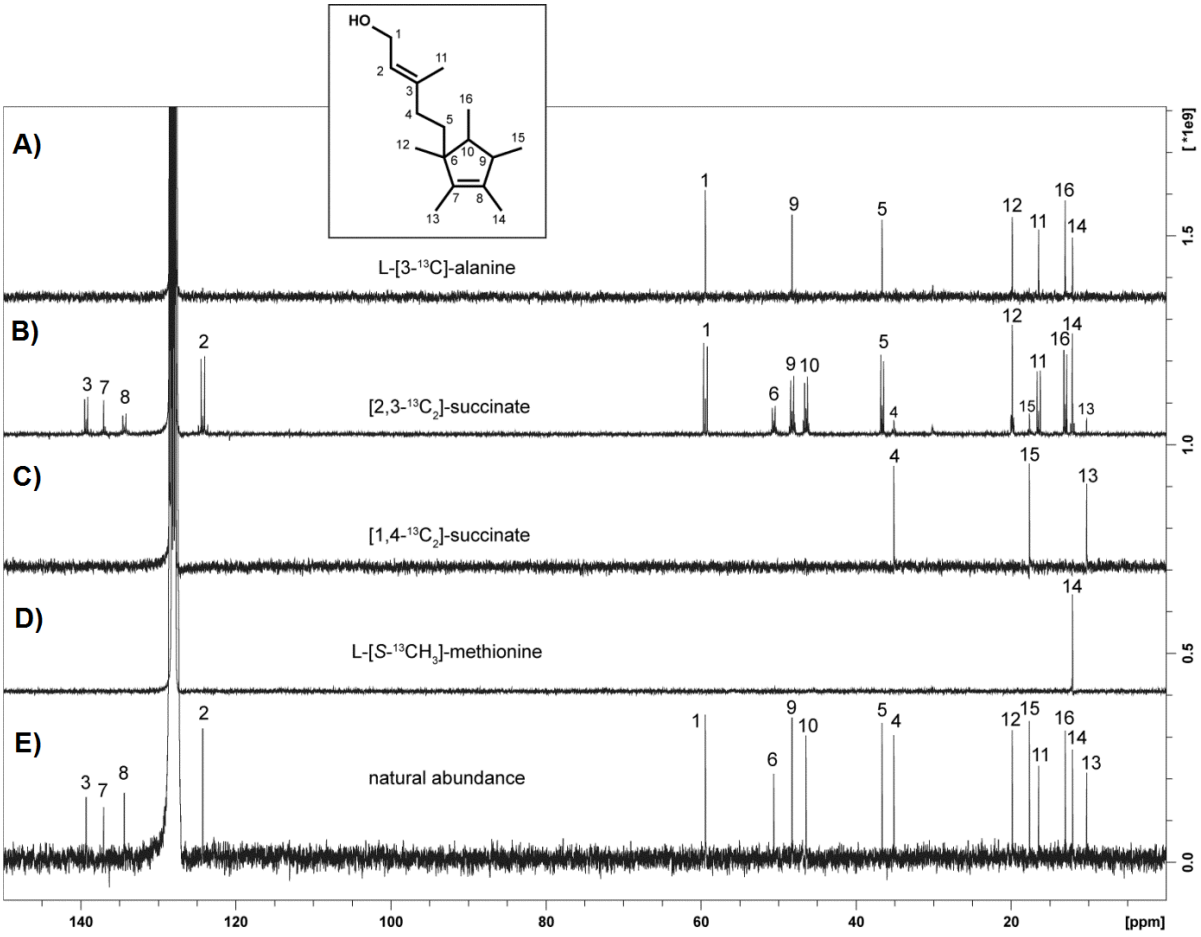


Figure 3.21: ^{13}C NMR spectra of the novel alcohol. The compound was produced by the terpene cyclase mutant in *Serratia plymuthica* 4Rx13 was cultivated in a minimal medium supplemented with different stable isotope labeled precursors: **A)** L-[3-^{13}C]-alanine, **B)** [2,3- ^{13}C]-succinate, **C)** [1,4- ^{13}C]- succinate, **D)** L-[S-$^{13}CH_3$]-methionine, and **E)** natural abundance. Headspace volatiles were eluted with D6-benzene every 24 h over 96 h and measured by broadband decoupled ^{13}C NMR spectra (100 MHz). Peak number: position in the novel alcohol. (constructed by Dr. S. v. Reuss).

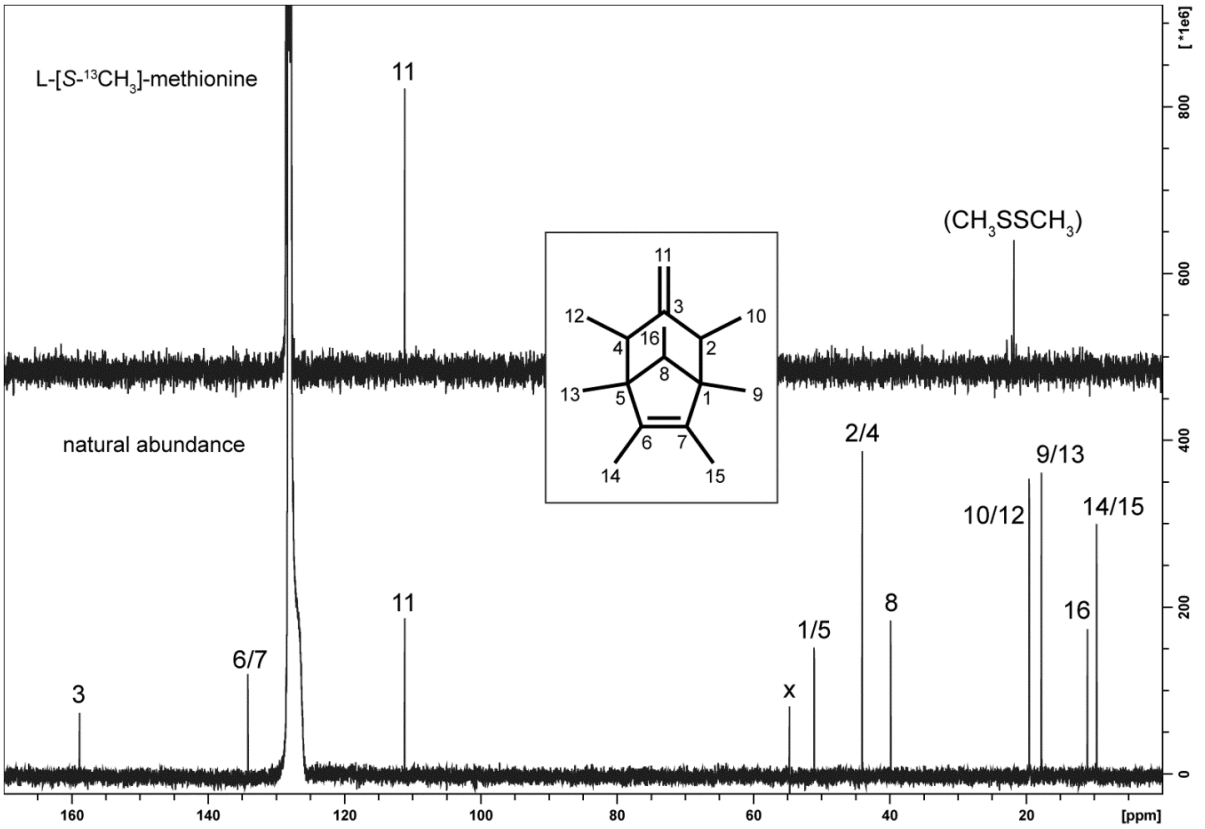


Figure 3.22: ^{13}C NMR spectrum of sodorifen. The compound was produced by the wildtype in *Serratia plymuthica* 4Rx13 was cultivated in a minimal medium supplemented with L- [*S*- $^{13}CH_3$]- methionine. Headspace were eluted in D6- benzene every 24 h over 96. Samples were measured by broadband decoupled ^{13}C NMR spectra (100 MHz). Top: incorporation of ^{13}C label into sodorifen (C-11), bottom: natural abundance, Peak numbers: position in sodorifen.

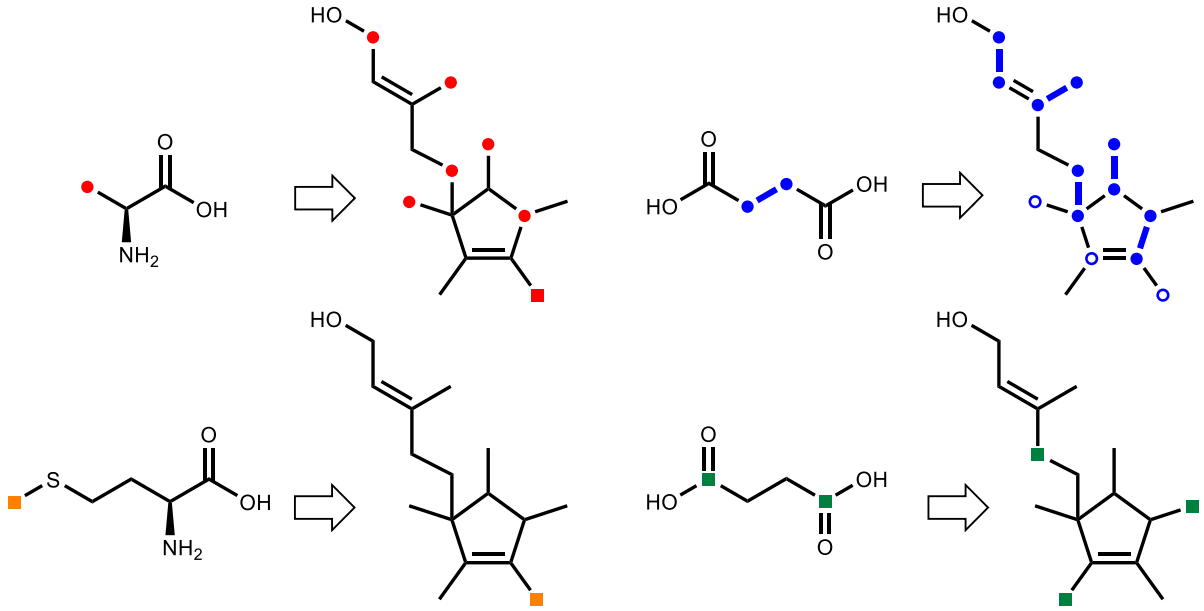


Figure 3.23: Incorporation of ^{13}C labels into the novel alcohol from stable isotope labeled precursors deduced from ^{13}C NMR spectra. Color code: green cube carbon incorporated by [1.4- $^{13}C_2$]-succinate, blue: C2-unit of [2.3-$^{13}C_2$]-succinate, red: C-unit of L- [3- ^{13}C]-alanine and orange: methyl group derived from L-[*S*-$^{13}CH_3$]-methionine. Full circle: duplet signal, empty circle singlet signal (constructed by Dr. S. v. Reuss).

The mass spectra of sodorifen and the new compound indicated differences in the methionine incorporation (**Figure 3.24**), which is in agreement with data derived by NMR spectrometry (**Figure 3.21D and 3.22**). First of all, the comparison of the mass spectra of both unlabeled sodorifen (**Figure: 3.18B**) and the new compound (**Figure: 3.18C**) with those obtained upon feeding with L-[*S*-$^{13}CH_3$]-methionine showed the successful incorporation due to the appearance of an additional signal. Unlabeled sodorifen has a molecular ion mass of *m/z*=218 but shows *m/z*=219 for [M+1] after feeding with labeled methionine. However, no change of the characteristic high intensity fragments at *m/z*=134,136 and 137 occurred, indicating that the isotope label does not reside within the pentamethylcyclopentenyl unit that gives rise to these ions (**Figure 3.24A**). However, for the new compound the incorporation of L- [S- $^{13}CH_3$]- methionine resulted in a shift of the dominating fragment ion from *m/z*=138 to *m/z*=139, indicating that the label is part of the pentamethylcyclopentenyl unit. (**Figure 3.24B**). Thus, the incorporation of methionine for both sodorifen and the novel alcohol differs.

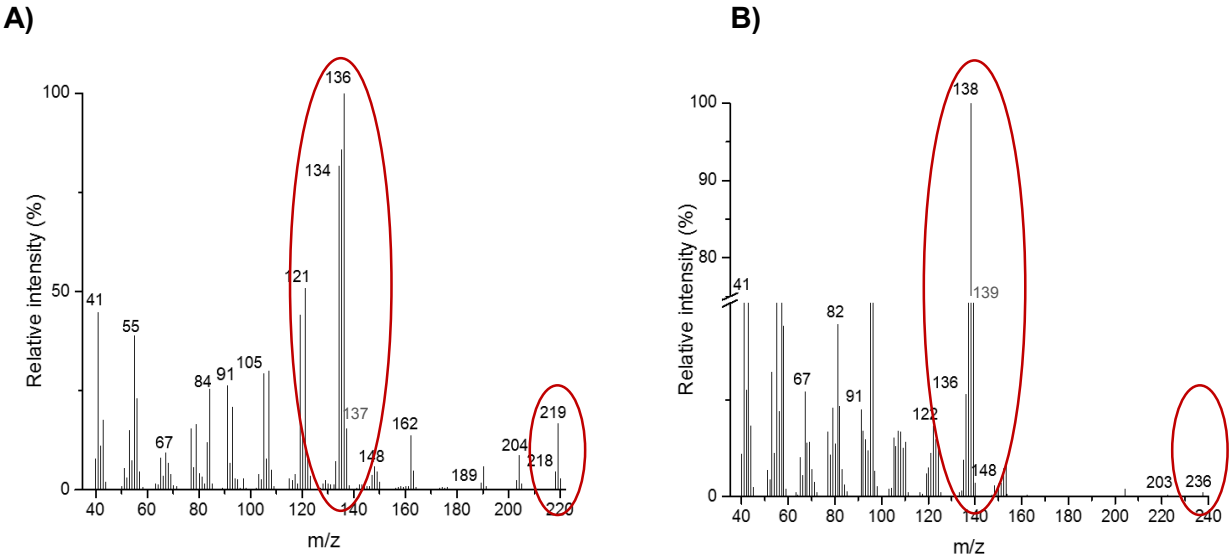


Figure 3.24: Mass spectra of sodorifen and the novel alcohol of *Serratia plymuthica* 4Rx13. Incorporation of L-[*S*-$^{13}CH_3$]-methionine into sodorifen **(A)** of the wild type and the novel alcohol **(B)** of the terpene cyclase mutant. Red circles: Characteristic signals are discussed in the text.

3.3.4 Proposed biosynthesis of the new compound

According to the acquired mass spectra and NMR-spectra obtained upon incorporation of labeled compounds and the elucidated stereochemistry a biosynthetic pathway was postulated for the novel alcohol. Geranyl pyrophosphate (GPP) is generated by the DOXP- pathway (**Figure 3.25***)*. For the following reactions, it is proposed that GPP and Isopentenyl pyrophosphate (IPP) react, however, not by the usual head to tail reaction but by head to middle reaction. Subsequently, two methyl groups are rearranged to build a cyclo pentene. The intermediate is methylated and the resulting cation reacts via a sequence of 1.2-methyl, 1.2-hydride, 1.2-methyl rearrangements and a deprotonation to form the novel alcohol (**Figure 3.25**).

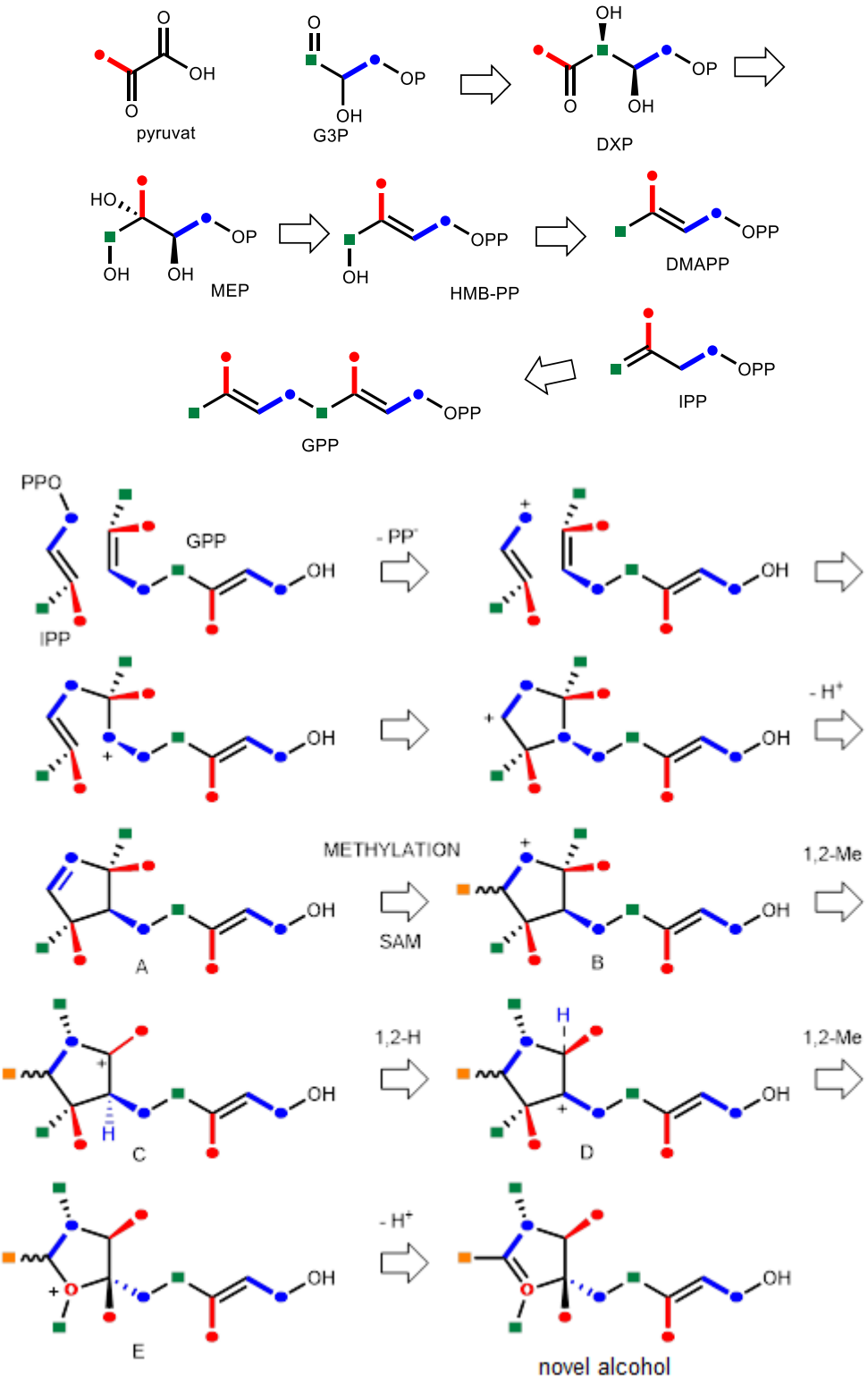


Figure 3.25: Proposed biosynthesis of the novel alcohol. Based on feeding experiments of the terpene cyclase mutant with isotope labeled precursors in *Serratia plymuthica* 4Rx13 a pathway could be obtained. Color code: green cube carbon incorporated by [1.4-$^{13}C_2$]-succinate, blue: C_2-unit of [2.3- $^{13}C_2$]-succinate, red: C_2-unit from alanine with red dot indicating carbon labeled by L- [3- ^{13}C]-alanine; and orange: methyl group derived from L-[*S*-$^{13}CH_3$]-methionine. Biosynthesis of GPP synthesized via the DOXP pathway and proposed sequence of reactions for the biosynthesis of the new alcohol (constructed by Dr. S. von Reuss).

3.3.5 Implications for the biosynthesis of sodorifen

It was observed that both sodorifen and the new compound are produced from three isoprene C_5-units plus one methionine derived C1-unit. Although the actual biosynthesis of sodorifen is still unknown till now it was hypothesized that it might resemble the biosynthesis of the novel alcohol (**Figure 3.25 and 3.26 right**).

Nevertheless, the biosynthesis of sodorifen is different in many aspects compared to the biosynthesis of the new compound. Most importantly the structural similarity (cyclopentene unit) does not correlate with a common biosynthetic origin due to the different position of the methionine-derived label in both sodorifen and the novel alcohol, which was demonstrated by the incorporation of labeled methionine (**Figure 3.21D; 3.22**). In **Figure 3.26** the proposed biosynthesis of sodorifen (left) and another possibility for the biosynthesis of the novel alcohol (right) is illustrated. Both pathways share a similar C15 intermediate (most likely a FPP related compound) that gets methylated by a SAM dependent methyl transferase and undergoes a series of stereoselective 1,2- methyl and 1,3-rearrangements. Indicated by dashed lines are putative non-head to tail fusion steps with subsequent methyl shifts that result in the large number of methyl groups in both sodorifen and the novel alcohol. While one of these steps is assumed to be involved in the biosynthesis of the new alcohol (**Figure 3.25**) the biosynthesis of sodorifen requires at least three of these steps. Suggesting that all three isoprene units would be linked via non-head tail fusion. Furthermore, a yet unresolved rearrangement of the C_2-unit composed of a succinate carboxyl unit and an alanine C2-position appear to occur in the sodorifen biosynthesis, which is hitherto not understood. Finally, a 1,3-rearrangement of a bicyclic pentalene skeleton might reveal the unique carbon skeleton found in sodorifen.

However, the proposed biosynthesis for the novel alcohol in **Figure 3.25** is the one, which is more likely because it contains no unlikely reactions, beside one unusual non-head tail fusion, and agrees with the obtained data.

Due to the fact that beside the sodorifen cluster further genes/enzymes are needed to synthesize this unusual natural compound, the complete biosynthesis of sodorifen including all enzymatic steps are presently not clarified.

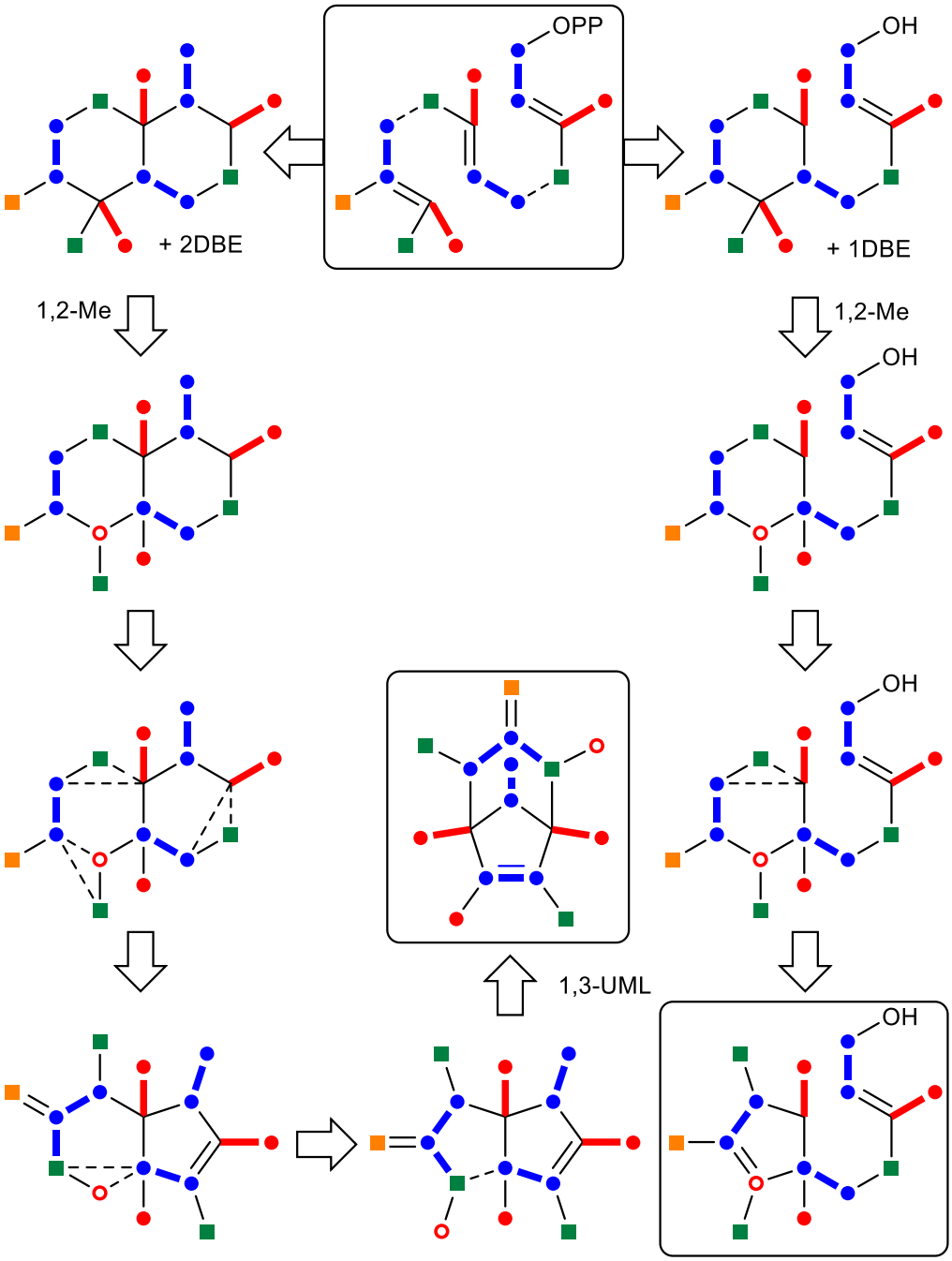


Figure 3.26: Proposed biosynthesis of sodorifen (left) and another possibility for the novel alcohol (right) in *Serratia plymuthica* 4Rx13. The biosynthesis is based on the incorporation of different stable isotope precursors. Color code: green cube carbon incorporated by [1.4-$^{13}C_2$]-succinate, blue: C-unit of [2.3-$^{13}C_2$]-succinate, red: C-unit of L-[3-^{13}C]-alanine and orange: methyl group of L-[*S*-$^{13}CH_3$]-methionine. (constructed by Dr. S. v. Reuss).

4. Discussion

4. 1 Involvement of the terpene cyclase in sodorifen biosynthesis

The comparative genome and transcriptome analysis of sodorifen-producer and non- producer, yielded in a list of candidate genes. The mutagenesis was applied for the genes *SOD_c13130* and *SOD_c44800*, which led to no change in sodorifen emission, while the knockout of the gene *SOD_c20750* revealed a sodorifen-negative phenotype. For the first time a gene was identified that was involved in the biosynthesis of the unusual natural product sodorifen produced only by certain *Serratia plymuthica* isolates.

4.1.1 Search for candidate genes

It has previously been shown that the bacterium *S. ply.* 4Rx13 mostly produces the novel compound sodorifen (Reuss et al., 2010). However, this compound has only recently been classified and its biosynthetic pathway is still unknown. Many research groups have worked towards elucidating new products (Blasse et al., 1965; Hardt et al., 2001; Böttcher et al., 2008) and metabolic pathways (Kuo et al., 1995; Lombó et al., 2006; Shirai et al., 2010) over the last few decades. In the present study, a multilayered approach was used to find candidate genes that encode enzymes that are likely to be involved in the biosynthesis of sodorifen.

Genome analysis has previously been used successfully to elucidate the 2C- methyl D- erythritol 4-phosphate (MEP) pathway (Rodríguez-Concepción and Boronat, 2002). In the present study, comparison of the genome data of sodorifen- producer isolates and closely related non-producer isolates provided some insight into candidate genes for sodorifen biosynthesis.

Genome analysis has been used in combination with transcriptome analysis to find the genes that are involved in a specific pathway for the bacteria *Yersina pestis* (Chauvaux et al., 2007) and *Ralstonia eutropha* H16 (Peplinski et al., 2010). This approach indicated 11 genes, which are specifically transcribed and might present new virulence factors. Another study of the bacterium *Ralstonia eutropha* H16, which produces poly 3-hydroxybutyrate (PHB) was examined (Peplinski et al., 2010). The studies contained a genome-wide transcriptome analysis to detect genes that are differentially

transcribed during PHB biosynthesis by the comparison of PHB-negative mutants with the wild type.

Therefore, in the present study, the transcription levels of genes in the sodorifen- producer isolate 4Rx13 and the closely related non-producer isolate AS9 were compared to generate a list of potential candidate genes. The transcriptome data showed a general distribution of strongly and weakly transcribed genes between 24 h and 48 h. Theoretically, all proteins and enzymes that are required for the basic development and survival of an organism are synthesized during primary metabolism (Mann, 1987) within the first 24 h. By contrast, specialized metabolites that are advantageous to the organism but not necessary for its survival are usually synthesized at a later stage of growth (Llorens et al., 2010). Since sodorifen is emitted during the stationary phase, it is believed to be a specialized metabolite. Thus, applying the two distinct criteria for the transcriptome analysis outlined above, a list of 21 candidate genes was compiled.

4.1.2 Mutagenesis and complementation of the terpene cyclase

The terpene cyclase gene *SOD_c20750* was found to be involved in sodorifen biosynthesis, which is interesting as it is also involved in terpenoid metabolism. Therefore, sodorifen might belong to the terpene group of chemicals.

Although this terpene cyclase gene is also present in isolate AS9, the transcriptome data revealed that it is not expressed, which could explain its inability to produce sodorifen–and the same could also be true for isolates AS12 (**supplement S6**) and AS13 (data not shown), which also have the gene but do not emit sodorifen. It would be interesting to compare the volatile blends of other bacteria (e.g., *Streptomyces* spp.*, Pseudomonas agarici,* and *Burkholderia pyrrocinia*) in the future.

To verify the involvement of the terpene cyclase in sodorifen biosynthesis, the knockout mutant of *SOD_c20750* was complemented with a recombinant plasmid containing the wild-type gene of the terpene cyclase, following which the function of the gene was successfully restored. This approach had previously been used in other organisms, such as the archaeon *Methanosarcina acetivorans* C2A (Zhang et al., 2002). The restoration of the function of dysfunctional genes by recombinant plasmids is common in bacteria, e.g., *E. coli* and *Pseudomonas putida* (Shanley et al., 1986), and

homologous genes have also been used to restore function in *Saccharomyces cerevisiae* (Minet et al., 1992).

The complementation of the terpene cyclase mutant was not straightforward, however, because after several attempts it was found that a strong and specific promoter was needed. As a first attempt, the wild-type gene was cloned into the vector pUC19, which harbored the *lac* promoter of *E. coli.* However, following the introduction and induction of the gene on the plasmid within the terpene cyclase mutant, it still remained unable to produce sodorifen. Therefore, it was speculated that either the transcription system of *S. ply.* was not able to recognize the promoter of *E. coli*, or that the protein-protein interaction of the cAMP receptor protein and RNA polymerase for the transcription was disturbed (Flatow et al., 1996) due to aberrant promoter binding sites. It is also possible that the binding of the transcription factors was not tight enough and so transcription did not take place. This implies that the different genetic background and variants in the consensus sequence may have been too great for transcription, meaning that the transcription system in *E. coli* is not similar enough to *S. ply.* Therefore, it was decided that a native promoter was required.

Secondly, a construct consisting of the wild-type gene and the native *gap-dh* promoter, cloned downstream of the gene, was generated. However, this still resulted in a sodorifen-negative phenotype. In this instance, it was thought that the promoter was not strong enough, which probably resulted in a low initiation frequency (Pátek, 2005) and consequently a low transcription level, so that the quantity of enzyme produced was not sufficient to synthesize sodorifen. Previous heterologous expression studies have shown that the *lac* promoter is weak with only low levels of proteins produced (Terpe, 2006). Therefore, it is likely that the same holds true for the *gap-dh* promoter, resulting in the lack of sodorifen emission.

Therefore, a third attempt was made in which a strong promoter was selected with the help of the transcriptome data. This approach led to successful complementation of the mutant, resulting in the recovery of sodorifen emission. To our knowledge, this is the first successful demonstration of complementation in *S. ply.* 4Rx13. However, the quantities of sodorifen produced by the complemented mutant were very small, possibly due to different levels of regulation that likely do not apply for the plasmid, including: i) global regulation of the metabolic pathway, ii) the individual regulation of certain genes or iii) the existence of feedback inhibition.

Studies have shown that plasmid gene expression is destabilized by a promoter that is too strong (Hannig and Makrides, 1998). It is also possible that the fine-tuning of mediators was missing, so that although the quantity of protein derived from the gene on the plasmid was sufficient, other compounds were not synthesized in sufficient quantities, resulting in minimal sodorifen emission. Additionally, it is possible that the mediator complex, a multi-subunit assembly, which is required for regulatory expression of most RNA polymerases (Poss et al., 2013), might be composed incorrectly or is not induced due to missing proteins or coactivators.

On the other hand, the overproduction of a protein as a result of continually transcribed genes can lead to a metabolic burden and result in growth inhibition (Donovan et al., 1996), although this was not observed for the complemented terpene cyclase mutant. Sodorifen itself also did not appear to be toxic for the cells and high levels were not detrimental for the organism. Feedback inhibition could also explain the low sodorifen emission. It cannot be proven that the recombinant plasmid was introduced only once, and so multiple copies were probably present after transformation. Thus, it can be assumed that high amounts of the protein were generated, which would have accumulated in the cells and may have been degraded.

The exact reasons for the difficulties experienced in complementing the mutant remain highly speculative for *S. ply.* 4Rx13 due to the lack of information about the molecular basis of the interaction between transcription factors and the regulation of sodorifen biosynthesis. Therefore, it is recommended that binding studies investigating transcription factors within the promoter region and detailed studies on the regulation of genes should be carried out to shed further light on the underlying mechanisms.

4.1.3 Characterization of the terpene cyclase

The gene *SOD_c20750* is annotated as a terpene cyclase, clearly defining the function of this enzyme. However, the actual involvement and mechanism of action of this gene in the biosynthesis of sodorifen remains ambiguous.

There is a wealth of knowledge and research concerning terpene cyclases in plants (Hohn et al., 1989; Degenhardt et al., 2009; Chen et al., 2011). However, little is known about terpene cyclases in bacteria and even less is known about sesquiterpenes. Up until now, only three sesquiterpene cyclases (Cane et al., 1994, 2006; Jiang et al., 2006, 2007), one diterpene synthase (Dairi et al., 2001; Hamano et al., 2002), and a

new group of monoterpenes (Komatsu et al., 2008) have been described biochemically, all of which are only found in the bacterial genus *Streptomyces.* It has also been shown that the cyanobacterium *Nostoc punctiforme* contains a specialized sesquiterpene cyclase (Agger et al., 2008).

Finding additional bacterial synthases by blast analysis is not sufficient because low levels of mutual sequence similarity exists between bacterial terpene synthases (Yamada et al., 2015). Yamada and coworkers (2015) applied an alternative genome mining strategy and were able to identify 262 bacterial terpene synthases. Thus, bacterial terpene synthases are also widely distributed in bacteria (Yamada et al., 2015). However, the majority associates with *Actinomycetales* microorganisms and only a minor fraction of gram-negative bacteria, mostly *Cyanobacteria* and *Myxobacteria* harbor terpene synthases according to the results of Yamada (2015). Additional bacterial synthases have also been found using BLAST analysis, but these have not been examined in detail. Therefore, the closer investigation of the terpene cyclase and its products that follows is speculative, as elucidation and characterization of bacterial terpene synthases is still in its infancy due to the huge structural and functional diversity that exists. Terpene cyclases are enzymes that catalyze the final step in isoprenoid metabolism. Therefore, it is tempting to speculate that the building blocks for sodorifen biosynthesis are generated within the DOXP pathway, and that the terpene cyclase is a specialized enzyme for the cyclization of the prenyl chain. The exact mechanism of action of the terpene cyclase of *S. ply.* 4Rx13 is currently unknown, as neither the type of cyclization that occurs nor the participation of the enzyme in closing the ring to form sodorifen could be clarified. Although the biochemical mechanism of action of terpene cyclases is well known and crystal structures even exist for some bacterial terpene cyclases (Lesburg et al., 1997; Aaron et al., 2010; Köksal et al., 2012), it is difficult to predict the correct native 3D-structure of terpene cyclases on the basis of their amino acid sequence (Rabe and Dickschat, 2013) due to the lack of examples for comparison. The sesquiterpene cyclase of *Streptomyces coelicolor* A3 has been described in detail (Aaron et al., 2010), but this is a rare example in the literature.

Only a few bacterial terpene cyclases have been investigated *in silico* or with site- directed mutagenesis to find typical motifs and for comparison with other bacterial synthases (Felicetti and Cane, 2004; Aaron et al., 2010; Smanski et al., 2012).

Here, it was shown that sodorifen is most likely a terpenoid, as it was synthesized with the support of the terpene cyclase. The molecular formula of sodorifen ($C_{16}H_{26}$) also suggests that it is a terpene as, according to the “isoprene rule” that was established by Wallach (1887), terpenes typically contain only carbon and hydrogen (empirical formula $(C_5H_8)_n$), which matches the composition of sodorifen. If n = 3 in the empirical formula, a compound with the formula $C_{15}H_{24}$ would result. However, it is possible that a methyl group is added to a sesquiterpene intermediate, leading to $C_{16}H_{26}$, or a diterpene precursor ($C_{20}H_{32}$) is shortened by the elimination of C atoms.

Therefore it was speculated that at least a sesquiterpene synthase or a diterpene synthase is required for the synthesis of sodorifen. At the present state it is a disadvantage that only a few bacterial sesquiterpene synthases and their products are known in detail, e.g. from *Streptomyces* species and *Nostoc* (Nakano et al 2011a). A possibility to analyse the products of terpene cyclases or cluster is by heterologous expression in the genetically engineered bacterium *Streptomyces avermitilis* (Komatsu et al., 2013). It was shown that the heterologous expression of the silent biosynthetic cluster in *S. avermitilis* resulted in the production of novel terpenes (Yamada et al., 2015). They identified 11 new diterpenes and two new sesquiterpenes from *Streptomyces* microorganisms. Interestingly, *S. clavuligerus* and *S. lactacystinaeus*, which are terpene non-producer under natural conditions, showed the expression of terpenes in the engineered *S. avermiltilis*. Thus, the heterologous expression allows the initiation of producing otherwise silent biosynthetic gene cluster. However, to apply this approach for *S. ply.* AS9 and enable this isolate to produce sodorifen, different aspects have to be considered: i) the whole cluster for the biosynthesis of sodorifen has to be elucidated and ii) the regulation in *S. ply.* 4Rx13 has to be encoded. The comparison to other microbial species could give hints about its nature and mechanism of action.

The BLASTp search for the terpene cyclase of *S. ply.* 4Rx13 resulted in high similarities among species within the genus *Serratia* (99% for isolates AS9, AS12, AS13, S13 and A30), but extremely low similarities with other bacteria species (43.4% for *Pseudomonas chlororaphis* O6, 41% for *Burkholderia pyrrocinia*, and 27.3% for *Streptomyces pratenensis*) exist. This supports the hypothesis that different terpene cyclases are highly species specific and probably have distinct substrate specificities, allowing them to catalyze different reactions to synthesize a huge variety of compounds. Although bacterial sesquiterpene cyclases have no significant amino acid

sequence identities among bacterial strains or plant enzymes, typical motifs can be found (Nakano et al., 2011). This is also true for the terpene cyclase of *S. ply.* 4Rx13, which contains a slightly different DDxxxDE motif but does not contain the NSE/DTE motif that is present in other plant and bacterial terpene synthases (Christianson, 2006; Nakano et al., 2011a; Yamada et al., 2014). It is possible that an alternative sequence (NDxxSxE) is present in the isolate 4Rx13, reflecting an improvement that allows the acceptance of an unusual substrate or the production of specific compounds–although comparisons can only currently be made with sesquiterpene synthases found in *Streptomyces* and *Nostoc* (Nakano et al., 2011b). In contrast, it is possible that the terpene cyclase of *S. ply.* 4Rx13 belongs to the class II enzymes, which only possess the DDxx(D/E) motif (Meguro et al., 2013).

Additional experiments will be necessary to reveal the relationship between the terpene cyclase of *S. ply.* 4Rx13 and sodorifen synthesis. The generation of a crystal structure of the terpene cyclase would help us to understand the mechanisms occurring inside the active pocket, as well as the binding mechanisms. However, these experiments will only be possible once the substrate of the enzyme is known and available. The location of the active pocket and the involvement of amino acid residues that are responsible for the reaction mechanism could then be targeted using site- directed mutagenesis to investigate whether changes in single amino acids lead to novel compounds. The exact classification of the terpene cyclase found in *S. ply.* 4Rx13 remains speculative at this point. Various aspects are still unknown, such as whether it is classified as a monoterpene, sesquiterpene or diterpene cyclase, and whether it is a single- or multi-product enzyme. Therefore, heterologous overexpression, purification, and biochemical characterization will be crucial for providing information about its function. The establishment of an enzyme assay would also clarify its mechanism of action, and different substrates could be used [isopentenyl pyrophosphate (IPP), geranyl diphosphate (GPP), neryl diphosphate (NPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP)] to investigate whether common substrates are converted by this new terpene cyclase.

It is highly likely that the new terpene cyclase of *S. ply.* 4Rx13 is involved in the biosynthesis of sodorifen, and although the entire pathway is not yet known, some speculation is possible. Firstly, it is well-established that terpenoid synthesis is connected to the IPP production pathway. In plants, the mevalonate pathway in the cytosol is widely used, while in plastids, the DOXP pathway is present–and in bacteria,

either one or the other exists (Rohmer et al., 1999; Rohdich et al., 2001). Based on the sequenced genome (Weise et al., 2014), it is known that *S. ply.* 4Rx13 possesses enzymes of the DOXP pathway. In addition, the gene *SOD_c20770*, which was annotated as a DOXP-synthase, was highly expressed in this isolate and found within the list of candidate genes. Thus it can be concluded that *S. ply.* 4Rx13 most likely produces the precursor IPP via DOXP pathway similar to *Bacillus subtilis, Campylobacter jejuni, E. coli, Heliobacter pylori* and others (Rohdich et al. 2001)

4.1.4 New compound produced by the terpene synthase mutant

The VOC profile of the terpene cyclase mutant showed the absence of sodorifen but the presence of another dominant compound. The mass spectrum of this compound is similar to sodorifen, but some characteristic masses are different. Therefore, the elucidation of the structure of this compound is the focus of chapter three. The relationship between this compound and sodorifen also needs to be investigated to determine whether the new compound is an intermediate of the biosynthetic pathway that accumulated due to the dysfunction of the enzyme in the mutant or an alternative byproduct that was produced by other enzymes to avoid an accumulation of superfluous compounds. Chemical analysis of this compound through the incorporation of labeled compounds and NMR spectrometry could also help to clarify the role of the terpene cyclase.

However, the investigation concerning the ecological role of the compound would be curious. The effects of microbial volatile mixtures or single compounds on microorganisms were tested in various studies by co-cultivation experiments, which only allows the diffusion of volatiles (Kai et al. 2007, 2010; Wenke et al., 2012). The influence of volatile organic compounds on the interactions of plant and fungi (Fiers et al., 2013) or bacteria and fungi (Garbeva et al., 2014) were investigated under natural cultivation conditions like soil, sand or with artificial root exudates. It was shown that emitted microbial volatiles affect growth, antibiotic production or gene expression in other organisms. Therefore, testing the effect of the emitted product of the mutant in dual culture would be interesting. It could be examined if the new compound shows an effect on microorganisms or plants and compare the data to the studies of the wild type. Investigations with *S. plymuthica* 4Rx13 demonstrated that the emitted volatiles inhibit the growth of many fungi and *Arabidopsis thaliana* (Vespermann et al., 2007).

Especially, dimethyl disulfide and ammonia were shown to be the bioactive compounds, while the application of the main compound sodorifen showed no effect on *A. thaliana* (Kai et al., 2010). For other soil bacteria the influence of volatiles was shown as well. The co-cultivation of soil bacteria *Collimonas pratensis*, *S. plymuthica*, *Paenibacillus sp.* and *Pedobacter* inhibited the growth of *Pseudomonas fluorescens* Pf0-1 (Garbeva et al., 2014). Another study by Splivallo (2007) confirmed the influence of fungal volatiles, which are released to regulate the interactions with other organisms. Volatiles of the symbiotic fungi *Tuber borchii*, *Tuber melanosporum* and *Tuber indicum* fruiting bodies inhibited the growth in *A. thaliana* and induced an oxidative burst. The application of 1-Octen-3-ol and *trans*-2-octenol induced the oxidative stress, while 1- Octen-3-ol had the strongest inhibitory effect on the plant (Splivallo et al., 2007).

4.2. Analysis of the sodorifen cluster genes

The terpene cyclase mutant was unable to produce sodorifen and enabled a cluster of four genes to be found, which were hypothesized to be relevant for the biosynthesis of sodorifen. Mutagenesis of the other three genes in this cluster also resulted in a changed phenotype. Therefore, a detailed analysis of this cluster and its products in *S. ply.* 4Rx13 and other species was performed.

4.2.1 Investigation of the cluster genes and complementing genes

Mutagenesis of the four genes in the sodorifen cluster proved that three of the genes were directly involved in sodorifen biosynthesis, as the loss of their function resulted in an inability to produce sodorifen. By contrast, the mutation of the *dxs* gene led to a significantly reduced sodorifen emission (30% of the wild-type emission).

A second *dxs* gene (*SOD_c09180*) was also found to be present in the genome of *Serratia*, which probably complemented the single and the double knockout mutants. Alignment of the *dxs* genes (SOD_c20770 and SOD_c09180) showed that they have the same gene size but an overall protein motif similarity of only 58%. Interestingly, the double-knockout mutant was still able to produce sodorifen but showed an even greater reduction in sodorifen emission (12.5% of the wild-type emission). There are three possible explanations for this: i) a third gene exists in the genome that is able to complement the function of the *dxs* gene of the cluster, but not as efficiently as the original gene, ii) the *dxs* gene is not directly involved in sodorifen emission or iii) the involvement of the two *dxs* genes in the production of sodorifen differs due to their independent regulation and transcription efficiencies.

Further analysis of the location of the second *dxs* gene revealed a small cluster of three genes, all of which have the same transcription direction and appear to belong to the DOXP pathway. The other two genes within this cluster were a farnesyl diphosphate synthase (*ispA* gene) and a putative oxidoreductase. These genes had the same structure as found in *E. coli* by Sponsel (2001), who speculated that the proximity of *dxs* to *ispA* is due to their functional relationship and involvement in the same pathway. To analyze the role of these two genes, mutants were generated. It was not possible to generate a mutant lacking the farnesyl diphosphate synthase (*ispA*), indicating that this is an essential gene for the organism. Since isoprenoids are essential for building

ubiquinone, cholesterol, carotenoids and components of the cell membrane for the growth and survival of many organisms. Mutation of the oxidoreductase had no effect on sodorifen emission, which was expected due to the presence of multiple putative oxidoreductases within the genome of *S. ply.* 4Rx13.

As the double *dxs* knockout mutant of *S. ply.* 4Rx13 (*SOD_c20770* and *SOD_c09180*) is able to survive the functional loss of both enzymes without growth impairment, it seems mandatory that a third gene with a complementary function exists. Subsequently, the genome was investigated once again to search for another gene that could complement the *dxs* gene function. A search based on gene annotations was not successful, possibly due to the incomplete manual annotation of the genome. Therefore, all hypothetical proteins were checked for the protein motifs that are typically present in the DOXP-synthase. No similarities were found, but this does not exclude the possibility that a gene may exist that is able to partly complement the *dxs* activity.

The *dxs* gene encodes transketolase-like proteins, which are thought to be able to catalyze the formation of DOXP (Sprenger et al., 1997). If this is also the case in *S. ply.* 4Rx13, the emission of sodorifen could still be observed due to complementation by transketolases, but may be reduced due to a decreased substrate turnover. The isolate *S. ply.* 4Rx13 contains two transketolases, *SOD_c33980* and *SOD_c39100*, the respective enzymes of which contain two of the three transketolase domains of the *dxs* gene but lack the DOXP-synthase domain. Since, a transketolase is able to catalyze the formation of xylulose-5-phosphate, it could be subsequently reduced to deoxy-xylulose-5-phosphate. Thus, the product IPP would be present and sodorifen could be produced even when the two DXS enzymes are dysfunctional. It should be noted, however, that this scenario is speculative at this stage, with the generation of single and double mutants required for verification.

The discovery of two distantly related isogenes demonstrates a new complexity in the genome of *S. ply.* 4Rx13. Isogenes have previously been described in roots colonized by arbuscular mycorrhizal fungi, and have been shown to have distinct functions and expression levels (Walter et al., 2013). DXS1 is probably responsible for housekeeping functions (primary metabolism), while DXS2 is associated with the synthesis of special isoprenoids (secondary metabolism)–although each gene can complement the function of the other. Therefore, the expression levels of these genes should distinguish them from each other. For *S. ply.* 4Rx13, it was expected that the

expression level of the housekeeping gene would be the same over time and might be expressed at a medium level, while the production of secondary metabolites would likely start after the exponential growth phase. It was found that the expression of the *dxs* gene outside the cluster matched the expression profile of a housekeeping gene, with low NPKM values (65 after 24 h and 82 after 48 h), while the *dxs* gene (*SOD_c20770*) within the sodorifen cluster showed high expression levels (1049 after 24 h and 1118 after 48 h), suggesting that this gene is probably involved in secondary metabolism.

An alternative scenario is that an additional pathway or a different branch point could be used to bypass the dysfunctional enzymes in the mutants and provide the missing intermediates for the DOXP pathway, which are needed for the biosynthesis of sodorifen. Thus, an alternative route beside isopentenyl isomerase (Arigoni et al., 1999; Rodríguez-Concepción et al., 2000; Sponsel, 2001) might exists to synthesize 1-desoxy-D-xylulose-5-phosphate as shown for the mevalonate pathway in archaea (Lombard and Moreira, 2010). For the archaea *Haloferax volcani* it was shown that two new enzymes are involved, which catalyze an alternative terminal reaction of the mevalonate pathway (van Nice et al., 2014).

To investigate the involvement of the DOXP pathway in the synthesis of sodorifen, other enzymes of the DOXP pathway should be targeted. Previous studies have shown that it is not possible to create a knockout mutant of the 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase gene (Piepenborn, Master's thesis, 2013) and DOXP reductase (Piepenborn, laboratory, 2013) in *S. ply.* 4Rx13–presumably due to the loss of these genes being lethal for the bacteria. Therefore, it was not possible to study the influence of these genes on sodorifen emission.

Usually two intermediates, IPP and DMAPP, are required for the synthesis of terpenoids. Another approach is to observe the effect of manipulating the ratio of IPP and dimethylallyl diphosphate (DMAPP) on sodorifen emission, as these are the only intermediate compounds required for the synthesis of terpenoids (with the exception of a small group of hemiterpenes that originate from either IPP or DMAPP; Rohdich et al., 2005). Eubacteria, which exclusively use the DOXP pathway to produce terpenes, have specialized isomerases that act as salvage enzymes in order to optimize the molar ratio of IPP and DMAPP depending on the metabolic conditions (Laupitz et al., 2004). The isomerase idi is able to catalyze the reaction for the interconversion of IPP and DMAPP, and studies on *E. coli* have shown that this isomerase has a low activity

and that the deletion of the *idi* gene is not lethal (Wolff et al., 2002), and similarly Charon (2000) also stated that the *idi* gene is not essential. This gene is also present in *S. ply.* 4Rx13 (*SOD_c20170*). The knockout of the *idi* gene in isolate 4Rx13 had no effect on sodorifen emission (**supplementary Figure S7**). This finding also suggests that the terpenoid products in *S. ply.* 4Rx13 can be produced from either IPP or DMAPP, as is the case for hemiterpenes.

A second enzyme with the ability to convert IPP and DMAPP (IspH) was also found in *S. ply.* 4Rx13, which would allow the pathway to continue to function despite the knockout of *idi* (Rohdich et al., 2005). However, the transcriptome data showed that the expression levels of the *idi* gene in both the sodorifen-producer and non-producer isolates (4Rx13 and AS9, respectively) were very low (NPKM = 9 after 24 h and 13 after 48 h), which excludes it from having an involvement in sodorifen biosynthesis, as only highly expressed genes were considered potential candidate genes for mutagenesis. Nevertheless, it would be interesting to investigate the effect of a double knockout of the *idi* gene and the *IspH* gene in *S. ply.* 4Rx13 on sodorifen production, as this could provide additional information about the involvement of the DOXP pathway in its biosynthesis.

4.2.2 Organization of the sodorifen cluster

Many of the genes in bacterial genomes are not randomly localized but rather appear in clusters and are organized in operons. These clusters form functional units often because they belong to the same pathway or are the basis for the same regulatory pathway (Overbeek et al., 2000; Newcomb et al., 2011). The sodorifen cluster in *S. ply.* 4Rx13 consists of four genes with only small interspace regions (under 50 bp) or overlapping genes (*dxs* gene with the methyl transferase), indicating a cotranscription of the genes (Moreira et al., 2012). The promoter is located directly upstream of the cluster, as demonstrated by the successful complementation of the mutants by cloning the area 500 bp upstream of the cluster into the vector.

Linkage of the terpene cluster genes of *S. ply.* 4Rx13 with sodorifen production is an important step in elucidating the biosynthetic process for this novel compound. The four genes contained within this cluster are certainly not sufficient to synthesize such a complicated structure as sodorifen. However, for the first time it has become clear

that sodorifen is derived at least partially from the terpenoid pathway and is therefore a terpene.

The sodorifen cluster has a similar structure to the terpene synthase cluster found in *S. clavuligerus*. This latter cluster is a small cluster that is involved in the biosynthesis of natural products, which also consists of a terpene cyclase and a methyl transferase, and is flanked by an oxidoreductase in the opposite orientation from the rest of the genes (Hu et al., 2011). Hu and coworkers demonstrated that the heterologous expression of the terpene synthases of *Streptomyces clavuligerus* produces cadinene and muurolol. The product of the corresponding biosynthetic genes of the cluster remained unknown. Thus, it can be speculated that this small cluster contains essential genes for the production of natural compounds. Since, sodorifen has a complicated structure, however, it can be assumed that other genes are also involved in its synthesis.

More genes that are involved in sodorifen biosynthesis need to be elucidated in future studies, however, which will be no trivial task given our limited knowledge about the general organization of the terpenoid biosynthetic pathway in bacteria (Tetzlaff et al., 2006). It is possible that the remaining genes will not appear in a cluster (DeJong et al., 2006), but rather will be scattered within the genome. However, it does seem that the distribution of small operons within the genome is common in bacteria, with small clusters often being found–for example, the two-gene operon found in *Streptomyces coelicolor*, which is responsible for the biosynthesis of the volatile compound methylisoborneol by Wang and Cane (2008). Thus, further investigations are essential to locate the additional genes that are involved in sodorifen biosynthesis.

A putative oxidoreductase, putative serine protease, and an adenosine deaminase are located in direct proximity and upstream of the sodorifen cluster genes. These genes are not potential candidate genes for sodorifen biosynthesis, however, due to their different orientation on the DNA strand. The genes downstream from the cluster belong to the arabinose biosynthesis pathway and were also excluded from being involved in the biosynthesis of sodorifen.

4.2.3 The sodorifen cluster in other species of bacteria

The presence of a sodorifen cluster with a similar cluster organization and similar genes was investigated in other species of bacteria. In addition, the correlation

between the presence of the cluster within the genome and the ability to produce sodorifen should be analyzed. This search revealed two bacteria that contain genes of the sodorifen cluster with a similar structure and an analogous order: *Streptomyces tsukubaensis* NRRL 18488, which has three of the four potential orthologous genes (the terpene cyclase, methyl transferase, and DOXP-synthase); and *Pseudomonas chlororaphis* O6, which contains all four orthologous genes with similar lengths, gene interspaces, and gene arrangements. The proteins exhibited an identity of 41–65% at the amino acid level. Both bacteria have yet not been tested regarding sodorifen emission. However, despite both of these bacteria containing a terpene cluster, it is highly likely that neither is able to produce sodorifen. There are several possible explanations for this, including i) the cluster is not sufficient for the biosynthesis of sodorifen, ii) the substantial differences at the amino acid level could result in a different protein product, iii) it is possible that the genes are silenced, and iv) the regulation could be remarkably different or suppressed.

A similar finding has previously been obtained for *Streptomyces* spp., which are known to emit geosmin and 2-methylisoborneol (Hu et al., 2010; Wang and Cane, 2008), but in which *S. griseus* has almost no detectable production of these compounds despite harboring the complete set of genes (Yamada et al., 2015). Although *Sterptomyces griseus* harbors the complete set of genes for geosminbiosynthesis, almost no detectable production was observed (Yamada et al., 2015). These results indicate the involvement of regulation events being decisive whether the products are synthesized or not. Another example is provided by *Streptomyces avermitilis,* which contains a biosynthetic cluster for the production of pentalenolactone; however, when this cluster was integrated into the non-producer *Streptomyces lividans*, it was still unable to produce the same product but did produce pentalenic acid (Tetzlaff et al., 2006). This indicates that regulatory processes are involved in determining whether the products are synthesized or not.

4.2.4 Regulation of sodorifen biosynthesis

Little is currently known about the regulation of sodorifen biosynthesis. It is known that the production of many secondary metabolites is dependent on the growth phase and is stringently regulated (Takano et al., 1992; Gramajo et al., 1993), with the late growth phase often being correlated with changes in environmental cues such as temperature,

pH, stress and nutrient supply that result in substantial metabolic alterations. For example, Williamson et al. (2006) showed that bacteria only invested in the production of prodiginine under appropriate conditions.

Feeding experiments have previously been used successfully to gain an insight into the synthesis and regulation of specialized metabolites in microorganisms (Olano et al., 2008; Sørensen and Giese, 2013). In *Serratia* spp., feeding experiments with varying carbon sources resulted in substantially different levels of sodorifen emission (Weise, dissertation, 2013).

It was found that succinate gave the maximum sodorifen emission and that alanine was also a good substrate, while methionine resulted in a moderate growth behavior and low emissions, and glucose produced no emissions in *S. ply.* 4Rx13. These differences in sodorifen emissions between substrates could be due to differences in i) uptake efficiencies, ii) metabolizing efficiencies or iii) regulation mechanisms.

The assumption that the DOXP pathway is involved in the biosynthesis of sodorifen led to pyruvate being used in these feeding experiments. However, this resulted in only a small emission of sodorifen. The most likely explanation for this is that pyruvate is not efficiently taken up by the cells and that the intermediates, such as glyceride- aldehyde-5-phosphate (G3P), are limited–G3P is preferentially channeled into the citric acid cycle rather than into the DOXP pathway, which would lead to a low sodorifen emission.

It is also possible that succinate acts as an activator while glucose acts as a repressor. Although glucose is usually a well-used carbon source for growth, various studies have demonstrated that it can act as a repressor of the production of secondary metabolites such as prodigiosin, penicillin, and neomycin (Drew and Demain, 1977). Therefore, it also probably acts as a repressor for sodorifen production.

Other studies have also shown that the availability of different carbon sources can have a regulatory effect on certain processes. The best example of this is catabolite repression in *E. coli* (1977; Postma et al., 1993; Stülke and Hillen, 1999), whereby the presence of two carbon sources results in a complex regulation so that the preferred carbon source is used first, and the sequential use of these carbon sources results in a delayed growth as the organism switches between the two sources. Glucose may act as a repressor in sodorifen biosynthesis.

Similarly, a switch in growth behavior was also observed for *S. ply.* 4Rx13 when it was first grown on glucose and then succinate was added to the medium. After the

depletion of glucose, succinate utilization started and correlated with the sodorifen emission (Kai and Piechulla, unpublished results). Furthermore, the growth of *S. ply.* 4Rx13 in minimal medium supplemented with glucose was faster than on succinate indicating that the catabolic enzymes for the depletion and conversion of glucose are already available and thus allows faster growth (Harder and Dijkhuizen, 1982), whereas the metabolism has to adapt for the consumption of succinate and so there is slower growth. Thus, the difference in consumption of succinate and glucose suggests that catabolite repression also occurs in this species (Moses and Yudkin, 1968).

The biological and ecological reasons for this suppression of the production of sodorifen are not clear, but it is possible that it is not necessary to invest in the synthesis of sodorifen under suitable growth conditions due to the optimal nutrient supply and absence of negative environmental cues. Further analyses are required to clarify the relationship between succinate and glucose consumption and sodorifen emission, however. The results so far indicate towards a relevance of sodorifen production under a glucose deficient nutrient situation.

The genes of the sodorifen cluster appear to be specifically regulated, with differences in the regulation of the transcription levels of the *dxs* genes within and outside the cluster. When *S. ply*. 4Rx13 was grown on succinate, the metabolism favored the production of sodorifen and higher transcription levels of the involved genes were achieved. Although, the second *dxs* gene can complement the function of the first, a weaker phenotype with a decreased sodorifen emission was observed in the knockout of the gene within the cluster, which corresponds with the lower transcription level. This difference in regulation could be the reason for the absence of sodorifen in isolate AS9 despite it containing the same genes. This isolate may have low expression levels because i) the regulator of the cluster is not present or got lost, ii) more than one element for regulation is missing, iii) the production of sodorifen is energetically too costly or iv) sodorifen is not advantageous in its environment. Given the high similarity at the genome and protein level of isolates AS9 and 4Rx13, it is unlikely that isolate AS9 lacks the regulator or the regulatory mechanism. Rather, it is most likely that the regulator is suppressed or blocked. The regulation of sodorifen-related gene expression appears to be highly complex, and so it may not be sufficient simply to enable the transcription of the sodorifen cluster in isolate AS9. It is also possible that the expression of the cluster genes results in the production of another compound

(Nakano et al., 2011). With regard to the expression of these genes in distantly related species, it may be difficult for the organism to fold the necessary proteins correctly or high amounts of the proteins could to be toxic for the organism or could be enclosed in inclusion bodies, as often occurs in *E. coli* –although protocols for the recovery of the proteins do exist (Tsumoto et al., 1998; Villaverde and Carrió, 2003). Therefore, determining why isolate AS9 lacks sodorifen emissions is the subject of ongoing research.

In the present study, it was not possible to locate the regulator of the sodorifen cluster. In general two level of regulation exist: i) the lowest level, the regulatory genes affect only a single biosynthetic pathway or cluster and ii) highest level, genes perform pleiotropic control on both development and secondary metabolites, which was investigated in *Streptomyces* spp. (Champness and Chater, 1994). However, the number of regulators and their position can differ, and in some cases the regulator is located directly in front of the cluster and in other cases located distantly from the cluster within the genome (Olano et al., 2008).

Usually, biosynthetic genes for secondary metabolites are grouped in a cluster and the pathway specific regulator is part of the cluster (Liu et al., 2013). These pathway- specific regulators are usually confined to genes in the same cluster (Bibb, 1996). Studies of Janga and coworkers (2007) showed that often short distances between regulators and their corresponding effector genes exists. An example for these regulators are the LAL-regulators (Large ATP-binding regulators of the LuxR family), which were identified in antibiotic and other secondary metabolite gene cluster from *actinomycetes* (Guerra et al., 2012). Also in *Streptococcus mutans* the transcriptional regulator SMU.1745c was found in direct proximity to its biosynthetic cluster (Faustoferri et al., 2015).

However, aberrant concepts of co-regulation of divergent sets of genes were found in *E. coli* (Janga et al., 2007). It was speculated that the direct proximity to the transcriptional regulator is not needed. Thus, biosynthetic cluster are often the target of multiple levels of regulation (Arias et al., 1999) and therefore makes direct proximity of all genes controlled by the same regulator not necessary. The distance between gene cluster and regulator influences also their activity due to the “just-in-time principle”. This principle describes that the response time of the activation of the promoter is shorter, the smaller the distance of enzymes. This concept was shown for the biosynthesis of amino acids (Zaslaver et al., 2004). Thus, for some cluster a fast

response was probably evolutional favored and therefore the regulator and the cluster were grouped together. This is often the case for smaller operons (Janga et al., 2007). For a global regulation, however, a different scenario was assumed by Janga and coworkers (2007), which postulates that transcription factors of large regulons are strongly transcribed and get to their target by diffusion, which makes the direct location of both regulator and genes dispensable.

Studies in bacteria demonstrated that the regulation processed can be highly complex Thus, genes can be described from more than one promoter (Bibb et al., 1985; Buttner et al., 1987). Amongst others *Streptomyces coelicolor* is a very well-studied organism regarding regulatory processes, which synthesizes various antibiotic (Huang et al., 2005). In this species, it has been shown that the production of antibiotics is not evenly controlled. The bacterium *S. coelicolor* is able to produce at least four different antibiotics (actinorhodin, undececylprodigiosin, methylenomycin and calcium- dependent ionophore) (Hopwood et al., 1995), which underlie different regulation mechanisms including pathway-specific and pleiotropic regulation (Bibb, 1996). The regulation of the produced antibiotics by *S. coelicolor* differ among the antibiotics. Both actinorhodin and undececylprodigiosin are controlled by the pathway-specific regulatory genes *actII*- ORF4 and *Red*D, which are located in direct proximity. Additionally, the regulators *afs*R and *afs*B have a pleiotropic effect on the biosynthesis of both antibiotics, while the biosynthesis of the other antibiotics methylenomycin and calcium-dependent ionophore are unaffected (Bibb, 1996). Studies by Liu and coworkers (2013) encoded the regulation of the antibiotics in *S. coelicolor* and indicated that actionrhodin is the target of at least eight known regulatory proteins. Contrary, to the regulation of the latter, the cryptic polyketide is regulated differently by the autoregulator gamma-butyrolactone, which accumulates to a certain concentration in the culture and binds to a cytoplasmic protein and causes its dissociation to activate another protein (Liu et al., 2013). Thus, the coordinated regulation of specialized metabolites is highly complex in *S. coelicolor,* the same could be assumed for sodorifen in *S. ply.* 4Rx13.

No regulator has yet been identified that is in direct proximity to the sodorifen cluster in *S. ply.* 4Rx13, and it is possible that the regulator is pleiotropic. The cluster of *S. ply.* 4Rx13 is flanked upstream by a regulator, which, based on its annotation, likely regulates arabinose metabolism. The knockout of this regulator had no effect on sodorifen emission. Thus, the regulator of the sodorifen cluster is probably not directly

associated with the cluster, but rather is present at a distant location in the genome, which suggests a multilayered regulatory system.

Therefore, it is likely that a pleiotropic regulation applies at least for the sodorifen cluster identified in *S. ply.* 4Rx13. It is likely that the genes of the sodorifen cluster are regulated equally, however, by a pleiotropic regulator, but the other genes involved in sodorifen biosynthesis could be regulated very differently. Nevertheless, a global regulator has to be involved for cooperation of all genes within the biosynthesis of sodorifen. A global or pleiotropic regulation of the sodorifen cluster complicates the finding of regulators. The regulator may not only activate the transcription of the genes of the sodorifen cluster but other metabolic pathways and therefore, a multilayered regulatory network is involved. Thus, the elucidation of the regulatory system of sodorifen biosynthesis in *S. ply.* 4Rx13 is the target of further studies (Magnus and Piechulla, unpublished.

4.3 Proposed biosynthesis of the new compound and sodorifen

4.3.1 Incorporation of isotope labeled precursors into the novel alcohol

Previous studies demonstrated that feeding labeled compounds can help to elucidate the production of secondary metabolites such as geosmin and 2-methylisoborneol (Bentley et al., 1981; Spiteller et al., 2002). Therefore, the novel alcohol emitted by the terpene cyclase mutant and sodorifen were investigated by feeding isotope labeled precursors and respective products were analyzed by NMR spectroscopy.

The appearance of the novel alcohol in the VOC profile of the terpene cyclase mutant made it a promising candidate for an intermediate in the biosynthesis of sodorifen. Therefore, feeding experiments similar to those performed previously with the wild type of *S. ply.* 4Rx13 were done to propose a biosynthetic pathway for sodorifen (Weise, dissertation, 2013). The analysis of two-dimensional NMR-spectra such as *dqf*-COSY, HSQC, and HMBC, as well as H, H-NOESY spectra resulted in the elucidation of the structure and relative configuration of the novel alcohol. The comparison of the sodorifen structure and the novel alcohol indicated a large degree of similarity given by the polymethylated cyclopentene moiety. However, the novel alcohol carried less methyl groups than sodorifen and instead contained two internal methylene units.

For further understanding the involvement of the genes of the sodorifen cluster, the other two mutants with a sodorifen negative phenotype were investigated. The compound emitted by the methyl transferase mutant was identified as farnesyl acetone. However, the involvement of the methyl transferase and IPP- isomerase in the sodorifen biosynthesis are not yet clear and are not further discussed in this thesis.

Due to the close structural similarity of sodorifen and the novel alcohol, it was hypothesized that the novel compound might represent either a direct intermediate of sodorifen biosynthesis or alternatively is a byproduct of the reaction. Thus, the biosynthesis of the novel alcohol was elucidated using incorporation experiments with stable isotope labeled precursors (1.4-$^{13}C_2$-succinate; 2.3-$^{13}C_2$-succinate; L- [3 - ^{13}C]- alanine, and L-[*S*-$^{13}CH_3$]-methionine). Therefore, a comparison of the incorporation patterns observed for sodorifen (Weise, dissertation, 2013) with the novel alcohol, was possible.

The feeding experiments revealed that succinate and alanine are suitable substrates for the synthesis of sodorifen. It was demonstrated that succinate as the sole carbon source led to a high sodorifen emission, while methionine by itself was an unsuitable carbon source (Weise, dissertation, 2013). A minimal medium supplemented with alanine and threonine revealed a substantial sodorifen emission. It was hypothesized that methionine was responsible for the high degree of methylation within the sodorifen molecule.

The incorporation of labeled succinate into both sodorifen and the novel alcohol were successful and no severe differences in the incorporation pattern were observed. However, the labelling with methionine revealed distinct differences between the two compounds. Surprisingly, the labeled methyl group was found at a different position in sodorifen than the novel alcohol. Sodorifen showed a ^{13}C label at the double bond of the exocyclic ring, while the methyl group of the novel alcohol was labeled at the olefin group of a cyclopentyl ring. Despite, the observed structural similarity between sodorifen and the novel alcohol, a different position of the methyl group incorporated into both compounds made it very unlikely that they originate from the same biosynthetic pathway.

4.3.2 Proposed biosynthesis of the new compound

Combining the results of the incorporation patterns obtained from various feeding experiments indicated that the structural units that make up the novel alcohol are generated by an already known pathway. The labelling pattern of the cyclopentene ring within the attached sidechain is in excellent agreement with a DOXP pathway derived terpenoid, although it was apparent that the reactions are common compared to other known molecules. An exception is the reaction of GPP and IPP, which is unusual because it does not consist of a head to tail reaction leading to a cyclopentenyl intermediate. The novel alcohol is therefore probably not formed by a novel pathway or novel enzymes and makes the postulated biosynthetic pathway highly likely.

4.3.3 Comparison of sodorifen and the novel alcohol

The results of the feeding experiments with isotope labeled precursors for both sodorifen and the novel alcohol revealed: Firstly, the novel alcohol is not a direct

intermediate in the sodorifen biosynthesis although structural similarity exist. Secondly, both compounds are highly methylated, however, the methyl group residing from methionine appeared at a different position. Thirdly, the incorporation patterns suggested the involvement of the terpenoid pathway and classified the novel alcohol as a sesquiterpene derivative, while several steps of the biosynthesis of sodorifen remain elusive.

Although the novel alcohol is a byproduct of the reaction and not an intermediate of sodorifen biosynthesis, it is interesting to observe how *S. ply.* 4Rx13 compensates for the loss of the terpene cyclase. The knockout of the terpene cyclase resulted in the loss of the ability to produce sodorifen. Instead of stopping the sodorifen biosynthesis and releasing an intermediate, a new compound was produced that dominated the volatile spectrum. Due to the first speculation that the novel alcohol was an intermediate of the biosynthesis, which was proven wrong by the experiments described earlier, another possibility has to be assumed. Another hypothesis is that the terpene cyclase mutant might channel the C15 precursor into a different biosynthesis direction such as a metabolic bypass to form the novel alcohol. The structural similarity of both compounds is probably due to earlier common metabolic steps.

4.3.4 Proposed function of sodorifen

Beside the absence of sodorifen in the knockout mutant, other compounds normally present in the wild type spectrum were not affected. Interestingly, the emission of the novel alcohol is also quite high and dominates the spectrum like sodorifen in the wild type. Therefore, it can be speculated that a part of precursors build till this point are at least partially used for the novel alcohol, which implies common first steps within the biosynthesis of both sodorifen and the novel alcohol. It is also possible that the novel compound is built with least energy input and emitted to avoid an accumulation of products. It seems that the release of carbon and hydrogen in the form of sodorifen or a similar compound, in case of the mutant, is important for the bacterium. Therefore, the question occurs, what benefit exists for the bacterium to release high amounts of carbon in a carbon restricted environment.

In the laboratory the sodorifen emission is high when *S. ply.* 4Rx13 is grown on a protein rich medium or a minimal medium supplemented with succinate. Although, the

sodorifen emission under natural conditions is unknown. It is possible that the microbial community is affected by the release of the volatile sodorifen, which could have a beneficial, a harmful or a neutral effect.

In general, the interactions between microorganisms in the same habitat are positive negative or neutral (Wheatly, 2002). It was assumed that volatiles function as growth inhibitors of bacteria and fungi (Kai et al., 2007; Vespermann et al., 2007; Zou et al., 2007; Weise et al., 2012), influence the metabolism of microorganisms in the same habitat (Kai et. al., 2009; Garbeva et al., 2014a) but may also functions as growth promoters (Wheatley, 2002; Garbeva et al., 2014a). Nevertheless, the biological and ecological function for many volatiles, including sodorifen, is unknown. Various approaches were undertaken but it was not possible until now to assign a function to sodorifen. It was postulated that the emission of volatile compounds are advantageous to easily dispose waste products and the compounds cannot be accumulated in the cell or direct proximity in high quantities due to their volatility. As a consequence it is convenient for the bacterium to release the compounds, which often have a low energetic value (Kai et al. 2010). Thus, it was speculated that sodorifen might serve as a valve to release superfluous carbon and hydrogen. This is a possible scenario concerning the structure of sodorifen containing 16 carbon atoms and 26 hydrogens but lacks any heteroatoms.

Another aspect is that other microorganisms could benefit from the released volatiles, including the highly reduced compound sodorifen. In fact, the rhizosphere is a nutrient rich area and is favored by many organisms but they also live in competition with each other. The surrounding soil is less attractive due to its nutrient poor conditions, nevertheless microorganisms are able to survive by the utilization of alternative sources. Different studies assumed that released volatiles, are able due to their volatility to travel large distances through soil and used as the sole carbon or/and energy source by other microorganisms (Owen et al, 2007, Garbeva et al., 2014a; Schulz-Bohm et al., 2015). A similar purpose of function might apply to the novel alcohol since it is also released in high amounts.

Therefore, the bacterial community in the soil with *S. ply.* 4Rx13 and other organisms should be investigated regarding growth promoting or inhibiting effects related to sodorifen emission levels, which occur under natural conditions. Rare studies exist that imitate natural conditions in soil and allow the analysis of the interaction and release of volatiles within authentic microbial communities (Garbeva et al., 2014a;

Bohm- Schulz et al., 2015). A first approach was done by Weise (dissertation, 2013), cultivating *S. ply.* 4Rx13 in a soil similar medium to observe whether changes of the volatile spectrum occur. Actually, the quantity of sodorifen emission decreased with the medium mimicking natural conditions. Alternatively, it cannot be excluded that sodorifen may function as an infochemical. Therefore, further experiments with the wild type isolate and the terpene cyclase mutant e.g., in dual culture could show different effects on other microorganisms or plants due to the changed volatile spectrum.

Some volatiles are also extremely toxic and inhibit growth or are lethal for most organisms, however, a few microorganisms developed a possibility to metabolize also these compounds. A few microorganisms are able to detoxify volatiles and use them as energy source (Schulz-Bohm et al., 2015). The bacterium *Burkholderia* (Chen et al., 2015) and the fungus *Cladosporium sphaerospermum* are both able to grow and utilize the volatile aromatic hydrocarbon toluene as the sole source of carbon and energy (Weber et al., 1995; Prenafeta-Boldú et al., 2001), which could be useful for the industry for degradation of waste products.

Although, terpenes are presumed to be compounds, which are difficult to be degraded (Cookson, 1995), it was shown that *Pseudomonas fluorescens* and *Alcaligenes xylooxidans* are able to rapidly grow on and biodegrade α- pinene (Kleinheinz et al., 1999). Since sodorifen and the novel alcohol originate from the terpenoid biosynthetic pathway these compounds could also be useful growth substances for other organisms.

4.3.5 Implications for the biosynthesis of sodorifen

The conclusion, which can be drawn at this stage is that even though a structural similarity exists, the new compound is not an intermediate in the biosynthesis of sodorifen but a byproduct. It turned out that the biosynthesis of sodorifen is different in many aspects compared to the biosynthesis of the novel alcohol.

A comparative genome and transcriptome analysis led to a cluster of four genes, which are involved in the biosynthesis of sodorifen. The cluster contains beside the DOXP- synthase, a methyl transferase, an IPP-isomerase and a terpene cyclase. However, only one methyl group is attached by the methyl transferase, the others derive form reactions within the DOXP pathway. The actual function of the IPP- isomerase for the sodorifen biosynthesis is not yet clear. The analysis of the

product emitted by the methyl transferase revealed a farnesyl acetone. The question is whether it is an intermediate of the sodorifen biosynthesis or a byproduct. For the scenario as an intermediate a precursor like geranyl geraniol (C20 unit) would be necessary to generate a farnesyl acetone (C18 unit). Following a methylation by the methyl transferase to form a C19 unit, which would be converted to a C16 unit by multiple head-tail conjunctions. This mechanism is possible but it is highly unlikely that this sequence of reactions was developed during evolution due to a high input of energy and various cumbersome reactions. It is more likely that a farnesyl pyrophosphate (C15) is methylated to a C16 unit, which includes less metabolic steps and energy input by the organism. Another important argument against the farnesyl acetone as an intermediate in sodorifen biosynthesis is the appearance of one uncommon reaction. For both sodorifen and the novel alcohol the unusual non-head tail fusion was observed.

The presence of the terpene cyclase, DOXP-synthase and IPP-isomerase of the sodorifen cluster supported that sodorifen and the novel alcohol derive from the terpenoid biosynthetic pathway. The feeding experiments with stable isotope precursors further indicated that sodorifen and the new compound derived from three isoprene units plus one methionine derived C1-unit. Thus, both compounds are built from common precursors but at a later stage of the biosynthesis different reactions and rearrangements are performed. Thus, a different sequence of reactions and enzymes are involved to form two independent products.

To further decode the precise function of the enzymes of the sodorifen cluster regarding their substrates, enzyme assays are crucial. An enzyme assays of the terpene cyclase with the new alcohol as a substrate could lead to new insights into the biosynthesis of sodorifen and ultimately clarify whether the alcohol is or is not an intermediate. Potential substrates of the terpene cyclase are also GGP, NPP, FPP and GGPP, which might further support the terpenoid structure of sodorifen. From the reaction mechanism point of view, terpene cyclases are very interesting enzymes due to their ability to produce a large variety of products from only a few substrates (multi product enzymes). It is also possible, however, that based on its unique structure, an unusual substrate is converted for the production of sodorifen. To find out whether the methyl transferase is able to methylate FPP would be interesting–if so, the methylated FPP could be utilized as a substrate for the terpene cyclase to investigate the ability of the enzyme to convert this precursor into sodorifen. Terpene cyclases are

extraordinary enzymes because they can produce a wide range of products from only a few substrates (IPP, GPP, and FPP).

At the present state of the art it can be asserted that the elucidation of the biosynthesis of sodorifen is hitherto not fully clarified. However, a major step forward was done when the genes of the sodorifen cluster were verified to be involved in sodorifen biosynthesis. These four genes identified so far are definitely not sufficient to compose such a complicated molecule as sodorifen. Thus, further investigations for finding all genes involved in the biosynthesis of sodorifen have to be conducted in future studies.

5. Conclusions and further perspectives

5.1 Conclusions

The volatile compound sodorifen was previously detected in the headspace of *Serratia plymuthica* 4Rx13. Its structure ($C_{16}H_{26}$) displayed striking features (polymethylation, no heteroatoms), which is unique and novel compared to other natural compounds.

This thesis aimed to elucidate the biosynthesis of the secondary metabolite sodorifen. It was hypothesized that sodorifen is synthesized via a new pathway that likely requires novel enzymes, and so one of the primary goals of this research was to locate the genes that are involved in its biosynthesis.

By transcriptome analysis a cluster of four genes was found that consisted of a terpene cyclase, methyl transferase, DOXP-synthase, and IPP-isomerase. The involvement of these genes in the biosynthesis of sodorifen was verified by mutagenesis. The presence of the terpene cyclase and DOXP-synthase indicated that sodorifen is probably a terpene and the presence of the methyl transferase was in accordance with the polymethylated structure of sodorifen. This cluster alone is not sufficient for synthesizing a complicated molecule such as sodorifen and makes the detection of additional genes indispensable.

Another aspect of this work dealt with the elucidation of compounds produced by the mutants and their biosynthesis. The elucidation of the product emitted by the methyl transferase mutant was identified as farnesyl acetone ($C_{18}H_{36}O$), while the terpene cyclase mutant produced a novel alcohol. The structure of the novel alcohol showed a high similarity to sodorifen. Thus, it was speculated that this novel alcohol could be a potential intermediate in the biosynthesis of sodorifen. The feeding experiments with isotope labeled precursors (succinate, alanine and methionine) and analysis with NMR spectroscopy revealed that the novel alcohol produced by the mutant of the terpene cyclase is not an intermediate of the sodorifen biosynthesis but a byproduct. However, it was assumed that earlier steps in the biosynthesis of both sodorifen and the novel compound are similar (till C15 precursor FPP). Thus, the precursors derive from the DOXP pathway. A biosynthesis for the novel alcohol of the terpene cyclase mutant was proposed on the basis of the obtained NMR data. The biosynthesis contains no unusual reactions, except one unconventional non-head tail fusion, which is also assumed for the biosynthesis of sodorfen. Contrary, to the novel alcohol the

biosynthesis of sodorifen was not fully clarified since the connection and rearrangement of the IPP units is not clear. The enzymes which are assumed not to follow common head-tail reaction are not yet found. The involvement of the farnesyl acetone produced by the methyl transferase mutant in the biosynthesis of sodorifen has to be investigated in further studies. However, it is unlikely at this point that the farnesyl acetone is an intermediate in the biosynthesis of sodorifen.
Although, the sodorifen biosynthesis is not fully elucidated, the identification of the sodorifen cluster was an important step forward.

5.2 Further perspectives

This cluster alone is not sufficient for synthesizing a complicated molecule such as sodorifen, however, and so additional enzymes need to be detected to elucidate the entire biosynthetic process and to support or refute the current theory.
Analysis of the transcription levels of genes under growth conditions with glucose as the sole carbon source could be helpful for revealing additional genes. It is thought that glucose suppresses the genes involved in sodorifen biosynthesis, and so these growth conditions should result in low expression of these genes in the sodorifen producer *S. ply.* 4Rx13.
The main focus here should be on genes that are transcribed at a low level or not transcribed at all from the start of growth, as the theory is that the genes will be suppressed for the entire time that glucose is utilized as the main carbon source. By contrast, the expression of these genes should not change in isolate AS9 whether it is grown on glucose of succinate, as this isolate is not able to produce sodorifen.
A combined approach of feeding succinate and glucose would likely be even more successful for investigating changes in the expression levels of genes involved in sodorifen biosynthesis. The bacteria will first utilize glucose, during which time the genes involved in sodorifen biosynthesis will likely be suppressed. However, as soon as the glucose is exhausted, succinate will need to be used for metabolism, which will result in a high expression of the genes involved in sodorifen biosynthesis. Thus, genes that have no or very little change in expression can be ignored as they are likely to be housekeeping genes or involved in general metabolism. Therefore, the bacterium *S. ply*. 4Rx13 and isolate AS9 as the control should be cultivated in a minimal medium

complemented with glucose and succinate and samples for RNA isolation and sequencing are taken.

Finally, it would be interesting to know more about the function of sodorifen. To date, no specific function has been assigned to this novel compound, although it was speculated that it could be a waste product that functions as a valve for the release of superfluous carbon and hydrogen (Kai et al., 2010). This theory is supported by the structure of sodorifen, which only contains carbon and hydrogen atoms without any heteroatoms, and by the fact that no significant effect of sodorifen on other organisms has yet been shown. Another theory is that sodorifen is involved in inter- and intra-species communication, such as through QS, although the possibility that it serves as a molecule for extinguishing signals (i.e., quorum quenching) has also not yet been excluded. In this context the regulation of the sodorifen biosynthesis is also of interest because it could enlighten under which environmental conditions and cues the synthesis is stimulated or repressed. Even though it was hitherto not possible to assign a function to sodorifen, it is not excluded that the compound was advantageous for the bacterium during evolution to adapt to specific habitats or niches. The latter is supported by the observation that the sodorifen emission was only found in a few isolates of *Serratia plymuthica*, indicating that the ability to emit sodorifen seems to be unique in the bacterial world.

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Abbreviations

%.....percentage
°C……degree Celsius
µF……Microfarad
µg……Microgram
2D……two dimensional
A……Ampere
aa……amino acid
aLRT…….. approximation of the standard Likelihood Ration Test
Amp……Ampicillin
antiSMASH……antibiotics and Secondary Metabolite Analysis Shell
BiBLAST……biddirectional Basic Local Alignment Tool
BLAST……. Basic Local Alignment Tool
$CaCl_2$……calcium chloride
CFU……colony forming unit
cm……centimeter
CRP……..cAMP receptor protein
DIG-dUTP……Digoxigenin-X-5-aminoallyl-2-deoxyuridine-5-triphosphate
DMAPP……Dimethylallyl pyrophospahte
DMM……Davis-Mignoli Minimal Medium
DNA……deoxyribonucleic acid
DOXP……1-Deoxy-D-xylulose 5-phosphate
DSMZ……Deutsche Stammsammlung von Mikroorganismen und Zellkulturen GmbH
DXS……1-Deoxy-D-xylulose 5-phosphate Synthase
E. coli……*Escherichia coli*
EMBL……European Bioinformatics Institute
et al……et alii
eV……electro volt
G2L……Göttingen Genome Laboratory
G3P……glycerinaldehyde 3-phosphate
GC-MS……Gas chromatography-Mass spectrometer
GPP……geranyl pyrophosphate
h……hour
HF……high fidelity
HMB-PP……(E)-4-Hydroxy-3-methyl-but-2-enyl-pyrophosphate
idi……isopentenyl diphosphate isomerase
IS……internal standard

IPP……..isopentenyl pyrophosphate
IPTG……..…….Isopropyl b-D-1-thiogalactopyranoside
ispA……..……farnesyl pyrophosphate synthase
Kan……Kanamycin
kb……..……..…….kilobase
kV…….kilovolt
LB…….……Luria Bertani
m/z…….mass-to-charge ratio
Mb…….Megabase
MEP……..……..…..2-C-methyl-D-erythritol-4-phosphate
Mg……..……milligram
$MgCl_2$……..Magnesium chloride
min……..minute
ml……..…….milliliter
mM……..…..millimolar
MPI…….Max Planck Institute
mRNA……..……..….messenger ribonucleic acid
ms……..…..millisecond
mVOC……..……microbial volatile organic compounds
NaCl…….……..sodium chloride
NaOH……..……sodium hydroxide
NB……..……Nutrient Broth
NCBI……..…Naional Center for Biotechnology Information
ng..…….nanogram
NIST……..……..National Institute of Standards and Technology
NMR……..……nuclear magnetic resonance
NPKM…….Nucleotide activities Per Kilobase of exon model per Million mapped reads
OD…….optical density
ORF…….open reading frame
PCR……..……..polymerase chain reaction
Pfam……protein families
pH……. *potenia hydrogenii*
RNA……..……..ribonucleic acid
RNAP……..……Ribonucleic acid polymerase
RNA-Seq……..…… Ribonucleic acid sequencing
Rpm…….……..Revolutions per million
RT…….....Retention time

SAM……..S-Adenosyl methionine
SCAM…….……strain collection of antagonistic microorganisms
SDS…….……..Sodium dodecyl sulfate
S. ply……...…….*Serratia plymuthica*
SOD……..……..*Serratia odorifera*
SPME……..……Solid phase micro extraction
TPS……terpene cyclase
TraV……..…Transcriptome Viewer
UV……..…..ultraviolet
USA……....United States of America
V……...Volt
v/v……...…….volume/volume
VOC……...…volatile organic ecompound
Ω…….Ohm
&……..…….and

Selbstständigkeitserklärung

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Dajana Domik Rostock, 27. November 2015